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Human milk feeding as a complex system

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Human milk feeding as a complex system

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Defining the lipid profiles of human milk, infant formula, and animal milk: implications for infant feeding

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Background: Breastfed infants have lower disease risk compared to formula-fed infants, however, the mechanisms behind this protection are unknown. Human milk has a complex lipidome which may have many critical roles in health and disease risk. However, human milk lipidomics is challenging, and research is still required to fully understand the lipidome and to interpret and translate findings. This study aimed to address key human milk lipidome knowledge gaps and discuss possible implications for early life health.

Methods: Human milk samples from two birth cohorts, the Barwon Infant Study ($n = 312$) and University of Western Australia birth cohort ($n = 342$), were analysed using four liquid chromatography-mass spectrometry (LC–MS) methods (lipidome, triacylglycerol, total fatty acid, alkylglycerol). Bovine, goat, and soy-based infant formula, and bovine and goat milk were analysed for comparison. Composition was explored as concentrations, relative abundance, and infant lipid intake. Statistical analyses included principal component analysis, mixed effects modelling, and correlation, with false discovery rate correction, to explore human milk lipidome longitudinal trends and inter and intra-individual variation, differences between sample types, lipid intakes, and correlations between infant plasma and human milk lipids.

Results: Lipidomics analysis identified 979 lipids. The human milk lipidome was distinct from that of infant formula and animal milk. Ether lipids were of particular interest, as they were significantly higher, in concentration and relative abundance, in human milk than in formula and animal milk, if present in the latter samples at all. Many ether lipids were highest in colostrum, and some changed significantly through lactation. Significant correlations were identified between human milk and infant circulating lipids (40% of which were ether lipids), and specific ether lipid intake by exclusively breastfed infants was 200-fold higher than that of an exclusively formula-fed infant.

Conclusion: There are marked differences between the lipidomes of human milk, infant formula, and animal milk, with notable distinctions between ether lipids that are reflected in the infant plasma lipidome. These findings have potential implications for early life health, and may reveal why breast and formula-fed infants are not afforded the same protections. Comprehensive lipidomics studies with outcomes are required to understand the impacts on infant health and tailor translation.

KEYWORDS

breastfeeding, breastmilk, metabolomics, DOHaD (development origins of health and disease), fat

Introduction

In the first months of life, human milk provides the infant with a multitude of nutritive and bioactive components, including lipids, which make up approximately 3–5% of human milk (w/w) (1). Not only does the lipid portion provide the majority of energy (approximately 50%) to the breastfed infant, it also delivers potentially bioactive species with critical roles in early life (2–4). The human milk lipidome is complex, comprised of numerous lipid classes, including triacylglycerols, sphingolipids, gangliosides each made up of hundreds of individual lipid species, many of which can be difficult to measure (2). Of the macronutrient components, the percentage of total lipids in human milk displays the highest interindividual variation. The percent of lipids is also related to the amount of milk in the breast at the time of collection (and thus feedings patterns), resulting in high variation (5). The degree to which the specific lipid species that comprise human milk vary between individuals and over time is unclear.

Breastfed infants have better short- and long-term health outcomes than formula-fed infants (6). The compositional differences between human milk and infant formula are likely responsible, at least in part, for these effects. There is emerging evidence that the human milk lipidome contributes to some of the benefits afforded by breastfeeding, decreasing the risk of obesity, diabetes, and non-communicable diseases (7). The potential mechanisms include anti-infection and anti-inflammatory actions by fatty acids (8–11), sustenance of beige adipose tissue by alkylglycerols and thus decreased risk of obesity (12, 13), and establishment of healthy metabolism and lipid regulation by lipid metabolites such as 12,13-diHOME (14–16). Circulating lipid dysregulation is also a demonstrated key risk factor for obesity and related non-communicable diseases (17). In an analysis of over 600 plasma lipids in 1,074 infants (Barwon Infant Study), we previously found that 90% of circulating lipids at 6 months of age were significantly associated with any breastfeeding at 6 months of age (18).

Limited understanding of the human milk lipidome, and its variation, restricts the interpretation and translation of research in this field. To date, this has not been profiled in the same detail as human blood. As interest in the human milk lipidome increases, there is a critical need for improved profiling and understanding of its composition and variance. In this study, we utilised human milk samples from two birth cohorts to address these knowledge gaps, through (1) comprehensive profiling of the human milk lipidome, (2) comparison of human milk with infant formula and animal milk, (3) assessment of inter-and intra-individual variation, (4) investigation of concentration, relative abundance, and intake, and (5) assessment of human milk and corresponding infant circulating lipids at 6 months. We discuss the potential implications of our findings, and the future direction of human milk lipidomics to further enhance understanding, interpretation, and translation of lipidomics in this field.

Methods

Cohort samples

The Barwon Infant Study (BIS) is a birth cohort study assembled using an unselected antenatal sampling frame in the Barwon region of Victoria, Australia (19). Women were recruited during pregnancy and excluded from the study if infants were born premature or developed serious illness. The human milk for BIS included samples collected at 1 month ($n = 247$), 6 months ($n = 32$), and 12 months ($n = 33$), from women who were breastfeeding (exclusively or mixed, a total of 287 dyads). Pre-feed samples were collected from one breast, at the start of each visit or at the end (approximately 2 hours) if the infant was recently fed. Participants were given the option to manually express or to use a breast pump for collection of samples. Lipidomics profiling has been reported previously on infant plasma at ages 6 and 12 months (18). Ethics approval was obtained by the Barwon Health Human Research and Ethics Committee (HEC 10/24).

The University of Western Australia Longitudinal Cohort (UWAC) is a birth cohort from Perth, Western Australia, Australia. Women who intended to exclusively breastfeed for 6 months were recruited for this study during pregnancy and excluded if infants developed serious illness or were no longer exclusively breastfeeding at 6 months (20). The UWAC included 17 healthy exclusively breastfeeding mother-infant dyads. Monthly sample and growth data was collected at birth ($n = 16$), 1 month ($n = 54$), 2 months ($n = 50$), 3 months ($n = 104$), 4 months ($n = 33$), 5 months ($n = 40$), and 6 months ($n = 45$). Monthly sample types include morning, noon, and evening samples, and pre- ($n = 60$) and post-feed ($n = 44$) samples at 3 months post-partum, providing coverage of known sources of lipid variation. In month three, daily infant milk intake was also measured with 24 h test weighing (21). Ethics approval was obtained by The UWA Human Research Ethics Office (RA/4/20/4023), and all participants provided informed written consent. All human milk samples were stored refrigerated (4°C, BIS) or frozen (<0°C, UWAC) for

<24 h before being transferred to a laboratory freezer (−80°C) for storage until thawing (at room temperature) and preparation for analysis.

Other samples for comparison

Commercially available infant formula was included for comparison with human milk, bovine milk-based ($n = 6$), goat milk-based ($n = 2$) and soy based ($n = 2$). Each formula type was reconstituted in water as per the directions. The fat from each of these infant formula samples is derived predominantly from vegetable and plant oils. Commercially available bovine ($n = 2$) and goat milk ($n = 1$) were also included for comparison.

Lipid extraction

Single phase lipid extraction is commonly carried out using a chloroform methanol method, however, to reduce preparation time and increase throughput (amenable to automation), we used single phase butanol and methanol extraction, after establishing efficacy (Supplementary File S1 and Table S1) (22). Lipids were extracted from 10 µL samples using 100 µL extraction solvent butanol: methanol (1,1, v/v) containing 10 mM ammonium formate and internal standards. Samples were vortexed, sonicated for 1 h, centrifuged for 10 min (14,000x g, 20°C), and supernatant was transferred to 2 mL glass mass spectrometry vials with 250 µL inserts (Agilent Technologies) for analysis. All sample types were extracted using butanol: methanol, with some alterations (described for each analysis method in Supplementary Files S2–S5).

Liquid chromatography-mass spectrometry based lipidomics

Human milk, infant formula, and animal milk samples were analysed using a combination of four liquid chromatography-mass spectrometry based lipidomics methods, as per Table 1. All methods were targeted, using scheduled multiple reaction monitoring (MRM), as described below. For all methods, analyses were performed as single batches, with quality control samples (pooled plasma QC, pooled human milk QC, and blanks) included every 20 samples. Species were identified based on MRM precursor/product ion pairs and retention time, and chromatographic peaks were integrated manually using Mass Hunter (B.09.00, Agilent Technologies) software. The median blank concentrations were subtracted from each sample. Concentrations below the limit of detection were replaced by half the minimum measured value for that species. For subsequent statistical analyses, results from method 1 and 2 were combined (representing the whole lipidome), while results from methods 3 and 4 were both kept separate.

Lipidome LC–MS analysis

Lipidome analysis was carried out on an Agilent 1290 UHPLC system coupled with an Agilent 6495C triple quadrupole mass spectrometer (Supplementary File S2). Samples were extracted with butanol:methanol, as in *Lipid extraction*. Concentrations for each lipid species were calculated based on area under the chromatographic curve relative to the labeled internal standard concentrations (23). For the UWAC samples, the chromatography was extended and retention time windows shifted appropriately, to include lower-abundance short chain fatty acid containing TG (SCFA-TG) with the lipidome.

Triacylglycerol LC–MS analysis

Because the concentration of TG in milk were high relative to other lipid species, a separate analysis (Supplementary File S3) for TG was performed whereby samples were diluted (1 in 100) with milliQ water before lipids were extracted from 10 µL with butanol:methanol, as described above. Analysis of milk triacylglycerols was performed on an Agilent 6490 QQQ mass spectrometer with an Agilent 1290 series UHPLC system. Concentrations of each triacylglycerol were calculated based on chromatographic peak area relative to the labelled triacylglycerol internal standard (23).

Total fatty acid LC-MS analysis

For the analysis of total fatty acids, milk samples were saponified to release all fatty acids prior to analysis (Supplementary File S4). Mass spectrometry analysis was as described for method 1. Concentrations of each fatty acid was calculated based on chromatographic peak area relative to deuterated fatty acid internal standard concentrations.

Alkylglycerol LC–MS analysis

Because we noted that the amount of TG(O) species was significant in human milk, we also analysed the alkylglycerol composition to quantitate the total TG(O) species. Lipid extracts for alkylglycerol analysis were saponified (Supplementary File S5), generating alkylglycerols from ether lipids, predominantly TG(O). Mass spectrometry analysis was as described for method 1. For quantification of alkyl glycerol species, a deuterated monoacylglycerol (MG 18:1d7) was used as an internal standard. Response factors for alkylglycerol species against MG 18:1d7 were calculated using serially diluted synthetic alkyl glycerol species in a range 1–300 µM and a fixed amount of MG 18:1d7. The efficiency of saponification was assessed by the residual triacylglycerol in saponified samples.

Infant intake comparison

Due to the complications introduced by sampling (and thus high variability between samples), infant lipid consumption (intake) was assessed at 3 months, comparing exclusively breastfed infants

TABLE 1 Description of the four LC–MS methods used to achieve comprehensive analysis of samples.

Method	Description	Samples analysed
1) Lipidome	Total lipidome excluding triacylglycerols	BIS human milk, UWAC human milk, animal milk, infant formula
2) Triacylglycerol	Extended method to cover triacylglycerols	BIS human milk, UWAC human milk, animal milk, infant formula
3) Total fatty acid	Total fatty acids that comprise the lipidome	BIS human milk, animal milk, infant formula
4) Alkylglycerol	Alkylglycerol composition, from alkyldiacylglycerols (TG(O))	BIS human milk, animal milk, infant formula

(UWAC) and an exclusively formula-fed infant. For the exclusively breastfed infants, total intake (in mL) was multiplied by mean sample concentration (pmol/mL) at 3 months (24). For an exclusively formula-fed infant, sample concentrations (pmol/mL) were factorised by the number and volume of feeds (at 3 months) on bovine milk infant formula package. Lipid intake was expressed as pmol/day.

Statistical analyses

Due to the complexity of human milk lipids, lipidomic measurements were expressed as concentrations, relative abundance (proportion of the total lipid content, calculated from the molar concentrations), and intake (per day, at 3 months post-partum). Paired *t*-tests were used to compare lipid extraction methods (butanol:methanol with chloroform:methanol). Full lipidome and extended triacylglycerol results were combined for analyses, but fatty acid and alkylglycerol analyses were performed independently. Concentration and relative abundance values were log transformed prior to modelling. Principal component analysis was performed on lipidomic measures for all sample types, to visualise the major axes of variation. Unpaired *t*-tests were used to compare mean ether lipid content in the different sample types and assess if they were different. Linear mixed-effects models were used to identify trends in human milk lipids between time points (time of day, pre- or post-feed, and month post-partum). Human milk lipid (class or species) concentration or relative abundance were modelled, with sample timing as a fixed effect and individual ID was a random effect to account for intra-individual variation. Pearson correlation was performed on 637 matched BIS plasma lipids measured at 6 months of age (previously published (18)), and BIS human milk lipids measured at 1 month. Pearson correlation was also performed between the DHA-containing TG and linoleic acid (LA)-containing TG ratio in human milk and infant plasma. Linear regression was performed to compare the intakes of exclusively breastfed and exclusively formula-fed infants at 3 months of age, with results expressed as fold-differences. Benjamini and Hochberg adjustment was made to account for multiple comparisons in all analyses (false discovery rate, FDR), with adjusted $p < 5 \times 10^{-2}$ considered significant (25). Statistical analyses were conducted using R Studio (version 4.1.2). Unless otherwise stated, values are presented in the text as mean ± standard deviation (SD).

Results

A total of 312 milk samples were analysed from BIS (Supplementary Table S2) with each of the four methods (Table 1). At the 1 month milk sample collection, 34.2% (67/196 who answered) reported exclusively breastfeeding, at 6 month collection 6.7% (2/30 who answered) reported exclusively breastfeeding, and at 12 months all participants (excluding one) were breastfeeding at least one feed per day. UWAC (Supplementary Table S3) was comprised of exclusively breastfeeding dyads and was used to look comprehensively at sampling and longitudinal human milk trends. A total of 342 longitudinal samples from UWAC were analysed.

Liquid chromatography-mass spectrometry allows comprehensive human milk lipidomics

Combining the lipidome and triacylglycerol LC–MS methods (methods 1 and 2), allowed measurement of 979 lipid species (Supplementary Table S4). The median human milk QC CV for each method was 8.8 and 11.7%, respectively, and all CV were <20%. In all human milk samples, approximately 99% of the total lipidome (w/w) was comprised of triacylglycerols (74 ± 17% of the total lipidome), free fatty acids (12 ± 10%), diacylglycerols (11 ± 6%), and monoacylglycerols (2 ± 1%).

Samples were saponified and total fatty acids were measured (method 3), from C8:0 to C24:6 (Supplementary Table S5). The median CV for the fatty acid method was 14.5%, and all CV were <20%. The total fatty acid component of human milk was comprised primarily of C18:1 (40 ± 5%), with C16:0 (18 ± 2%), C18:2 (8 ± 2%), and C18:0 (8 ± 2%), C14:0 (7 ± 3%), C12:0 (6 ± 2%), and all others comprising ≤2% of the total fatty acids measured.

Similarly, alkylglycerols (method 4) (from TG(O)) were also measured, via sample saponification (Supplementary Table S6). The median CV for the alkylglycerol method was 15.4%, and all CV were <26%. This method was able to identify 18 alkylglycerol (AKG) species, ranging from AKG(12:0) to AKG(24:1). The three most abundant species (AKG(18:1), AKG(16:0), and AKG(18:0)) comprised over 90% of the total AKG content.

Human milk has a distinctive lipidome

Principal component analyses (PCA) was firstly used to obtain an overview of the relatedness of the lipid profiles of the infant formula and animal samples analysed in this study. This showed clear separation of all the human milk samples from all infant formula, and animal milk samples, for both total lipid species (Figure 1A) and total fatty acids (Figure 1B). The lipidome of infant formula of bovine, goat, and soy milk origin, and bovine and goat milk, differed from each other (Figure 1A), but clustered closer together when total fatty acid composition was assessed (Figure 1B).

Despite the total lipid content being very similar in all sample types (all $p > 5 \times 10^{-2}$, Figure 2A), there were marked compositional differences between human milk, infant formula, and animal milk, at both the lipid species and class level (Figures 2B,C and Supplementary Table S4). Human milk contained lower TG than other samples, however the sum of TG, FFA, DG, MG was 98.3% of the total lipid content, approximately 0.6–1.1% lower than bovine formula (99.4%), goat formula (99.2%), soy formula (99.0%), bovine milk (99.1%), and goat milk (98.9%). The 'other' lipid classes (Figure 2C) in infant formula and animal milk were particularly different from human milk. Specifically, the three most abundant 'other' classes in human milk comprised >0.9%, made up of COH (0.4%), TG(O) (0.3%), and PC (0.2%). In contrast, these three classes comprised 0.3% of bovine formula, 0.4% of goat formula, and 0.5% soy based formula, with PC making up the majority of this (0.2, 0.3, and 0.5% respectively). Measures for the animal milk samples were closer to that of human milk, with COH, TG(O), and PC comprising 0.5% of bovine milk and 0.7% of goat milk, with majority comprising COH (0.3%).

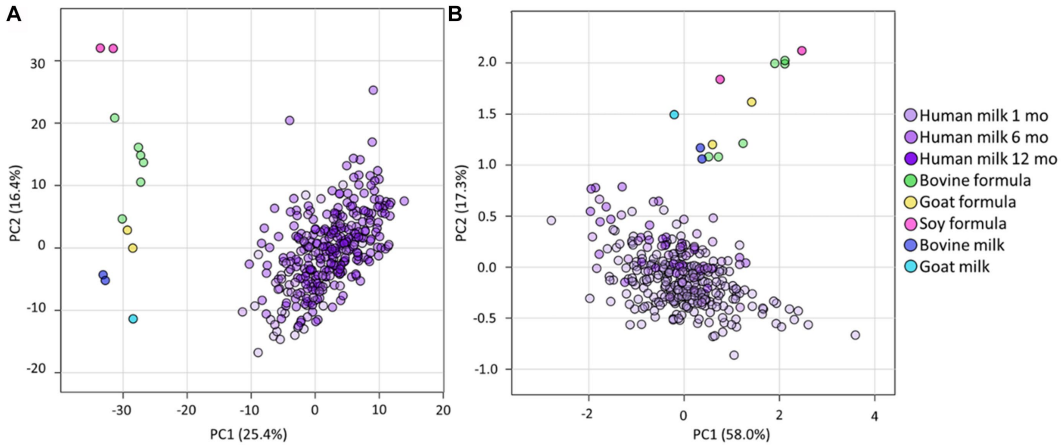

FIGURE 1
The lipidomes of human milk (BIS), infant formula, and animal milk. PCA of lipidomic measures for **(A)** the total lipidome and **(B)** the total fatty acid composition, for BIS human milk at 1 (Human milk 1 mo), 6 (Human milk 6 mo), and 12 (Human milk 12 mo) months, infant formula (bovine, goat, and soy), and animal milk samples (bovine milk and goat milk). All concentrations were log10 transformed prior to PCA.

Large differences in both concentration and relative abundance were also reflected in the FA analysis. The total fatty acid content varied significantly from human milk (106.3 ± 45.5 mM) and bovine formula (135.4 ± 0.1 mM, $p = 1.4 \times 10^{-2}$), soy formula (169.2 ± 76.3 mM, $p = 4.2 \times 10^{-3}$), and goat milk (166.6 mM, $p = 2.3 \times 10^{-2}$), but not from goat formula (135.4 ± 10.1 mM, $p = 1.2 \times 10^{-1}$), or bovine milk (117.5 ± 5.5, $p = 0.5.5 \times 10^{-1}$). C18:1 was the most prevalent fatty acid in all sample types, comprising 40% of the total fatty acid content of human milk, 37% of bovine and soy milk formula, and 45% of goat milk formula. C18:1 made up 24 and 26% of goat and bovine milk fatty acids, respectively (Supplementary Table S5). Odd-chain fatty acids, including C15:0, C17:0, and C19:1, were all lower in concentration in infant formula, regardless of milk base.

Human milk contains high levels of ether lipids

Ether lipids, those containing an ether or vinyl ether link, made up approximately 0.45 ± 0.19% of the total human milk lipidome, comprised of TG(O) (0.28 ± 0.09%), PE(P) (0.06 ± 0.03%), DG(O) (0.02 ± 0.02%), PC(O) (0.01 ± 0.003%), PC(P) (0.004 ± 0.002%), LPE(P) (0.001 ± 0.002%), LPC(O) (0.0001 ± 0.0001%), and LPC(P) (0.00009 ± 0.00007%) (Figure 3A). In contrast, total ether lipids comprised approximately 0.05 ± 0.005% bovine milk, 0.19% goat milk, 0.04 ± 0.004% bovine milk-based infant formula, 0.06 ± 0.009% goat milk-based infant formula, and 0.05 ± 0.007% soy-based infant formula (Figure 3B).

TG(O) comprised the majority of the ether lipids in human milk and were found in significantly higher proportions than in infant formula and bovine milk (all $p < 0.0001$), but were not different in goat milk ($p > 0.05$) (Figure 3C). Over 80% of the TG(O) class was made up of 10 species, including TG(O-52:2), TG(O-52:1), TG(O-50:1), TG(O-54:3), and TG(O-54:2), containing primarily C18:1 and C16:0 fatty acids. PE(P) lipids (Figure 3D) were the second most abundant ether lipid, comprising 15% of total ether lipid content. Sixteen different PE(P) species made up over 80% of the total PE(P), containing many polyunsaturated fatty acid species (PUFA) (Supplementary Table S4).

To have a more accurate measurement of the TG(O) species, milk samples were saponified to measure the alkylglycerol content (Figure 4A). These alkylglycerols originate from TG(O) species, and were made up of predominantly AKG(18:1), AKG(18:0), and AKG(16:0) (Figure 4C) (Supplementary Table S6). Compared to human milk 0.12 ± 0.1%, all infant formula (0.001–0.005%), bovine milk (0.004%), and goat milk (0.007%) all contained lower levels of AKG. However, goat milk contained concentrations closest to human milk (0.12 mM versus 0.11 ± 0.04 mM respectively) (Figure 4B).

The lipidome of human milk differs over lactation

Human colostrum has a distinct lipidome

Results for colostrum samples (UWAC) were analysed separately and compared to 1 month human milk samples. Total lipid content was similar in colostrum and mature milk (colostrum: 108 ± 59 mM vs. 1 month: 105 ± 61 mM; $p > 5 \times 10^{-2}$), yet there were distinct lipid class and species differences (Supplementary Tables S7, S8). For reference, 50–100 mM total human milk lipids is equivalent to 3–7 g/100 mL. Specifically, at the class level, colostrum was higher in TG(O) (1.3 times), LPE(P) (5 times), LPC(P) (3.3 times), PE(P) (2 times), and PC (2.5 times) (all $p < 5 \times 10^{-2}$). Only GQ1 (4.23-fold), FFA (3.20-fold), BA (2.75-fold), and DG (2.62-fold) increased significantly from colostrum to mature milk (all $p < 5 \times 10^{-2}$). The concentration of 35% of the individual species were significantly different between colostrum and one-month mature milk, which included many non-TG(O) ether lipids.

The human milk lipidome varies in concentration but not composition throughout a feed

There were significant differences between lipid species concentrations pre-feed and post-feed at 3 months post-partum (Supplementary Tables S9, S10). The concentration of 46% of the lipid

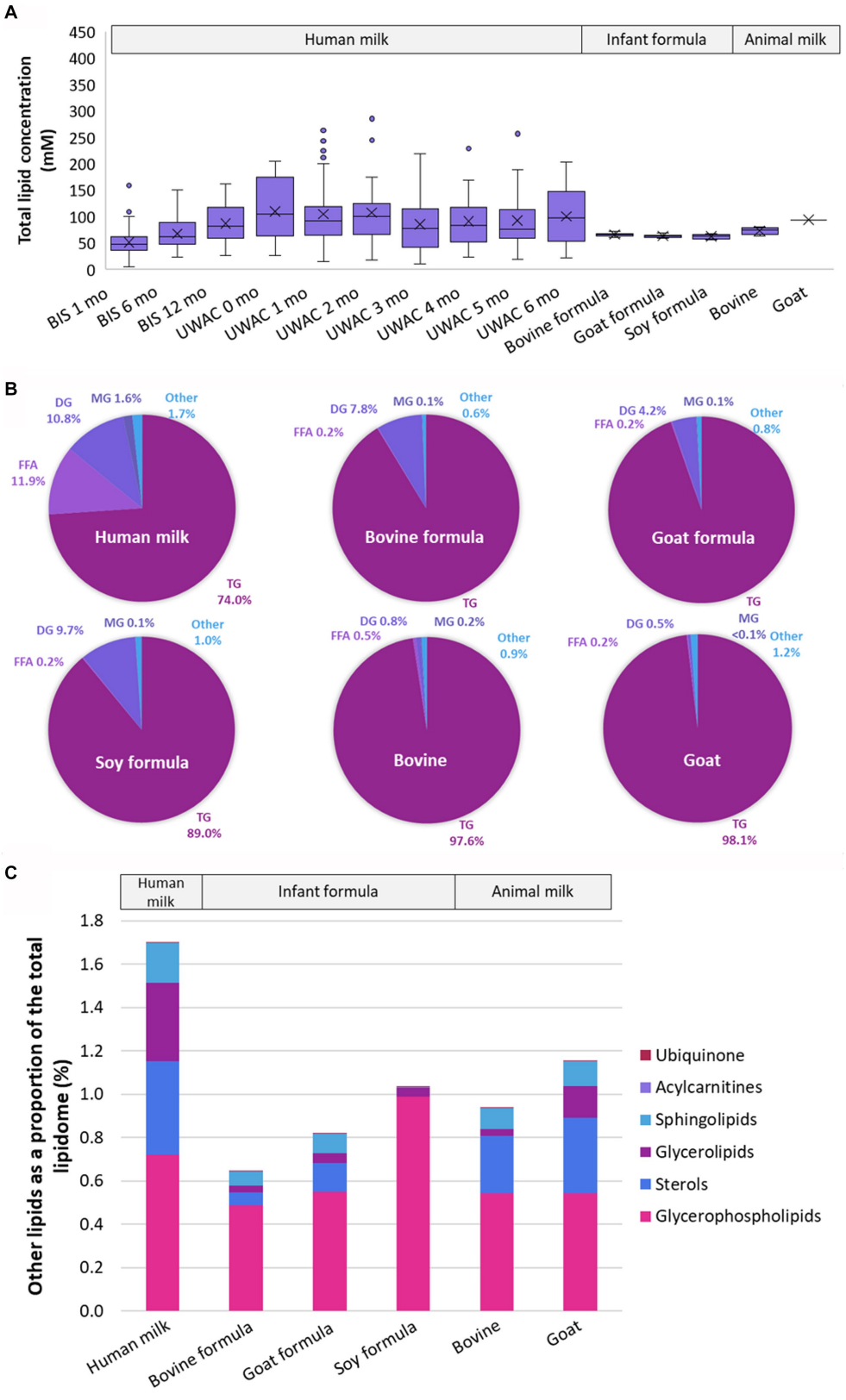

FIGURE 2
The lipidomes of human milk ($n = 654$), bovine ($n = 6$), goat ($n = 2$), and soy ($n = 2$) based infant formula ($n = 6$), bovine milk ($n = 1$), and goat milk ($n = 1$). **(A)** The total lipid concentration (mM) of animal milk, infant formula, and human milk samples. Boxes are median and lower (25%) and upper (75%) quartile values, crosses are mean values, whiskers are minimum and maximum, with outliers greater than 1.5× the interquartile range. **(B)** The mean relative abundance of lipids that comprise the human milk, bovine milk, goat milk, bovine milk formula, goat milk formula, and soy milk formula lipidomes, represented as class totals triacylglycerol (TG), free fatty acid (FFA), diacylglycerol (DG), monoacylglycerol (MG) and Other. **(C)** The mean

(Continued)

FIGURE 2 (Continued)
relative abundance (as a percentage of the total lipid content, calculated from molar concentrations) of other lipid classes in human milk, bovine milk, goat milk, bovine milk formula, goat milk formula, and soy milk formula. Lipid classes are listed in ascending order of magnitude for human milk and defined as Ubiquinone, Acylcarnitines (hydroxylated acylcarnitine and acylcarnitine), Sphingolipids (sphingomyelin, monohexosylceramide, dihexosylceramide, trihexosylceramide, ceramide, deoxyceramide, dihydroceramide, GM3 ganglioside, sphingosine, and sulfatide), Glycerolipids (alkyldiacylglycerol and monoalkyldiacylglycerol), Sterols (free cholesterol and cholesterol ester), Glycerophospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, alkenylphosphatidylethanolamine, phosphatidylserine, lysophosphatidylethanolamine, lysophosphatidylcholine, alkylphosphatidylcholine, alkenylphosphatidylcholine, lysophosphatidylinositol, phosphatidic acid, alkylphosphatidylethanolamine, lysoalkenylphosphatidylethanolamine, phosphatidylglycerol, lysoalkylphosphatidylcholine, and lysoalkenylphosphatidylcholine). Ubiquinone and Acylcarnitines comprise <0.002% in all sample types. Triacylglycerol, diacylglycerol, monoacylglycerol, and free fatty acid classes were not included.

species increased significantly (FDR adjusted $p < 5 \times 10^{-2}$), and one (TG(O-54:4)) decreased significantly ($p = 3.74 \times 10^{-2}$). Similarly, 11 out of 49 lipid class totals increased significantly ($p < 5 \times 10^{-2}$). For this reason, only pre-feed samples were included in the subsequent analyses. However, when we compared relative abundance, only 9% of the lipid species changed significantly, and no significant class changes were observed between pre- and post-feed samples (Supplementary Tables S11, S12).

The human milk lipidome does not change throughout the day

While 18 of the lipid classes increased significantly in concentration throughout the day, only 4 species (dhCer(d18:0/24:0), PC(P^{-18}:1/22:6), AC(18:1), and PC(O-16:0/22:6)) did (all $p < 5 \times 10^{-2}$, Supplementary Tables S13, S14). When relative abundance was modelled, no significant changes occurred for the lipid classes, and only 3 species (dhCer(d18:0/24:0), PC(O-42:5), and PC(O-44:6)) increased (all $p < 5 \times 10^{-2}$, Supplementary Tables S15, S16).

The human milk lipidome changes significantly throughout lactation

Both absolute concentration and relative abundance of human milk lipids were compared across 6 months lactation. The total lipid content did not change over the full lactation period ($p = 5.08 \times 10^{-1}$; Figure 5A). However, significant concentration changes occurred for sixteen of the lipid classes, eight classes increased significantly from 1 to 6 months, and eight classes decreased significantly from months one to six (Supplementary Table S17). The greatest concentration increases per month were measured for GQ1 (1.24-fold, $p = 2.33 \times 10^{-21}$), dimethyl-CE (1.19-fold, $p = 1.86 \times 10^{-3}$), SCFA-TG (1.16-fold, $p = 1.59 \times 10^{-3}$), and GM3 (1.14-fold, $p = 4.09 \times 10^{-7}$), while ether lipids including LPE(P) ($p = 1.75 \times 10^{-3}$), LPC(O) ($p = 6.47 \times 10^{-3}$), and TG(O) ($p = 8.16 \times 10^{-3}$), were all lower each month (all approximately 1.1 times). Other ether lipids, such as PE(P), did not change significantly through lactation (0.95-fold, $p = 6.06 \times 10^{-2}$). At the species level, 171 lipids changed significantly, with most, 121/171, decreasing (Supplementary Table S18). The analysis of lipid classes as a proportion of the total lipid content (Supplementary Table S19) revealed similar findings, increases for SCFA-TG (from approximately 0.06 to 0.16% of the total lipid content, Figure 5B), GM3 (increased from 0.003 to 0.006%, Figure 5C), no change to TG(O) (Figure 5D), and decreases to PE(P) (0.035 to 0.027%, Figure 5D) and LPE(P) (0.00219 to 0.00143%, Figure 5E). At the species level (Supplementary Table S20), 180 species changed significantly, approximately half decreasing (93/180) and half increasing (87/180).

Human milk lipids correlate with plasma lipids

Matched lipids were compared between the BIS human milk samples at 1 month lactation and corresponding infant plasma samples at 6 months of age. Significant positive correlations ($p < 5 \times 10^{-2}$) existed between the relative abundance (as a proportion of the total lipid content) of 122 of 637 lipid species (uncorrected), and 51 of 637 after FDR correction. This included primarily lipid species containing PUFAs, such as 22:6, 22:5, and 20:5. Notably, 40% of the significantly correlated lipids in human milk and plasma, after FDR correction, were ether lipids (Supplementary Table S21). The ratio of DHA-containing TG to linoleic acid (LA)-containing TG was positively correlated between milk and infant plasma (Pearson correlation = 0.37, $p = 3.43 \times 10^{-7}$).

Exclusively breastfed infants have a different lipid diet to that of exclusively formula-fed infants

To further understand infant lipid dietary differences between human milk and infant formula, in the context of early life, we compared the infant lipid intake at 3 months, for an infant exclusively breastfed or exclusively formula-fed. This was calculated with concentrations and the milk intake from UWAC, and infant formula preparation instructions. Firstly, milk intakes for exclusively breastfeeding infants were 741 ± 163 mL/day, while formula intake was 850 ± 77 mL/day. Overall specific lipid species intake varied widely between breastfed infants and separated distinctly from that of exclusively formula-fed infants, however total lipid intake was not different ($p = 9.2 \times 10^{-1}$). Significant differences existed between most lipid classes (Figure 6, Supplementary Tables S22, S23), excluding only total PC, FFA, AC, PC(O), TG, PE, DG, and Hex2Cer. Most lipid classes were consumed in higher amounts by exclusively breastfed infants. Almost all ether lipid species were fed to the exclusively breastfed infant in significantly higher amounts, however, this analysis excluded those species that were not able to be measured in infant formula.

Discussion

It is critical that human milk lipidomics continues to improve and advance, in order to effect meaningful research interpretation and translation, to understand and improve early life health. In this study we profiled the lipidome of 654 human milk samples from two birth cohorts, BIS and UWAC, to advance current lipidome understanding. The key findings from this study were (1) the human milk lipidome differs from that of infant formula, animal milk, and is rich in ether lipids, (2) human milk lipids exhibit longitudinal trends, and (3) the human milk lipidome impacts infant circulating lipids.

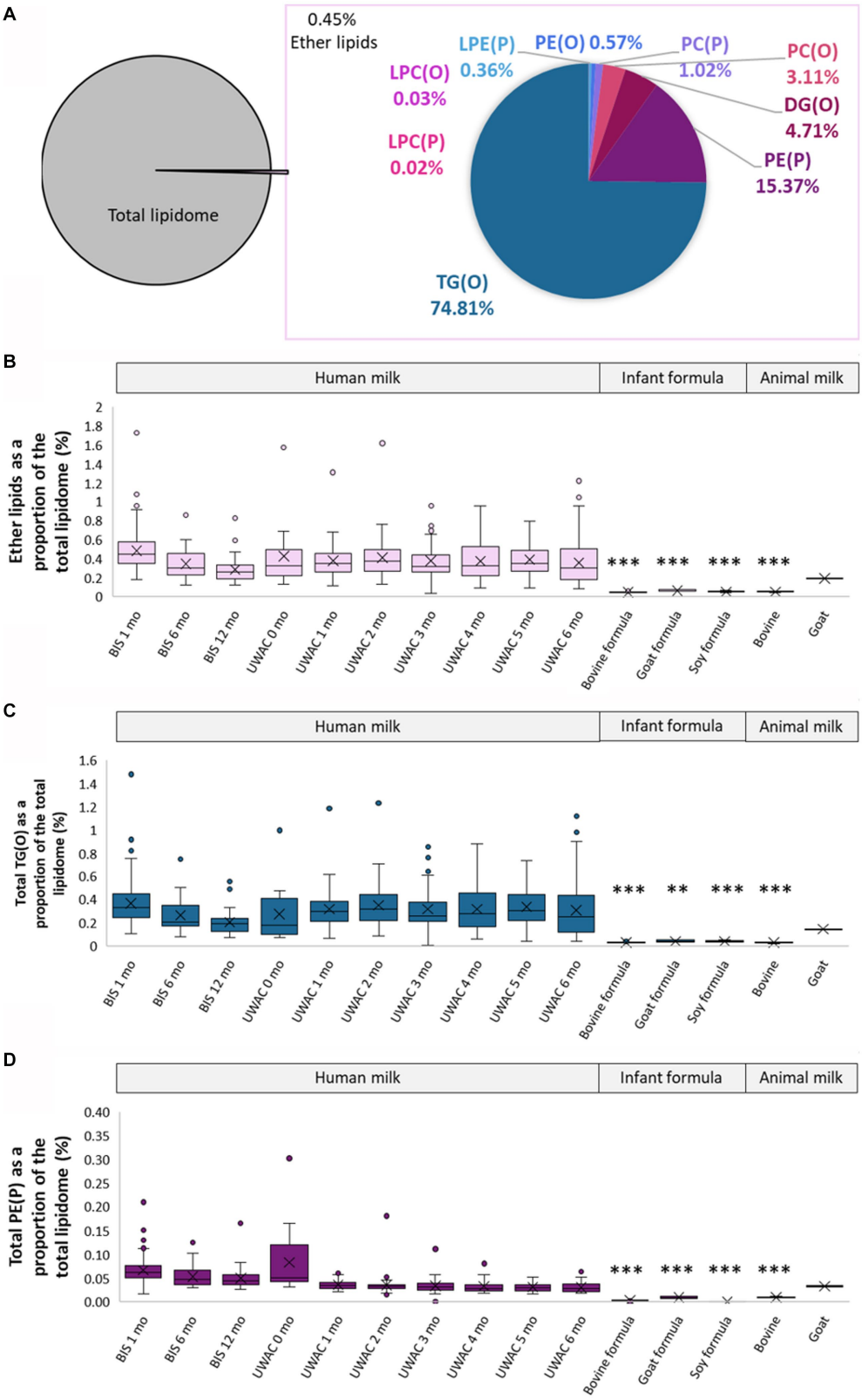

FIGURE 3
Ether lipids are different in human milk, infant formula, and animal milks. **(A)** Total ether lipids as a proportion of the total lipidome, and the proportion of each class of ether lipids. **(B)** Total ether lipids as a proportion of total lipidome (%, calculated using the molar concentrations) in BIS and UWAC human milk samples, infant formula, and animal milk. **(C)** Total TG(O) as a proportion of total lipidome (%) in BIS and UWAC human milk samples, infant formula, and animal milk. **(D)** Total PE(P) as a proportion of total lipidome (%) in BIS and UWAC human milk samples, infant formula, and animal milk. Boxes are median and lower (25%) and upper (75%) quartile values, crosses are mean values, whiskers are minimum and maximum, with outliers greater than 1.5× the interquartile range. Significant differences between the mean values of human milk and other sample types are represented by *($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$).

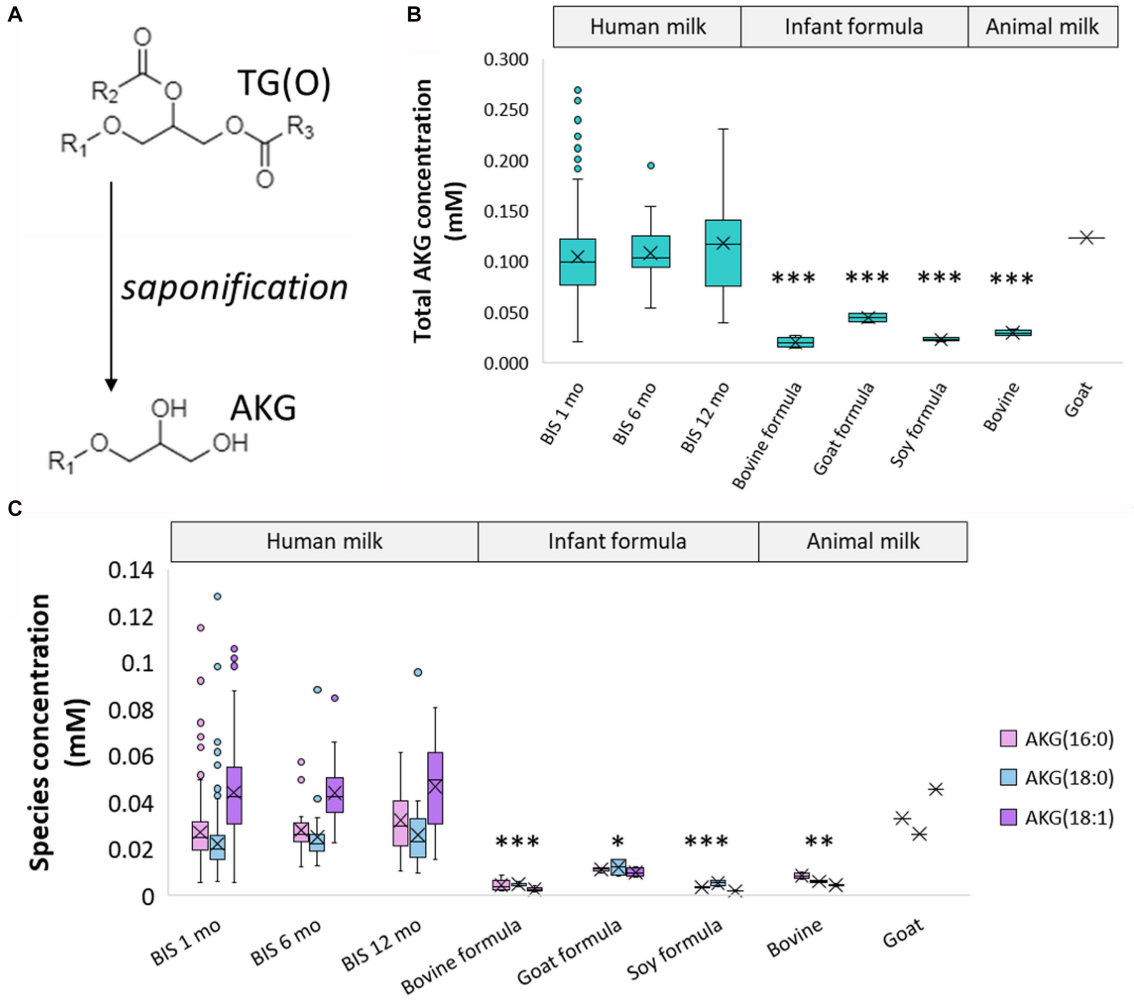

FIGURE 4
Alkylglycerol analysis of human milk TG(O). **(A)** Akyldiacylglycerol (TG(O)) were saponified to alkylglycerol (AKG) for further measurement. **(B)** Total concentration of the alkylglycerol content of human milk, infant formula, and animal milk. Significant differences between human milk and other sample types are represented by *($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$). **(C)** Concentration of the three most abundant AKG in human milk, infant formula, and animal milk. The lowest significant differences between each AKG in human milk and other sample types are represented by *($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$). Boxes are median and lower (25%) and upper (75%) quartile values, crosses are mean values, whiskers are minimum and maximum, and single points are outliers greater than 1.5× upper quartile.

The human milk lipidome differs from that of infant formula, and animal milk, and is rich in ether lipids

Marked lipidome differences were identified between human milk samples and infant formula and animal milk, with distinct separation for both the lipidome (Figure 1A) and the total fatty acid composition (Figure 1B). This was despite the total lipid concentration being similar for human milk and infant formula samples (Figure 2A), which is likely a reflection of energy requirements for formulation of infant food. The clear difference between animal milk and corresponding infant formula is also likely a reflection of preparation processes for infant formula, removing a large portion of the native lipids and/or adding a blend of vegetable oils. This human milk analysis adds many additional species to existing works, including 204 ether lipids from PC(O), PC(P), LPC(O), LPC(P), PE(O), PE(P), DG(O), and TG(O) classes, which are low abundance and thus difficult to measure (2, 10, 20, 26–29). At the lipid class level (Figure 2B), TG comprise the majority of human milk and all infant formula, although the higher proportion of FFA, DG, and MG in human milk may be a result of triacylglycerol lipolysis in human samples (infant formula was made fresh for analysis and does not contain lipase enzymes). Nevertheless, the 'other' portion of the lipidome comprises approximately 1.7% of human milk, and between 0.6 and 1.0% in infant formula (Figure 2C). These other lipids are of high interest as potential bioactive lipids with highly important functional roles in early life, many of which are clearly enriched in human milk compared to infant formula. The substantial differences in early life dietary lipids may contribute to why breastfed infants have increased risk protections when compared to formula-fed infants (6, 28).

Ether lipids are one of the 'other' lipid classes found in much higher (typically more than 10-fold) abundance in human milk than infant formula (Figure 3B). Ether lipids in human milk were first

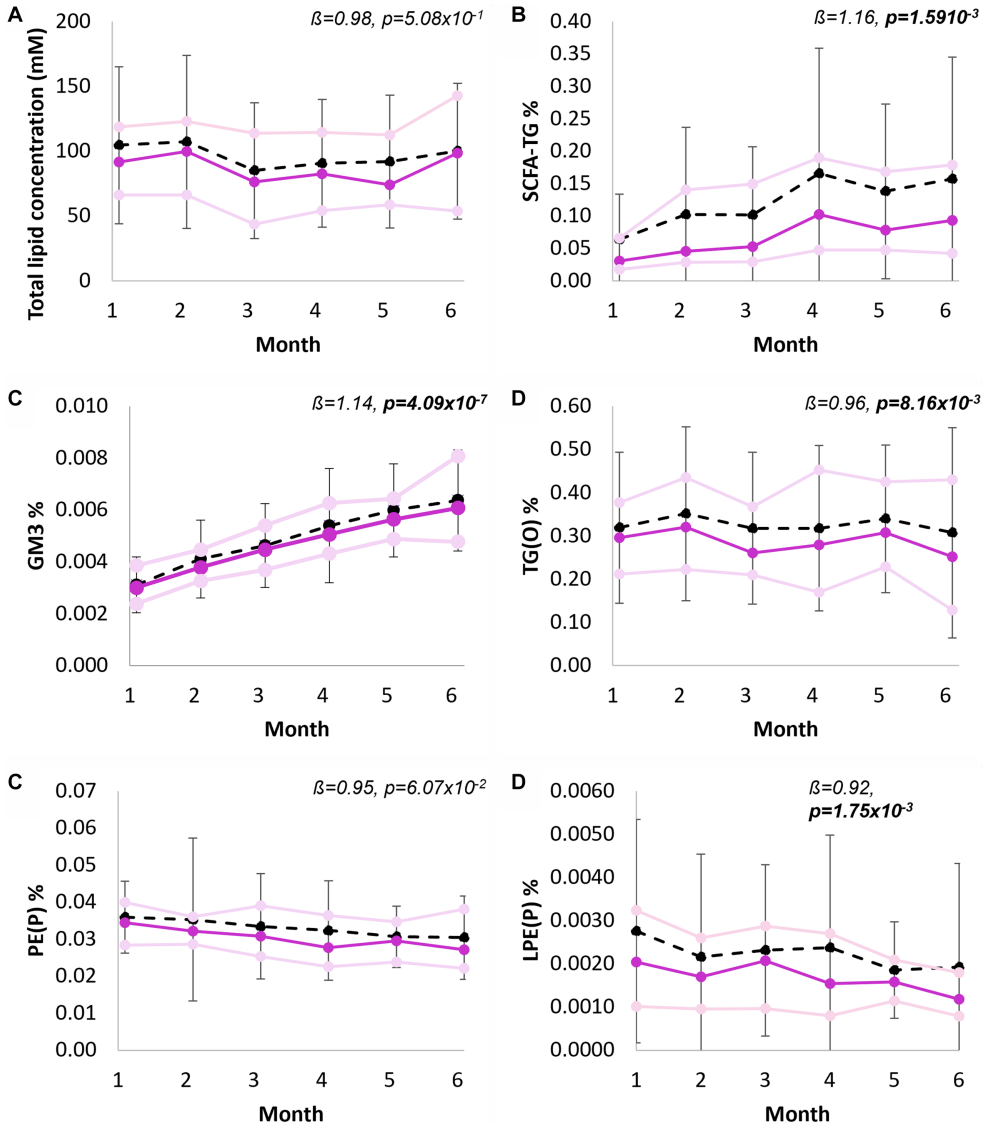

FIGURE 5
Longitudinal trends of human milk lipid classes, from 1 to 6 months exclusive breastfeeding. **(A)** Total lipid concentration of human milk (mM), **(B)** Short chain fatty acid containing TG as percentage of total lipid content, **(C)** Total GM3 gangliosides as percentage of total lipid content, **(D)** Total TG(O) as percentage of total lipid content, **(D)** Total PE(P) as percentage of total lipid content, and **(E)** Total LPE(P) as percentage of total lipid content. Black line indicates mean and standard deviation, purple line indicates median, pink lines indicate first and third quartiles. Interpretation of the beta coefficient is fold change per month, *p* values are FDR corrected ($p < 0.05$ in bold). Percentage of total lipid content was calculated from molar concentrations.

identified in human milk in the 1970s, and have been the subject of increasing interest in early life research in recent years (13, 28, 30–32). In the limited studies that have previously covered ether lipids, species are commonly presented as peak area and/or relative to other species, not quantified. Previous work in BIS has shown that breastfeeding is positively associated with 90% of infant circulating lipids at 6 months of age, including ether lipids, some of which were up to 19-fold higher in breastfed infants than formula-fed infants at 6 months of age (18). Alkyldiacylglycerols (TG(O)) are the major ether lipid class (Figures 3A,C), which make up approximately 75% of the ether lipid composition and approximately 0.4% of the total human milk lipidome, but only <0.1% of the total lipids in infant formula (Figure 2C). To accurately quantitate the AKG composition of TG(O), samples were saponified (Figure 4), and AKG(18:0), AKG(16:0), and AKG(18:1) containing TG(O) were the most abundant, these three AKG species have previously been identified in similar concentrations in a single human milk sample (12), among many other long chain saturated and monounsaturated species. In mice, milk ether lipids are broken down into AKG and metabolised to platelet-activating factor by adipose macrophages, activating the IL-6/STAT3 signaling pathway, and impeding the conversion of beige adipose into white adipose tissue in the pups. Shortened presence of beige adipose tissue results in increased white adipose tissue accumulation, leading to a higher risk of obesity later in life. Thus, formula-fed infants may be missing out on many essential AKG that protect against obesity development. Higher amounts of beige adipose tissue have also been measured in breastfed infants, suggesting that this mechanism through which AKG are sustaining beige adipose and impeding early

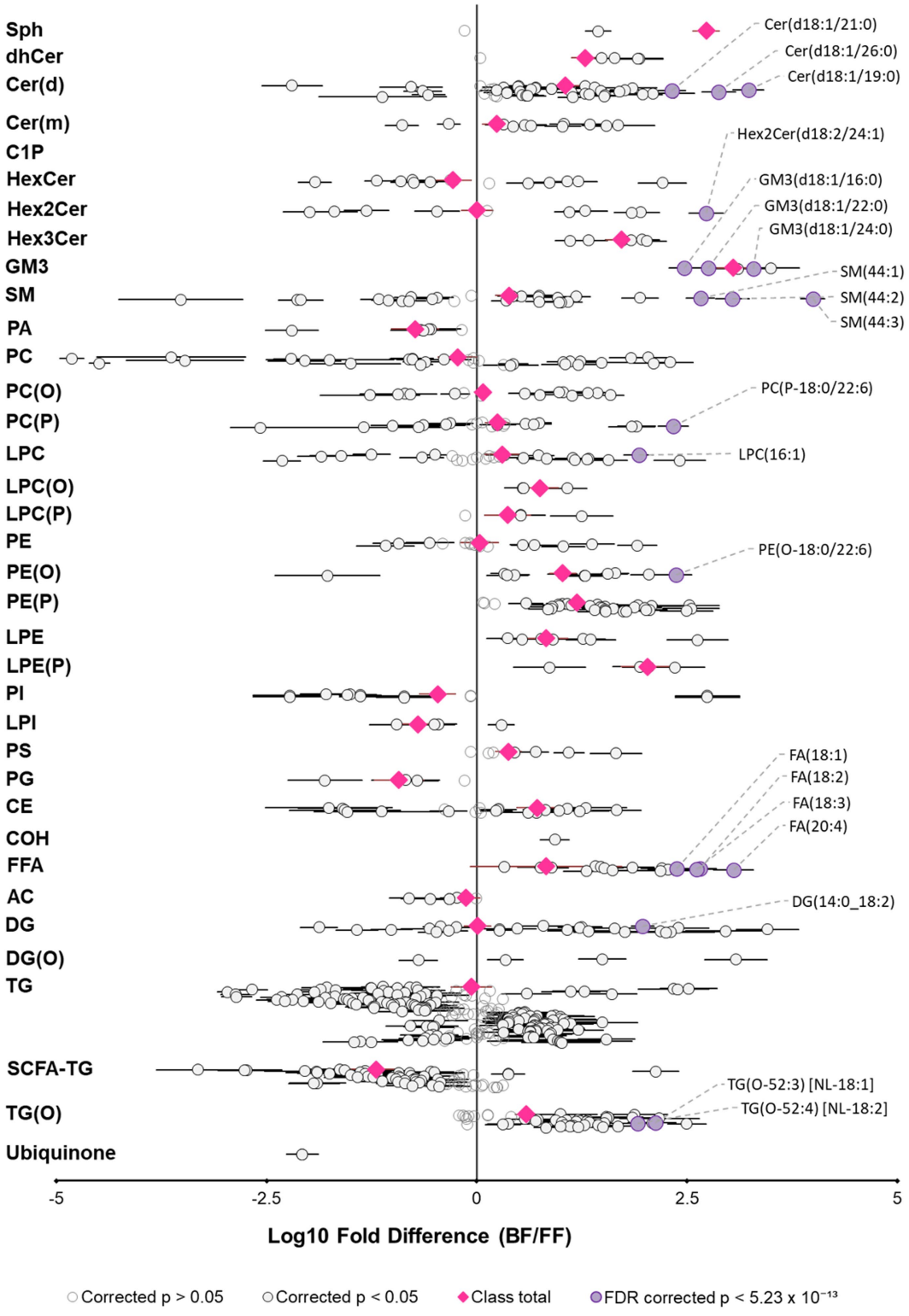

FIGURE 6
Exclusively breastfed and formula-fed infants consume different lipid diets. Fold difference of lipid intake (matched lipids) for exclusively breastfed infants relative to an exclusively formula-fed infant at 3 months of age. Each circle represents an individual lipid species, grey open circles represent $p > 0.05$, white closed circles represent $p < 0.05$, the top 20 lipid species that are higher for breastfed infants are shown in purple and labeled. Pink circles represent lipid class totals. All p values were corrected for multiple comparisons (Benjamini and Hochberg adjustment). Horizontal bars indicate 95% confidence intervals for significant species. Note, plotted species exclude lipids that were not measured in both sample types. Lipid classes are listed in biological order, abbreviations are sphingosine (Sph), dihydroceramide (dhCer), ceramide (Cer(d)), deoxyceramide (Cer(m)), ceramide-1-phosphate (C1P), monohexosylceramide (HexCer), dihexosylceramide (Hex2Cer), trihexosylceramide (Hex3Cer), ganglioside GM3 (GM3), sphingomyelin (SM), phosphatidic acid (PA), phosphatidylcholine (PC), alkylphosphatidylcholine (PC(O)), alkenylphosphatidylcholine (PC(P)), lysophosphatidylcholine (LPC), lysoalkylphosphatidylcholine (LPC(O)), lysoalkenylphosphatidylcholine (LPC(P)), phosphatidylethanolamine (PE),
(Continued)

FIGURE 6 (Continued)
alkylphosphatidylethanolamine (PE(O)), alkenylphosphatidylethanolamine (PE(P)), lysophosphatidylethanolamine (LPE), lysoalkenylphosphatidylethanolamine (LPE(P)), phosphatidylinositol (PI), lysophosphatidylinositol (LPI), phosphatidylserine (PS), phosphatidylglycerol (PG), cholesterol ester (CE), free cholesterol (COH), free fatty acid (FFA), acylcarnitine (AC), diacylglycerol (DG), monoalkyldiacylglycerol (DG(O)), triacylglycerol (TG), SCFA-containing triacylglycerol (SCFA-TG), alkyldiacylglycerol (TG(O)).

accumulation of white adipose, is occurring in humans (12). While the presence of TG(O) in infant formula was somewhat surprising, as it has not been published before, concentrations were significantly (6–10 fold) lower than those in human milk. There were many individual TG(O) species that we found in human milk that were not present in infant formula at all (including TG(O-48:1) and TG(O-54:4)). Further, the most abundant resulting alkylglycerols, AKG(16:0), AKG(18:0), and AKG(18:1) were essentially negligible in formula in comparison (Figure 4C). We also found that, of the species that were present, they were much lower than in human milk (such as TG(O-54:2) which was up to 100-fold lower). Lactating rats supplemented with AKG resulted in milk with higher AKG than those that were not (33). Human supplementation studies have shown that supplementation with specific AKG has significant impact on circulating and cellular plasmalogens, hence it will be important to define the exact functions and roles of specific TG(O) species in order to translate this work into early life supplementation to allow optimal health benefits (34).

Dietary TG(O) are known precursors to plasmalogens. Plasmalogens, alkenyl phosphatidylethanolamines (PE(P)), are the second-most abundant ether lipid class in human milk (Figure 3D). These are highly bioactive lipid species, and their unique structure allows them roles as antioxidants, in cell differentiation, in lipid regulation, and in metabolism. Circulating plasmalogens are lowered in obesity, type 2 diabetes, and other disease states in humans (35–37). Indeed, evidence is emerging on the role of plasmalogens in early life - total PE(P) has been negatively associated with fat mass and positively associated with free-fat mass, and alkenyl phosphatidylcholine (PC(P−18:0/18:0)) has been linked to preterm infants growing on a fast trajectory (13, 27, 31). Plasmalogens are a reservoir of long chain PUFAs, which we found to be the case in human milk also, with species including PE(P−18:1/22:6), and PE(P−16:0/22:4) (32). It is of interest that plasmalogens are abundant in the adult brain, yet relatively low in the newborn brain (38). Studies have shown that formula-fed infants have poorer cognitive outcomes than breastfed infants (39, 40). In formula supplementation studies, addition of DHA and AA, which are typically esterified on TG, did not improve cognitive function to the level of breastfed infants (41). These results suggest that the lipid species that carry the PUFAs are critical to ensure they contribute to the appropriate signalling mechanisms, propelling the need to understand the role of plasmalogens in early life and those present in human milk and breastfed infants.

While ether lipids were higher in human milk than in infant formula, the high variation exhibited between individuals was notable, and thus intake was also highly variable between exclusively breastfed infants (Figure 6). The variability of the human milk lipidome has been shown many times, with the lipid profile likely a combination of diet and genetics, as well as the total fat content of the sample (Supplementary Tables S9–S12) (2). While milk synthesis remains somewhat a mystery, lactating cells have been shown to express relevant genes involved in vinyl-ether addition (PEDS1) and fatty acid to fatty alcohol conversion (FAR1 and FAR2), and thus potentially have notable ether lipid synthesis capability (42, 43). It is unlikely that maternal diet contains ether lipids in appreciable amounts, but maternal diet will provide precursors such as PUFAs. Differential synthesis and levels in human milk may also contribute to the unclear relationship between breastfeeding and disease risk, as levels may not be fed to all breastfed infants in sufficient amounts.

Human milk lipids exhibit longitudinal trends

Longitudinal changes in the human milk lipidome are thought to occur to suit the infants' changing needs and have potential biological relevance, as well as implications for sampling in birth cohorts. This is clearly evidenced by the vast concentration increases between pre- and post-feed samples, such as TG species increasing up to 12-fold, adding complication to human milk studies. We identified several differences through lactation, from birth to 6 months, in the UWAC. Colostrum is critical for immune protection and development, and often considered low in lipids and energy, with smaller milk fat globules and high in immune factors and hormones (44). Our findings indicate that in fact, the total lipid content is the same in both colostrum and mature milk, and many potentially bioactive lipids are very high in concentration in colostrum, compared to mature milk at 1 month. Previously, TG(O) have also been identified to be significantly higher in colostrum (28). Though the infant receives a very small volume of colostrum in the first hours to days of life, the highly bioactive functions of these lipids may be critical. Ether lipids have been linked to immunity in adults, having structural and functional importance in immune cells, signposting the possibility of their role in early life immune protection (35, 45, 46).

Significant differences in both lipid concentrations and relative abundance were identified from months one to six of lactation. Indeed, longitudinal changes in human milk composition have been previously identified in bioactive components, including in human milk oligosaccharides (47). Relative abundance of TG(O), for example, did not change throughout lactation (1 to 6 months), while total PE(P) decreased. PE(P) species as a total of PE have been previously shown to decrease (13). While there is little data on lipid digestion, absorption, and metabolism in early life, it is possible that this is due to changes in infant requirement with an evolving gastrointestinal system – as pH decreases, enzymatic activity increases, and the intestinal barrier develops (48). AKG resulting from TG(O) lipolysis would survive even the low pH (as they do in adult supplementation), while PE(P) would be destroyed. In contrast, infant formula composition will not change over lactation. Compositional changes are not only relevant in understanding differences between breastfed and formula-fed infants. Some infants, including preterm infants or those who are very ill, receive donor milk. In Australia, lactating volunteers provide milk which is pooled and provided to these vulnerable infants. Depending on the time of donation, milk may not contain the required bioactive lipids for that infant. The exact

biological relevance of this is yet to be understood, however, because these infants are more vulnerable than their term counterparts, it is an essential consideration.

The human milk lipidome impacts infant circulating lipids

Positive correlations were identified between matched human milk and infant plasma lipids, with ether lipids accounting for 40% of the significantly correlated lipids. Previously, some human milk TG, PE, and FFA species have been correlated with infant circulating PC(O-36:4) (49). Nutrient transfer from mother to infant has been of interest for many other species and ratios (such as DHA and LA), and this novel finding suggests that dietary lipids may impact development of infant circulating ether lipids, which is essential to understand because we know that metabolic physiology is established early in life (7, 50). These results have important implications for infant nutrition and health, as they suggest that increasing the levels of ether lipids in maternal diet could lead to increased human milk ether lipid content and potentially alter infant circulating ether lipids. This could have downstream effects on infant metabolic health and disease risk, as early-life lipid metabolism has been linked to the development of chronic diseases such as obesity and type 2 diabetes later in life (51–53).

Strengths and limitations

To date, this is the most comprehensive human milk lipidomics study, utilising advanced lipidomics methodology to interrogate the complexity of the human milk lipidome. This study included a large sample size of human milk samples ($n = 654$), from both exclusively breastfeeding and mixed feeding dyads, allowing us to capture the variability of the lipidome, and compare it with infant formula and animal milk to explore differences that may contribute to infant health. The study's focus on ether lipids is novel and not able to be conducted in many biological samples due to their low abundance. Furthermore, this is the first example of extensive dietary lipid intake differences in early life, between breastfed and formula-fed infants, and is a novel way to consider potential subsequent health differences.

This research was limited in that health outcomes were not analysed, which is a key next step. Other milk components were also not considered, which may also have health implications. Consideration of a combination of different milk components will be a critical step to fully comprehend human milk composition and the role of human milk and breastfeeding in early life. Although our findings were comparable to the limited work published already, it is important to consider that (1) samples were stored for 24 h (fridge or freezer) prior to being stored at −80°C, and that for some participants this was done in their own home thus samples may have been subject to conditions that influenced lipid composition, (2) that timing between milk samples and infant plasma samples were not optimally timed and infant intake was not able to be considered, and thus correlation analyses were simple and limited, and (3) all study participants were based in Australia, thus potential ethnic, or more likely dietary, differences may mean that these findings are not representative of the entire world.

Conclusion

While many bioactive lipids found in human milk have been identified, there is still much to learn about their specific functions and how they contribute to the differences observed in health outcomes between breastfed and formula-fed infants. Ether lipids, which are present in higher concentrations in human milk compared to formula, may play an important role in infant health. Given the significant differences in the lipidome between human milk and formula, it is not surprising that formula-fed infants do not receive the same protections as breastfed infants. To address this issue, further research is needed to understand the specific role of human milk bioactive lipids in early life, for it to be translated into maternal supplementation, donor milk supplementation, or improvements in infant formula composition. Continuation of this research is essential to ensure that all infants have the best possible start in life.

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 author.

Ethics statement

This study received ethical approval from the Barwon Health Human Research and Ethics Committee (HEC 10/24). Ethics approval was obtained by The UWA Human Research Ethics Office (RA/4/20/4023), and all participants provided informed written consent. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

AG and DG: UWAC project administration. RS, PV, A-LP, and DB: BIS project administration. AG, SP, TW, NM, TD, KH, CG, AN, SB, and PM: methodology. AG and SP: acquisition and analysis. AG, TW, SB, and PM: interpretation. AG, TW, and SB: manuscript writing. AG, SP, TW, NM, TD, KH, CG, AN, DG, TM, RS, PV, A-LP, DB, SB, and PM: manuscript revision. AG, SB, and PM: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DG declares a potential conflict of interest (DG receives an unrestricted research grant from Medela AG, administered by the University of Western Australia). PM declares a potential conflict of interest (PM has licenced plasmalogen precursor supplement IP to Juvenescence 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

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1227340/full#supplementary-material

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Maternal inflammatory, lipid and metabolic markers and associations with birth and breastfeeding outcomes

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Background: Conditions *in utero* influence intrauterine and postnatal infant growth and a few studies indicate that maternal inflammation and insulin resistance might affect birth and breastfeeding outcomes. Furthermore, hormones in human milk (HM) may influence infant appetite-regulation and thereby milk intake, but the associations are less understood.

Objective: (1) To investigate associations between maternal inflammatory, lipid and metabolic markers and birth and breastfeeding outcomes, and (2) to assess predictors of maternal inflammatory, lipid and metabolic markers in pregnancy.

Methods: Seventy-one mother-infant dyads participating in the Mothers, Infants and Lactation Quality (MILQ) study were included in the present study. Fasting blood samples were collected around 28th gestational week, and HM samples at three time points from 1.0 to 8.5 months, where milk intake was assessed using 24-h test weighing. Maternal plasma inflammatory, lipid and metabolic markers included high-sensitive C-reactive protein (hs-CRP), tumor-necrosis factor-α (TNFα), interferon-γ (IFNγ), Interleukin (IL)-6, IL-8, high-, low-, and very-low-density lipoprotein (HDL, LDL, VLDL), total-cholesterol, triglycerides, leptin, adiponectin, insulin, C-peptide, the homeostasis model assessment of insulin resistance (HOMA-IR) and glucose concentration at t = 120 min following an oral glucose tolerance test. Of these, TNFα, IFNγ, IL-6, IL-8, leptin, adiponectin and insulin were also measured in HM samples.

Results: HDL in pregnancy was inversely associated with gestational age (GA) at birth and GA-adjusted birthweight z-score, whereas triglycerides and glucose (t = 120) were positively associated with GA-adjusted birthweight z-score. Higher hs-CRP, VLDL and triglycerides were associated with a higher placental weight. Furthermore, higher HDL, insulin, leptin and HOMA-IR were associated with longer duration of exclusive breastfeeding (EBF). Higher pre-pregnancy BMI was the main predictor of higher levels of hs-CRP, log-TNFα, leptin, insulin, C-peptide, and HOMA-IR.

Conclusion: Maternal lipid and metabolic markers influenced birthweight z-score and placental weight as well as duration of EBF. Furthermore, pre-pregnancy BMI and maternal age predicted levels of several inflammatory and metabolic markers during pregnancy. Our findings indicate that maternal lipid and metabolic profiles in pregnancy may influence fetal growth and breastfeeding, possibly explained by overweight and/or higher placental weight.

Clinical trial registration: https://clinicaltrials.gov/, identifier NCT03254329.

KEYWORDS

inflammatory markers, lipid markers, metabolic markers, *in utero* programming, pregnancy, breastfeeding, human milk composition

1. Introduction

Since the 1990s, Barkers theory of *in utero* programming has been well-established proposing that malnutrition during critical windows in fetal life predispose the offspring to increased risk of later disease (1, 2). Primarily low birthweight and small abdominal circumference at birth tend to be associated with higher infant total-cholesterol and lipoprotein levels in adulthood (3). These results are independent of gestational age (GA) at birth indicating that restricted fetal growth rather than premature birth affects plasma lipid levels. Through programming mechanisms causing appetite-regulation and energy homeostasis to become dysregulated during restricted intrauterine growth and following catch-up growth (4, 5), the infant is predisposed to an increased risk of later disease such as obesity and type 2 diabetes (6–9). This increased risk may persist throughout the life course and perhaps onto the next generation.

During pregnancy, the fetus has direct access to nutrients from the maternal circulation through the placenta. As such, maternal circulation represents the complete nutrient source for the fetus and concurrently reflects maternal nutritional and health status. Furthermore, obesity during pregnancy is associated with increased levels of C-reactive protein (CRP), interleukin (IL)-6, tumor-necrosis factor-α (TNFα) and leptin (10, 11). Higher levels of cytokines and CRP in women with normal weight or slight overweight have independently been associated with low birthweight (12, 13). Additionally, Swanson and colleagues found among an American population, that the increase in pre-pregnancy body mass index (BMI) from 1995 to 2004 was positively correlated with the increased placental weight in the same period (14). While a healthy pregnancy involves a slight increase in cytokine levels altering insulin sensitivity for the benefit of the growing fetus (15, 16), an excessive increase in these hormones might influence birth outcomes with unintended consequences possibly mediated through effects on the placenta.

Lower breastfeeding rates are seen for overweight and obese mothers and one of the possible explanations includes altered inflammatory and hormonal profiles which may interrupt breastfeeding (17, 18). Gestational diabetes mellitus (GDM) has additionally been proposed as an important risk factor for delayed or unsuccessful breastfeeding (19, 20) and even mild gestational hyperglycemia as a result of a healthy pregnancy may predict a shortened duration of breastfeeding (21). In Denmark, in the period of inclusion for the present study, GDM was diagnosed by a two-hour glucose level of ≥9.0 mmol/L following a 75 g oral glucose dose, i.e., an oral glucose tolerance test (OGTT) (22). Additionally, a homeostasis model assessment of insulin resistances (HOMA-IR) can identify pregnant women at risk of developing GDM, with higher HOMA-IR increasing the risk of GDM (23). Ley et al. reported positive associations between fasting glucose and HOMA-IR measured in pregnancy and HM insulin at 95 days postpartum indicating a larger window for a potential effect on the infant (24). Recent studies further indicate that the early cessation of EBF seen for mothers with overweight and/or GDM might be explained by altered glucose homeostasis and subsequent insulin resistance in pregnancy (18, 25, 26). Additionally, Walker et al. recently found increased hs-CRP and TNFα concentrations in HM of mothers with very low compared to normal milk output and suggested that TNFα inhibits fatty acid uptake in the mammary gland resulting in reduced milk production (27). As such, elevated levels of inflammatory, lipid and metabolic markers in pregnancy due to overweight may exert influence via the placenta as well as through the breastfeeding period, however, the evidence within a healthy population is sparse.

We aimed (1) to investigate associations between maternal inflammatory, lipid and metabolic markers and pregnancy and breastfeeding outcomes within a healthy population, and (2) to assess predictors of inflammatory, lipid and metabolic markers in pregnancy. We hypothesize that concentrations of inflammatory, lipid and metabolic markers are associated with birth outcomes such as placenta weight and birth weight z-score and breastfeeding outcomes such as duration of EBF and HM intake.

2. Methods and materials

2.1. Study design and participants

We included a subgroup of participants from the Mothers, Infants and Lactation Quality (MILQ) Study (28). The MILQ study is a multi-center cohort study including 1,000 mother-infant dyads and with the aim of developing reference values for micro-and macronutrient concentrations in HM. Data are collected in four sites (Bangladesh, Brazil, Denmark and The Gambia) of which data from Denmark are used in the present analysis.

Pregnant women less than 28 weeks of gestation were invited to participate, and informed consent was obtained. The study was conducted from February 2018 to December 2019 and took place at the Copenhagen University Hospitals, Rigshospitalet and Hvidovre

Hospital, as well as the Department of Nutrition, Exercise and Sports, University of Copenhagen.

Women were screened according to the following inclusion criteria for the MILQ study; being non-smokers and 18–40 years old with a pre-pregnancy BMI between 18.5 and 29.9 kg/m^2. They should have a low intake of fortified foods and only take vitamin-and mineral supplements recommended by the Danish Health Authorities. They were excluded if they expected twins or had preeclampsia, GDM and/or anemia. The latter was accepted if they were willing to take iron supplements.

2.2. Data collection

Participants attended one physical examination visit during gestational weeks 28–30 (Visit 0, V0), which included fasting blood samples at $t = 0$ and plasma glucose at $t = 60$ and $t = 120$ min following a 75 g oral glucose load (OGTT). Screening of the infants according to MILQ criteria took place 2–3 weeks after birth (Visit 1, V1), and mother-infant dyads followed the protocol of the MILQ study if eligible. The three postpartum examination visits of the MILQ study took place during the periods 1–3.49 months (Visit 2, V2), 3.5–5.99 months (Visit 3, V3) and 6–8.49 months (Visit 4, V4) postpartum (Figure 1). Furthermore, mother-infant dyads were excluded from the MILQ study if they were not exclusively breastfeeding (EBF) at V2, or had ceased breastfeeding at V3.

2.3. Sample collection and analyses

Fasting blood samples were collected at V0 and stored at −80°C until analysis. The pregnancy markers included hs-CRP, TNFα, IFNγ, IL-6, IL-8, LDL, HDL, VLDL, total cholesterol, triglycerides, leptin, adiponectin, insulin and C-peptide and were analyzed at Department of Clinical Biochemistry, Copenhagen University Hospital, Hvidovre, Denmark. The markers hs-CRP, LDL, VLDL, HDL, total-cholesterol, triglycerides, insulin and C-peptide were analyzed using Elecsy Reagents (Roche Cobas®, F. Hoffmann-La Roche Ltd., Rotkreuz, Switzerland), whereas TNFα were analyzed using DRG® TNF-α ELISA Kit (DRG International Inc., United States), IFNγ and IL-8 using the Invitrogen™ Human IFN gamma and IL-8 Ultrasensitive ELISA Kit (Thermo Fisher Scientific Inc., MA, United States), IL-6 using Human IL-6 High sensitive ELISA Kit (eBioscience, Vienna Austria), leptin using SPI-BIO (Montigny Le Bretonneux, France) and adiponectin using ELISA Kit (Sigma-Aldrich Inc., United States); all according to manufacturers' protocol. Plasma glucose following an OGTT was analyzed at the time of blood sampling. HOMA-IR was calculated using fasting insulin (pmol/L) × fasting glucose (mmol/L) divided by 135 (29, 30).

Mature milk samples were collected as full breast expressions using an electric pump and 250 mL collection bottles (Medela Symphony; Medela; Baar, Switzerland). Samples were collected at the three postpartum visits (V2-V4), and time since last meal of the mother and infant was recorded. From the full breast expression, a 30 mL sample was retained in an amber 50 mL polypropylene tube, and the remaining milk was offered for the mother to take home. Whole milk samples (1.5 mL) were mixed, homogenized and aliquoted into 2 mL amber screw cap tubes immediately after collection and frozen (−80°C) until analysis. Milk samples were analyzed for TNFα, IFNγ, IL-6, IL-8 and the hormones leptin and insulin using MSD U-plex immunoassays (Meso Scale Diagnostics, Rockville, United States). Milk adiponectin was analyzed using sandwich enzyme-linked immunosorbent assay and the human adiponectin duoset (DY1065) from R&D (Biotechne, Minneapolis, MN, United States). Samples were diluted 1:2 for inflammatory markers as well as leptin and insulin, whereas dilution was 1:10 for adiponectin. Assays were performed according to manufacturer protocols. Lower limits of detection were 12 pg./mL (leptin), 11 pmol/L (insulin) and 30 pg./mL (adiponectin), 1.0 pg./mL (TNFα), 3.4 pg./mL (IFNγ), 0.7 pg./mL (IL-6), and 0.3 pg./mL (IL-8). For non-detectable (ND) data, half of the lower cut-off concentration of the specific marker was used for statistical analyses. An internal reference sample was prepared by pooling aliquots of 80 samples and included in duplicates on each plate. The obtained values were used to determine assay variability. The intra assay coefficient of variability (CV) for insulin, leptin and adiponectin was 8.5, 9.5, and 11%, and inter assay CV-values were 18, 20, and 29%, respectively. For TNFα, IFNγ, IL-6, and IL-8, the intra assay CV-values were 28, 10, 18, and 8%, respectively, and inter assay CV-values were 66, 22, 18, 8, and 14%, respectively.

2.4. Milk intake

Infant milk intake was estimated at V2-V4 using the 24-h test weighing method and a digital scale (ADE M101000-01; ADE GmbH

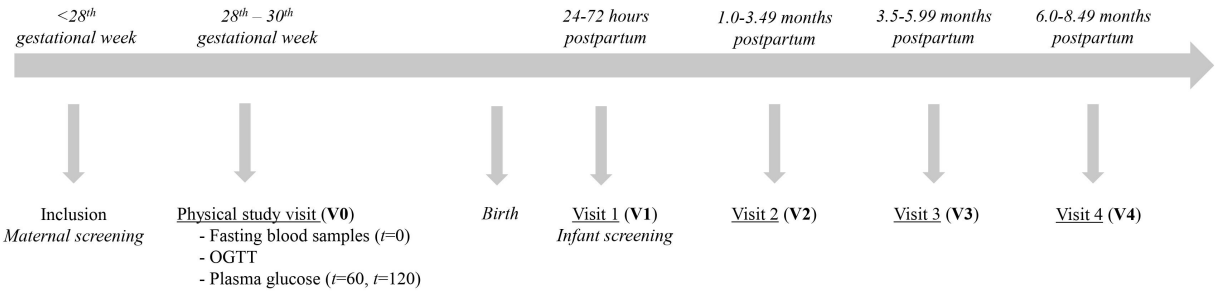

FIGURE 1
Study design and timeline of participation with collection at four study visits. GA = Gestational Age; OGTT = Oral Glucose Tolerance Test.

& Co., Hamburg, Germany) with the accuracy of 5 g for weights <10 kg and 10 g for weights >10 kg. The mothers were instructed to complete the test weighing protocol within the week following each visit by weighing the infants wearing the same clothes before and after each feed for 24 h plus one extra weighing. Total milk intake was defined as intake during the registered period, divided by the number of hours and multiplied by 24. Milk intake per kg bodyweight was estimated by dividing total milk intake with the weight of the infant measured at the visit. Feeds >400 g were regarded as outliers and set to missing, whereas logs with >3 missing feeds were regarded invalid and discarded from analyses. For logs with ≤3 missing feeds, the hot deck imputation method using neighboring weights from the same infant was applied.

2.5. Data from obstetric medical files

The following data were obtained from the medical files at the hospital; infant sex (female/male), birthweight (g), date of birth, placenta weight (g), parity (nulliparous/multiparous), mode of delivery (vaginal, elective/acute cesarean section), assisted births (induction, vacuum extraction), use of epidural or oxytocin during birth (yes/no), blood loss at birth (mL), Apgar score at 5 min.

2.6. Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD) for normally-distributed data and as median and interquartile range (IQR) for non-normally distributed data. Categorical variables are presented as counts and percentages. Normal distribution of data was checked using histograms and Quantile-Quantile plots prior to analyses. Non-normally distributed data were log-transformed prior to analysis and model estimates were back-transformed to percent change for reporting. Collinearity and equal variance of residuals were checked before reporting model estimates.

Our primary analyses included associations between inflammatory, lipid and metabolic markers (pregnancy markers) and pregnancy and breastfeeding outcomes, respectively, whereas our secondary analyses included assessment of maternal predictors of the pregnancy markers.

Linear regression analysis was applied in both the primary and secondary analyses.

Linear regression analysis was applied in the primary analyses with exposures including the pregnancy markers hs-CRP, TNFα, IFNγ, IL-6, IL-8, HDL, LDL, VLDL, total-cholesterol, triglycerides, leptin, adiponectin, insulin, C-peptide as well as a two-hour OGTT and HOMA-IR, while outcomes included GA at birth, placental weight, birthweight z-score and duration of EBF. Birthweight z-scores were calculated using the INTERGROWTH 21st Study software and thus birthweights were adjusted for gestational age at birth (31). Additionally, linear mixed-effect models (with subject ID as random effect) were used to investigate associations between the pregnancy markers and repeated measures of milk intake per kg bodyweight and HM markers (leptin, insulin, adiponectin, TNFα, IFNγ, IL-6, and IL-8) across lactation. Here, interaction terms between the pregnancy markers and visit as well as the pregnancy markers and infant sex were included to test if the associations between the pregnancy markers and milk intake per kg bodyweight and/or HM markers differed between postpartum visits and infant sex. Milk intake per kg bodyweight was chosen over total milk intake to acknowledge the potential driving effect of infant weight on milk intake (32). Models were made separately for each marker and each outcome of interest, and the following covariates were additionally included in the primary analyses: maternal pre-pregnancy BMI, age and parity. Infant sex was further included when investigating placental weight and HM markers across lactation, while infant age was included when investigating duration of EBF as outcome.

For the secondary analyses, maternal pre-pregnancy BMI, age and parity were included as exposures with each pregnancy marker as outcomes, i.e., separate models for each pregnancy marker.

Covariates was chosen *a priori* based on existing evidence, plausible biological explanations and Directed Acyclic Graphs (DAGs) constructed using dagitty.net (33).

Statistical analyses were conducted using R software (version 4.1.3; R Foundation for Statistical Computing) (34). The *lme4*-package was used to construct linear mixed-effect models. A p-values of <0.05 was chosen as the level of significance for additive covariates, whereas $p < 0.1$ were chosen for interactions.

3. Results

Out of the 383 mothers enrolled in the MILQ study, 288 were invited to participate in the substudy. Of these, 194 accepted to receive more information and 82 finally accepted participation and were enrolled. Among these, 11 dropped out resulting in 71 completing the study (attended blood sampling during pregnancy, V0) of which 46 also completed the MILQ study (attended physical examination Visits, V2-V4) (Figure 2). The participants who were excluded due to non-EBF at V2 or no BF at V3 did not differ significantly from the participants completing the MILQ study with respect to the inflammatory, lipid or metabolic markers in pregnancy or birth-related characteristics (data not shown).

Mothers had a mean age of 31.3 ± 4.0 years at inclusion and were healthy with a mean pre-pregnancy BMI of 22.9 ± 2.7 kg/m^2 (Table 1). None of the mothers was clinically diagnosed with GDM (plasma glucose following two-hour OGTT ≥9.0 mmol/L).

Infants were born with a median GA of 40.6 [39.4, 41.3] weeks and a birthweight of 3,638 ± 535 g and with only two born premature (GA 34 and 36). Fifty-six percent were males who were born 1.44 cm [0.21, 2.68] longer than the females, but similar birthweight ($p = 0.12$). Male and female offspring had similar total milk intake ($p > 0.05$), whereas females had a higher milk intake per kg bodyweight compared to males at V2 and V3 ($p \leq 0.018$) (Table 1).

Mean plasma concentrations of inflammatory, lipid and metabolic markers measured in pregnancy are presented in Table 2 together with medians of human milk concentrations of the respective markers. Human milk concentrations of TNFα, IFNγ, IL-6, IL-8, leptin and adiponectin decreased through lactation ($p < 0.05$), while concentrations of insulin remained constant in models only adjusted for infant sex (Table 2).

3.1. Primary analyses

3.1.1. Inflammatory, lipid, and metabolic markers and birth outcomes

Maternal HDL concentrations were inversely associated with GA at birth and infant birthweight z-score, whereas concentrations of

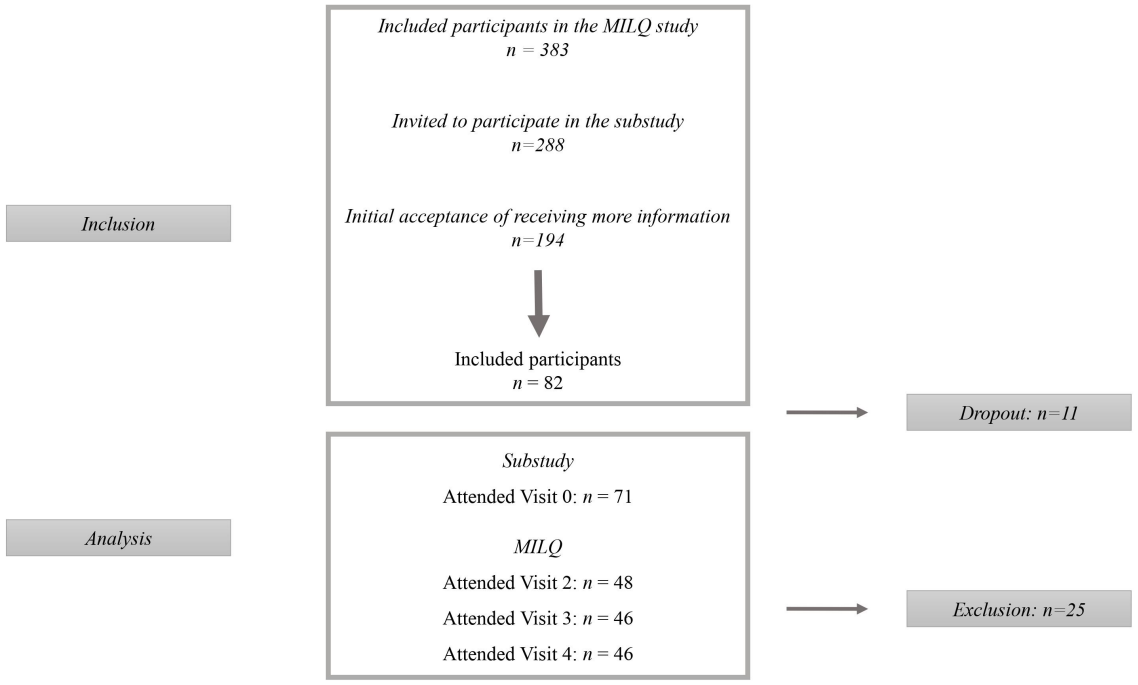

FIGURE 2
Flow diagram of participants. The present study is a sub-study to the Mothers, Infants, and Location Quality (MILQ) study, where participants were invited to participate after inclusion in the MILQ study.

triglycerides and glucose at $t = 120$ were positively associated with birthweight z-score (Table 3). Furthermore, log-hsCRP, log-VLDL and triglycerides were positively associated with placental weight (Table 3). For log-VLDL and log-hsCRP, a 10% increase in VLDL and hs-CRP concentrations resulted in a 2.1 g and 5.0 g increase in placental weight, respectively.

3.1.2. Inflammatory, lipid, and metabolic markers and breastfeeding outcomes

Higher HDL, insulin, leptin and HOMA-IR were associated with increased duration of EBF by 1.0, 0.02, 0.03, and 0.7 month per unit increase in each marker, respectively ($p_{all} \leq 0.048$) (Table 4). Furthermore, an interaction was present between visit*total-cholesterol when investigating human milk intake per kg bodyweight as outcome ($p_{tot\text{-}chol} = 0.062$) resulting in an increase of 8.5 mL per kg bodyweight for every mmol/L increase in total-cholesterol at V2 only ($p = 0.046$), but not at V3 or V4 ($p \geq 0.073$) (Table 4). A similar interaction was initially found between visit and insulin in pregnancy ($p = 0.089$) resulting in a lower milk intake per kg bodyweight per pmol/L increase in plasma insulin at V2 (data not shown). However, the interaction as well as association disappeared when adjusting for infant sex.

3.2. Secondary analyses

3.2.1. Predictors of inflammatory, lipid, and metabolic markers

Maternal pre-pregnancy BMI was positively associated with concentrations of log-hsCRP, log-TNFα, C-peptide, insulin, leptin and HOMA-IR in pregnancy (Table 5). For hs-CRP and TNFα, this resulted in an increase of 10% in both TNFα and hs-CRP, respectively, per 1 kg/m^2 increase in BMI. Maternal age was negatively associated with leptin and insulin, but positively associated with fasting glucose (Table 5). Parity was not associated with any of the markers.

4. Discussion

In this population of healthy women without obesity, we found significant associations between inflammatory, lipid and metabolic markers measured around the 28th week of pregnancy and pregnancy and breastfeeding outcomes. Several of the metabolic markers were significantly related to the birth outcomes placental weight, gestational age and birthweight z-score, whereas hs-CRP was the only inflammatory marker positively associated with placental weight. Several of the metabolic markers were furthermore positively associated with duration of EBF, while total cholesterol was positively associated with HM intake. Among maternal predictors, maternal pre-pregnancy BMI and age, but not lipid markers, were associated with certain inflammatory and metabolic markers.

4.1. Inflammatory, lipid, and metabolic markers and birth outcomes

Higher plasma HDL in pregnancy was associated with lower GA at birth as well as birthweight z-score, whereas higher triglyceride levels were associated with higher placental weight and birthweight z-score. Similarly, Okala and colleagues found lower plasma

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TABLE 1 Participant characteristics.

Maternal characteristics		All (n = 71)
Age, years		31.3 (4.0)
Pre-pregnancy BMI, kg/m^2		22.9 (2.7)
Parity		
Nulliparous		55 (77)
Multiparous		16 (23)
Gestational weight gain (kg)[a]		13.6 (4.6)
Maternal educational level[a]		
Short (<3 years)		9 (19)
Medium (3–4 years)		7 (15)
Long (>4 years)		32 (67)
Birth characteristics		
Induction of labor		17 (24)
Use of oxytocin		21 (30)
Use of epidural		10 (14)
Vacuum extraction		7 (10)
Cesarean section		9 (13)
Acute		6 (8)
Elective		3 (4)
Placental weight, grams		646 (148)
Blood loss, mL		508 (320)

Infant characteristics	Males (n = 40)	Females (n = 31)	All (n = 71)
GA at birth, weeks	40.6 [39.6;41.6]	40.6 [38.9;41.1]	40.6 [39.4;41.3]
Premature birth	2 (5)	0 (0)	2 (2.9)
Birthweight, g	3727 (497)	3522 (568)	3638 (535)
Birthweight z-score	0.7 (0.8)	0.6 (1.2)	0.7 (1.0)
Birth length, cm	52.8 (2.5)	51.3 (2.6)*	52.1 (2.6)
Apgar score, 5 min	10 [7;10]	10 [8;10]	10 [7;10]
Duration of EBF, months[a]	4.7 (0.7)	4.5 (1.2)	4.6 (1.0)
Total milk intake, mL			
Visit 2[a]	791 (149)	789 (88)	790 (125)
Visit 3	829 (252)	871 (147)	848 (210)
Visit 4	660 (197)	675 (205)	666 (198)
Milk intake, mL/kg bodyweight			
Visit 2[a]	131 (24)	157 (26)*	142 (28)
Visit 3	107 (32)	128 (24)*	116 (30)
Visit 4	73 (22)	84 (24)	77 (23)

Values are given as mean (SD), median [interquartile range] or counts (%). [a]Certain data were collected at the postpartum visit, where the sample size was n = 48. *Significant differences between sexes. BMI, Body mass index; GA, Gestational age.

triglyceride levels in gestational week 30 among mothers who gave birth to small-for-gestation (SGA) infants in rural Gambia (35). However, the authors found lower HDL in early and late pregnancy associated with a greater risk of giving birth to infants with low birthweight (LBW), which is contrary to our findings. Similarly, higher HDL has been associated with longer duration of pregnancy among mothers in Ghana (36), which is also contrary to our findings. Furthermore, mothers with infants born SGA and LBW in The Gambia also had lower BMI and lower gestational weight gain (GWG). A study by Ouyang et al. reported increased birthweight z-score among mothers with pre-pregnancy BMI ≥30 kg/m^2 compared to 18.5–24.9 kg/m^2 and among mothers with excessive compared to adequate GWG according to the Institute of Medicine guidelines (37). The authors found that associations attenuated when they adjusted for placental weight and suggest that the placenta might have a mediating effect on the association. Our findings could similarly indicate a mediated effect of placental weight in the positive associations between triglycerides and birthweight z-score. These and our results may reflect a dietary pattern of high-fat and/or high-carbohydrate intakes, which could affect both lipid profile, GWG, and thereby placental weight followed by increased intrauterine growth. Lastly, the conflicting results found in low-and middle-income countries and in the present study could reflect environmental and genetic differences. However, these suggestions are speculative and were not investigated in the present study.

In line with other studies, we found a positive association between two-hour glucose concentrations following an OGTT and birthweight z-score. This association is especially well-documented in studies of mothers with GDM and obesity (38–40). Yuan et al. found maternal factors such as pre-pregnancy BMI, GWG, glucose values at OGTT, HDL and LDL together with other metabolites predicted macrosomia infants in mothers with GDM (41). Although mothers in the present study did not have obesity or GDM, our findings could indicate similar mechanisms occurring across a wider range of maternal weight statuses in our population.

4.2. Inflammatory, lipid, and metabolic markers and breastfeeding outcomes

We found a positive association between total-cholesterol and milk intake per kg bodyweight at V2 and V3, but not at V4. Initially, an inverse associations was found between plasma insulin and milk intake per kg bodyweight at V2 only, but the association disappeared when adjusting for infant sex. Our results may support the findings from a case–control study including 42 mothers, where markers of metabolic health were reported to be worse in mothers with very low milk output (<300 mL/day) compared to nested controls (milk output ≥300 mL/day) and an external control group consisting of exclusively breastfeeding mothers (mean milk output 758 g/day) (42). HOMA-IR, BMI, fasting plasma concentrations of glucose, insulin and C-peptide were higher, whereas concentrations of triglycerides, HDL and prolactin were lower in mothers with extreme low milk output compared to the other groups. Although the sample size in the case–control study is low, these and our results indicate that poorer metabolic health and hormonal imbalance during pregnancy could affect milk production possibly through delayed *lactogenesis II* (26, 43–45). Nommsen-Rivers et al. further showed in a randomized controlled trial, that milk production increased by 60% when intervening with metformin compared to an increase of 20% in the placebo group, although the results were not significant (46). Improvement of milk production correlated strongest with earlier time after delivery and lower baseline milk production, although these results were also

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TABLE 2 Concentrations of inflammatory, lipid and metabolic markers measured in maternal plasma in gestational week 28–30 and in human milk between 1.0 and 8.49 months postpartum.

	Maternal plasma concentrations		Human milk concentrations[b]		
Pregnancy markers	n	V0 (Gestational week 28–30)	V2 (1–3.49 months)	V3 (3.5–5.99 months)	V4 (6–8.49 months)
Inflammatory					
hsCRP (mg/L)	67	2.7 [0.4;4.0]	–	–	–
TNFa (pg/mL)[a]	69	0.15 [0.15;0.35]	2.1 [1.2;2.3]	2.1 [2.1;2.1]	1.3 [0.4;2.1][¤]
IFNγ (pg/mL)[a]	70	0.8 [0.8;0.8]	9.9 [4.9;41.2]	3.8 [0.6;5.8]	2.6 [0.6;6.0][¤]
IL-6 (pg/mL)[a]	69	0.8 [0.5;1.9]	3.6 [2.1;6.4]	1.4 [0.3;2.6]	1.7 [0.5;2.8][¤]
IL-8 (pg/mL)[a]	70	1.4 [0.9;2.7]	146 [61;221]	133 [99;265]	244 [162;354][¤]
Lipid					
HDL (mmol/L)	68	2.0 (0.4)	–	–	–
LDL (mmol/L)	68	3.9 (1.0)	–	–	–
VLDL (mmol/L)	68	0.8 [0.7;0.9]	–	–	–
Total cholesterol (mmol/L)	67	6.7 (1.0)	–	–	–
Triglyceride (mmol/L)	68	1.9 (0.5)	–	–	–
Metabolic					
Leptin (ng/mL)[a]	67	25 (14)	0.13 [0.023;0.35]	0.056 [0.013;0.22]	0.052 [0.013;0.15][¤]
Adiponectin (μg/mL)[a]	68	4.0 (1.1)	0.0027 [0.0022;0.0041]	0.0018 [0.0012;0.0024]	0.0021 [0.0015;0.0030][¤]
Insulin (pmol/L)[a]	62	63.0 (26.4)	170 [129;210]	155 [120;203]	157 [118;207]
C-peptide (pmol/L)	68	693 (163)	–	–	–
Glucose (mmol/L) (OGTT, $t = 0$)	67	4.4 (0.4)	–	–	–
Glucose (mmol/L) (OGTT, $t = 120$)	67	6.0 (1.1)	–	–	–
HOMA-IR	62	2.1 (0.9)	–	–	–

Concentrations are presented as mean (SD) for normally distributed data and as median [IQR] for non-normally distributed data. [a]Half the lower detectable concentration has been used for non-detectable data in plasma and/or human milk. [b]Estimates are based on a reduced sample size ($n = 41–46$) due to fewer completing the MILQ study after birth. [¤]Significant change across lactation. HDL, High-Density Lipoprotein; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; hsCRP, high-sensitive C-Reactive Protein; IFNγ, Interferon-γ; IL, Interleukin; IQR, inter-quartile range; LDL, Low-Density Lipoprotein; SD, standard deviation; OGTT, Oral Glucose Tolerance Test; TNFα, Tumor-Necrosis Factor-α; VLDL, Very Low-Density Lipoprotein.

non-significant. This may indicate that improvement of insulin sensitivity could increase milk production, and that a stronger effect is seen in mothers with lower milk output early after delivery. As mentioned, associations in the present study attenuated when adjusting for infant sex. As males had a significantly lower intake per kg bodyweight, due to a greater weight than females, this difference between the sexes might drive the association between pregnancy markers and infant milk intake. As the sample size is rather small, it is possible that a small group of males with particularly low milk intake per kg bodyweight had mothers with high insulin in pregnancy, which could drive the association. Generally, estimates of milk intake per kg bodyweight in the present study were comparable to estimates recently published in a systematic review and meta-analysis (32), and are therefore likely to be valid. Lastly, lipid metabolism of the mammary glands during various conditions were not investigated and may be an important explanation for altered HM synthesis.

Our results further showed that plasma HDL, insulin, leptin and HOMA-IR during pregnancy were positively associated with duration of EBF. As higher insulin was initially associated with lower milk intake, and lower milk production may shorten the duration of EBF (47), the results are contrary to the expected. However, the participants were well-educated and motivated to breastfeed, which enhances the chances of successfully establishing and continuing breastfeeding. In addition, the mothers were offered breastfeeding counseling throughout the project period to support breastfeeding recommendations. It is plausible that mothers with breastfeeding complications, possibly due to overweight or altered metabolic profiles, might have used the counselors more and thereby overcame any complications resulting in longer duration of EBF.

Finally, plasma leptin in pregnancy was positively associated with HM concentrations postpartum. Similar findings for adiponectin were reported by Ley et al., who found positive associations between serum adiponectin measured in pregnancy and HM adiponectin at both 2 and 95 days postpartum (24). Other studies have shown positive associations between both pre-pregnancy BMI and plasma leptin, respectively, and HM leptin (48, 49), hence our results confirm previous findings.

TABLE 3 Associations between inflammatory, lipid and metabolic markers measured in maternal plasma in gestational week 28–30 and birth outcomes.

Pregnancy markers	(a) Gestational age at birth (weeks)			(b) Placental weight (g)			(c) Birthweight z-score		
	β	95% CI	*p*-value	*β*	95% CI	*p*-value	*β*	95% CI	*p*-value
Inflammatory									
Log-hsCRP	0.14	[−0.47 to 0.75]	0.65	56.38	[3.72 to 109.03]	**0.04**	−0.09	[−0.45 to 0.26]	0.60
Log-TNFα	0.20	[−0.23 to 0.63]	0.36	−1.02	[−40.05 to 38.01]	0.96	0.20	[−0.06 to 0.46]	0.13
Log-IFNγ	0.05	[−0.26 to 0.37]	0.74	−3.79	[−32.32 to 24.74]	0.79	0.04	[−0.16 to 0.23]	0.69
Log-IL6	−0.18	[−0.61 to 0.25]	0.40	−11.06	[−50.16 to 28.03]	0.57	−0.04	[−0.31 to 0.22]	0.74
Log-IL8	0.19	[−0.21 to 0.59]	0.34	4.95	[−33.52 to 43.41]	0.80	−0.06	[−0.31 to 0.19]	0.64
Lipid									
HDL (mmol/L)	−1.14	[−2.12 to −0.15]	**0.03**	−52.17	[−144.03 to 39.69]	0.26	−0.92	[−1.51 to −0.33]	**<0.01**
LDL (mmol/L)	0.06	[−0.32 to 0.44]	0.76	20.23	[−14.54 to 54.99]	0.25	−0.01	[−0.24 to 0.22]	0.93
Log-VLDL	0.01	[−0.21 to 0.24]	0.90	22.17	[1.90 to 42.43]	**0.03**	0.13	[−0.00 to 0.27]	*0.052*
Total cholesterol (mmol/L)	0.00	[−0.40 to 0.41]	1.00	16.32	[−21.73 to 54.36]	0.39	−0.06	[−0.30 to 0.18]	0.63
Triglyceride (mmol/L)	0.30	[−0.43 to 1.03]	0.42	98.51	[36.94 to 160.08]	**<0.01**	0.60	[0.17 to 1.03]	**<0.01**
Metabolic									
Leptin (ng/mL)	0.00	[−0.03 to 0.04]	0.77	1.18	[−1.82 to 4.18]	0.44	0.01	[−0.01 to 0.03]	0.49
Adiponectin (μg/mL)	0.16	[−0.19 to 0.51]	0.37	−15.87	[−47.50 to 15.76]	0.32	−0.20	[−0.41 to 0.01]	*0.065*
Insulin (pmol/L)	−0.00	[−0.02 to 0.01]	0.73	0.10	[−1.63 to 1.83]	0.91	0.00	[−0.01 to 0.01]	0.73
C-peptide (pmol/L)	0.00	[−0.00 to 0.00]	0.61	0.11	[−0.14 to 0.36]	0.39	0.00	[−0.00 to 0.00]	0.21
Glucose (mmol/L) (OGTT, $t = 0$)	0.46	[−0.54 to 1.45]	0.36	−2.77	[−91.86 to 86.32]	0.95	0.55	[−0.04 to 1.15]	*0.067*
Glucose (mmol/L) (OGTT, $t = 120$)	−0.04	[−0.39 to 0.31]	0.82	−8.98	[−40.17 to 22.21]	0.57	0.22	[0.03 to 0.41]	**0.03**
HOMA-IR	−0.01	[−0.48 to 0.46]	0.97	4.62	[−45.48 to 54.73]	0.85	0.11	[−0.23 to 0.45]	0.51

Linear regression models were adjusted for the following covariates: (a) maternal pre-pregnancy BMI, age and parity; (b) maternal pre-pregnancy BMI, age and parity; (c) maternal pre-pregnancy BMI, age, parity and infant sex. Sample size vary between 62 and 70 depending on available data on plasma concentrations and outcome data. *p*-values in bold indicate significance ($p < 0.05$), and *p*-values in italic indicate borderline significance ($p < 0.1$). HDL, High-Density Lipoprotein; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; hsCRP, high-sensitive C-Reactive Protein; IFNγ, Interferon-γ; IL, Interleukin; LDL, Low-Density Lipoprotein; OGTT, Oral Glucose Tolerance Test; TNFα, Tumor-Necrosis Factor-α; VLDL, Very Low-Density Lipoprotein.

4.3. Predictors of inflammatory, lipid, and metabolic markers

Our findings indicate that higher pre-pregnancy BMI and younger maternal age were the main contributors to elevated levels of inflammatory and metabolic markers in pregnancy. In addition, the positive association between hs-CRP and placental weight might be explained by increased pre-pregnancy BMI, which has been shown previously (50). However, concentrations of inflammatory and metabolic markers were similar to those in healthy pregnancies (51–53).

The lack of associations between pre-pregnancy BMI and lipid markers may seem surprising as free fatty acids (FFAs) secreted from

TABLE 4 Associations between inflammatory, lipid and metabolic markers measured in maternal plasma in gestational week 28–30 and breastfeeding outcomes.

Pregnancy markers	(a) Duration of exclusive breastfeeding (months)			(b) Human milk intake (mL/kg)			(c) Inflammatory and metabolic markers in human milk (conc.)		
	β	95% CI	*p*-value	*β*	95% CI	*p*-value	*β*	95% CI	*p*-value
Inflammatory									
Log-hsCRP	0.21	[−0.38 to 0.79]	0.48	−6.81	[−19.70 to 6.07]	0.30	–	–	–
Log-TNFα	−0.06	[−0.51 to 0.39]	0.80	−1.58	[−10.86 to 7.69]	0.74	−0.03	[−0.45 to 0.51]	0.92
Log-IFNγ	0.06	[−0.23 to 0.35]	0.70	1.18	[−4.80 to 7.16]	0.70	−0.20	[−0.51 to 0.11]	0.21
Log-IL6	−0.15	[−0.56 to 0.26]	0.46	0.63	[−8.71 to 9.96]	0.90	−0.11	[−0.50 to 0.28]	0.57
Log-IL8	−0.08	[−0.43 to 0.27]	0.64	−3.06	[−10.77 to 4.65]	0.43	−0.12	[−0.39 to 0.15]	0.39
Lipid									
HDL (mmol/L)	1.03	[0.07 to 2.00]	**0.04**	11.44	[−9.84 to 32.71]	0.29	–	–	–
LDL (mmol/L)	0.06	[−0.26 to 0.38]	0.70	2.79	[−3.97 to 9.55]	0.42	–	–	–
Log-VLDL	0.11	[−0.10 to 0.32]	0.31	1.83	[−2.52 to 6.18]	0.41	–	–	–
Total cholesterol (mmol/L)	0.20	[−0.16 to 0.55]	0.27	8.54	[0.14 to 16.93]	**0.046**	–	–	–
Triglyceride (mmol/L)	0.36	[−0.33 to 1.05]	0.29	4.58	[−10.48 to 19.65]	0.55	–	–	–
Metabolic									
Leptin (ng/mL)	0.03	[0.00 to 0.06]	**0.048**	−0.01	[−0.70 to 0.67]	1.00	0.03	[0.00 to 0.06]	**0.02**
Adiponectin (μg/mL)	−0.08	[−0.37 to 0.22]	0.60	0.86	[−5.21 to 6.93]	0.78	0.05	[−0.17 to 0.28]	0.64
Insulin (pmol/L)	0.02	[0.00 to 0.04]	**0.03**	−0.00	[−0.00 to −0.01]	0.44	−0.00	[−0.01 to 0.00]	0.81
C-peptide (pmol/L)	0.00	[−0.00 to 0.00]	0.12	−0.01	[−0.06 to 0.04]	0.69	–	–	–
Glucose (mmol/L) (OGTT, $t = 0$)	0.50	[−0.43 to 1.42]	0.29	6.75	[−12.03 to 25.52]	0.48	–	–	–
Glucose (mmol/L) (OGTT, $t = 120$)	−0.24	[−0.52 to 0.03]	*0.08*	−0.40	[−6.13 to 5.32]	0.89	–	–	–
HOMA-IR	0.68	[0.17 to 1.20]	**0.01**	1.06	[−9.23 to 11.35]	0.84	–	–	–

Models (a) were linear regression analyses, whereas models (b–c) were linear mixed-effect models with subject ID as random effect. Models were adjusted for the following covariates: (a) maternal age, pre-pregnancy BMI, parity and standardized age at the visit; (b) maternal age, pre-pregnancy BMI and parity and (c) maternal age, pre-pregnancy BMI, parity and infant sex. Sample size vary between 41 and 46 depending on available data on plasma concentrations and outcome data. *p*-values in bold indicate significance ($p < 0.05$), and *p*-values in italic indicate borderline significance ($p < 0.1$). *The estimate is given only for V2 as an interaction was present between total cholesterol and visit. HDL, High-Density Lipoprotein; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; hsCRP, high-sensitive C-Reactive Protein; IFNγ, Interferon-γ; IL, Interleukin; LDL, Low-Density Lipoprotein; OGTT, Oral Glucose Tolerance Test; TNFα, Tumor-Necrosis Factor-α; VLDL, Very Low-Density Lipoprotein.

the excessive adipose tissue are transported to the liver resulting in increased synthesis of triglycerides and VLDL particles, partly in favor of HDL (54, 55). However, as we do not have information on dietary intake or physical activity level at the time of blood sampling, which may have varied substantially depending on the condition of the pregnancy, these factors could have affected plasma lipid concentrations (56, 57). In non-pregnant individuals, higher intakes of, e.g., saturated fatty acids have been associated with higher

TABLE 5 Maternal predictors of inflammatory, lipid and metabolic markers measured in gestational week 28–30.

Pregnancy markers	Pre-pregnancy BMI			Maternal age			Parity		
	β	95% CI	*p*-value	*β*	95% CI	*p*-value	*β*	95% CI	*p*-value
Inflammatory									
Log-hsCRP	0.10	[0.04 to 0.16]	**<0.01**	−0.00	[−0.05 to 0.04]	0.90	0.36	[−0.04 to 0.76]	*0.076*
Log-TNFα	0.10	[0.01 to 0.18]	**0.03**	0.00	[−0.05 to 0.06]	0.88	0.41	[−0.16 to 0.97]	0.15
Log-IFNγ	0.10	[−0.01 to 0.21]	*0.068*	−0.00	[−0.08 to 0.07]	0.95	−0.07	[−0.80 to 0.66]	0.85
Log-IL6	0.07	[−0.01 to 0.16]	*0.076*	−0.03	[−0.08 to 0.03]	0.38	0.09	[−0.46 to 0.65]	0.73
Log-IL8	0.04	[−0.05 to 0.12]	0.40	−0.04	[−0.10 to 0.02]	0.24	0.21	[−0.36 to 0.79]	0.47
Lipid									
HDL (mmol/L)	−0.02	[−0.05 to 0.01]	0.24	−0.00	[−0.03 to 0.02]	0.82	0.05	[−0.17 to 0.28]	0.63
LDL (mmol/L)	−0.03	[−0.12 to 0.07]	0.57	0.00	[−0.06 to 0.07]	0.93	0.01	[−0.60 to 0.62]	0.98
Log-VLDL	−0.03	[−0.19 to 0.13]	0.72	0.08	[−0.02 to 0.19]	0.13	0.07	[−0.96 to 1.11]	0.89
Total cholesterol (mmol/L)	−0.05	[−0.14 to 0.05]	0.32	−0.01	[−0.07 to 0.06]	0.85	−0.14	[−0.76 to 0.48]	0.66
Triglyceride (mmol/L)	0.03	[−0.02 to 0.07]	0.29	0.01	[−0.02 to 0.04]	0.56	0.00	[−0.31 to 0.32]	0.99
Metabolic									
Leptin (ng/mL)	2.41	[1.31 to 3.51]	**<0.001**	−1.26	[−2.02 to −0.50]	**<0.01**	5.42	[−1.78 to 12.61]	0.14
Adiponectin (μg/mL)	−0.09	[−0.19 to 0.01]	*0.089*	0.00	[−0.06 to 0.07]	0.90	0.07	[−0.58 to 0.72]	0.84
Insulin (pmol/L)	5.04	[2.95 to 7.13]	**<0.001**	−1.74	[−3.20 to −0.28]	**0.02**	−4.65	[−18.72 to 9.43]	0.51
C-peptide (pmol/L)	33.25	[20.38 to 46.12]	**<0.001**	−7.15	[−16.07 to 1.77]	0.11	−14.39	[−98.98 to 70.20]	0.74
Glucose (mmol/L) (OGTT, $t = 0$)	0.02	[−0.02 to 0.05]	0.35	0.03	[0.01 to 0.06]	**0.02**	−0.03	[−0.26 to 0.21]	0.83
Glucose (mmol/L) (OGTT, $t = 120$)	0.07	[−0.04 to 0.17]	0.24	−0.00	[−0.08 to 0.07]	0.92	−0.19	[−0.85 to 0.48]	0.58
HOMA-IR	0.16	[0.09 to 0.24]	**<0.001**	−0.03	[−0.08 to 0.02]	0.21	−0.17	[−0.68 to 0.33]	0.49

Models included all three covariates: maternal pre-pregnancy BMI (kg/m^2), age (years) and parity (nulliparous vs. multiparous). Sample size vary between 62 and 70 depending on available data on plasma concentrations and outcome data. *p*-values in bold indicate significance ($p < 0.05$), and *p*-values in italic indicate borderline significance ($p < 0.1$). HDL, High-Density Lipoprotein; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; hsCRP, high-sensitive C-Reactive Protein; IFNγ, Interferon-γ; IL, Interleukin; LDL, Low-Density Lipoprotein; OGTT, Oral Glucose Tolerance Test; TNFα, Tumor-Necrosis Factor-α; VLDL, Very Low-Density Lipoprotein.

LDL-cholesterol through increased hepatic LDL secretion and reduced LDL clearance (58), whereas exercise has been shown to increase HDL-cholesterol (59). Additionally, fish oil supplementation was allowed during the project period, which may have reduced triglyceride levels and increased HDL cholesterol (60, 61). Combined with our findings regarding birth and breastfeeding outcomes, it is likely that women who took fish oil supplements were the same women with certain dietary intakes and physical active lifestyles, which contributed to enhanced lipid profiles as well as to a lower placental weight, birth weight z-score, and longer duration of EBF. However, lipid concentrations increase during healthy pregnancies as a results of increased estrogen levels (62–64), and our results were considered within the normal range for pregnant women.

Furthermore, increased FFA secretion from excessive adipose tissue impair insulin sensitivity resulting in reduced glucose uptake in the muscles and a compensatory increase in pancreatic insulin

secretion to maintain normoglycemia (16, 65). This mechanism might be reflected in our results showing higher pre-pregnancy BMI was associated with increased C-peptide, insulin and HOMA-IR, but not plasma glucose concentrations. This indicates that women with moderate overweight may be slightly insulin resistant, measured by HOMA-IR, but additionally compensate by having increased insulin production, measured by insulin and C-peptide. C-peptide is often used as a marker of pancreatic insulin secretion, as it is secreted into the plasma in equimolar amounts as insulin, while also having a longer half-life than insulin making it a more stable marker than plasma insulin (66). However, C-peptide concentrations in the present study are within the normal range of healthy adults, which indicate normal pancreatic insulin secretion as expected. Insulin resistance has further been reported in healthy pregnancies with a reduction in insulin sensitivity up to 27% in the third trimester (67), and it was not determined if the reduction in sensitivity in the present study was within normal range.

Positive associations were found between pre-pregnancy BMI and plasma leptin, which was expected as the adipokines leptin is secreted from adipose tissue (68). Furthermore, leptin secretion is stimulated by increased insulin levels (69), which could be a contributing mechanism in the present study for women with slight insulin resistance.

Finally, maternal age was inversely associated with leptin and insulin, also when adjusting for parity, but positively associated with fasting glucose although effect estimates were small. The latter was expected, as hyperglycemia and insulin resistance increase with age (70).

4.4. Strengths and limitations

The main strength of our study is the data collection covering both pregnancy as well as breastfeeding, and interesting results were found even in healthy mothers with normal-weight and slight overweight. However, factors related to pregnancy may influence the infant both in the short-term, e.g., birthweight and gestational age, but also in the longer term, e.g., through breastfeeding. The challenge lies in disentangling the influence of pregnancy on short-term outcomes from the influence of pregnancy on the long-term outcomes. It is likely that a series of mechanisms affect each other, whereby the outcome of interest is affected cumulatively. As only a few of these mechanisms are confirmed in the literature, caution must be taken when statistically analyzing data, especially to avoid retrieving biased estimates (71, 72). The use of birthweight z-score compared to using birthweight might seem less clinically relevant as most of the infants were born at term. However, as GA is likely to affect birthweight, despite a term birth, GA was considered relevant to adjust for. Adjusting separately for GA was considered inappropriate as GA might be mediating the influence on birthweight, and thus, birthweight z-score was chosen in analyses.

The study holds certain limitations of which the sample size is of most concern as 71 participants were included and only 46 completed the postpartum study. The study might be underpowered to confirm the results, especially regarding outcomes measured in the postpartum period. Furthermore, the effect estimates are relatively small with wide confidence intervals. These aspects reduce the external validity of the findings and the study should be replicated in a larger population in order to increase generalizability of the results. In addition, $n = 3$ had ceased EBF before the age of 3 months, while $n = 2$ were born prematurely (GA < 37 + 0). It is possible that certain associations were driven by a few participants in this small cohort. It could further be relevant to investigate the associations within the groups of normal- versus overweight to support the findings. However, this would require a larger sample size with evenly distributed groups. Furthermore, plasma concentrations of the inflammatory markers TNFα and IFNγ were below the detection limit for 72 and 76% of the samples, respectively. Half of the lower cut-off concentration was therefore used in analyses, which may have resulted in uncertain estimates. In that regards, assays used for HM analyses have not been validated in the HM matrix, neither in the present study nor in existing literature, and thus estimates of HM concentrations might have been affected, whereas associations are less likely to be affected. The high number of analyses additionally introduces a risk of chance findings. Applying correction for multiple testing, e.g., Bonferroni correction, could reduce this risk, however, this was omitted for the explorative purpose of the study. The strength of using several markers for, e.g., lipid profile is the possibility of finding consistent results across several markers. Each marker adds valuable information individually and, when combined, strengthens the findings and thereby the understanding of the underlying mechanisms.

Lastly, it is worth reiterating that the population was healthy pregnant women without obesity. Although certain associations were significant, the effect estimates were small, thus the clinical relevance can be questioned. However, the findings might be of relevance in other populations where associations might be stronger and/or estimates larger.

5. Conclusion

We showed that maternal metabolism during pregnancy was associated with several important birth-related and breastfeeding outcomes in this relatively small cohort of healthy Danish women. Mainly lipid markers were associated with birth outcomes such as birthweight z-score, whereas higher metabolic markers were associated with longer duration of exclusive breastfeeding.

Finally, pre-pregnancy BMI was the main predictor of metabolic markers involved in glucose homeostasis and insulin resistance, which is in accordance with current literature.

Despite the fact that the estimates are marginally significant, the findings provide information that can help to understand mechanisms behind early programming and thereby optimize short-and long-term health of infants. However, further studies are encouraged to confirm the findings and explore the pathways by which the associations occur.

Data availability statement

The datasets presented in this article are not readily available due to them containing information that could compromise research participant privacy/consent. Further inquiries can be directed to the corresponding author. Requests to access the datasets should be directed to SC, sch@nexs.ku.dk.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Capital Region of Denmark. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

LA, KM, SC, CM, AR, TG, and KR participated in designing and conducting the study. HF analyzed HM samples. SC and JL analyzed data statistically and wrote the manuscript. SC had primary responsibility for the final content. All authors have 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.

Publisher's note

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Glossary

BMI	Body mass index
CI	Confidence interval
CV	Coefficient of variance
DAGs	Directed acyclic graphs
EBF	Exclusive breastfeeding/Exclusively breastfed
GA	Gestational age
GDM	Gestational diabetes mellitus
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
HM	Human milk
Hs-CRP	High-sensitivity C-reactive protein
IFNγ	Interferon gamma-γ
IL	Interleukin
IQR	Inter quartile range
LDL	Low-density lipoprotein
MILQ	"Mothers, Infants and Lactation Quality" study
ND	Non-detectable
OGTT	Oral glucose tolerance test
PBF	Partial breastfeeding
SD	Standard deviation
TNFα	Tumor-necrosis factor-α
VLDL	Very low-density lipoprotein
V2, V3, V4	Visit 2, Visit 3, Visit 4
WHO	World Health Organization
WLZ	Weight-for-length Z-scores

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Maternal stress is associated with higher protein-bound amino acid concentrations in human milk

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Background: Maternal stress in the postpartum period affects not only the mother but also her newborn child, who is at increased risk of developing metabolic and mental disorders later in life. The mechanisms by which stress is transmitted to the infant are not yet fully understood. Human milk (HM) is a potential candidate as maternal stress affects various components of HM, e.g., fat and immunoglobulin concentrations. To date, it is unknown whether maternal stress also affects the amino acids (AAs) in HM, even though this nutrient is of extreme importance to child health and development. This study aimed to investigate whether and how maternal stress is associated with the AA composition of HM.

Methods: In this observational cohort study (Amsterdam, The Netherlands), lactating women were recruited in two study groups: a high-stress (HS) group; women whose child was hospitalized ($n = 24$), and a control (CTL) group; women who gave birth to a healthy child ($n = 73$). HM was collected three times a day, on postpartum days 10, 17, and 24. Perceived psychological stress was measured using validated questionnaires, while biological stress measures were based on hair, saliva, and HM cortisol concentrations. HM protein-bound and free AAs were analyzed by liquid chromatography and compared between groups.

Results: Maternal perceived stress scores were higher in the HS group ($p < 0.01$). The concentrations of protein-bound AAs in HM were higher in the HS group compared to the CTL group ($p = 0.028$) and were positively associated with HM cortisol concentrations ($p = 0.024$). The concentrations of free AAs did not differ between study groups and were unrelated to cortisol concentrations.

Conclusion: Findings from this prospective cohort study suggest that maternal stress in the postpartum period is associated with an altered human milk amino acid composition, which could play a role in the transmission of maternal stress effects to her child. The physiological implications of these stress-induced changes for infant development await future research.

KEYWORDS

breast milk, lactation, postpartum stress, amino acid, early life stress

1. Introduction

Maternal stress in the postpartum period not only affects the mother but may also have consequences for her newborn child. It has been shown that maternal stress occurring during this sensitive developmental period is associated with the infant's risk of developing a wide range of disorders, including metabolic and mental health disorders (1–3). Because the prevention of stressful maternal circumstances in the early postnatal period is generally difficult, a better understanding of the processes underlying these detrimental consequences for the infant is needed. Several mechanisms by which transmission of maternal stress to her infant occurs have been suggested, one of them being stress-induced changes in human milk (HM) composition (4, 5).

HM is the optimal source of nutrition for newborn infants (6–8). It is a highly complex fluid, consisting of over 100 different components that are influenced by many different factors (9). It has been demonstrated previously that maternal psychopathology and maternal stress in the postpartum period are associated with an altered composition of fatty acids in HM (10–16). However, whether maternal stress is also associated with changes in other HM nutrients, e.g., amino acids (AAs), is so far unknown. As AAs in HM are critical for infant growth and development and are necessary for almost all infant body processes (17), it is important to understand how they are affected by maternal stress. In addition, previous human studies point toward an effect of stress on AAs in the plasma, and previous animal experimental studies even suggest a change in milk composition under the influence of stress.

In both human and animal plasma, AAs decrease as a result of stress (18–20). As maternal plasma is the source from which AAs are transported into milk, lower maternal plasma AA levels, e.g., due to stress, may likely result in lower AA levels in milk (21). Whereas, human studies addressing this aspect are lacking, two studies in mice have shown that while maternal stress resulted in lower concentrations of AA in maternal plasma and reduced growth of the offspring, the concentrations of the AAs asparagine and alanine were increased in milk (22, 23). In another animal study, maternal stress during lactation lowered methionine levels in the offspring brain and plasma and induced cognitive deficits later in life, which, notably, could be partly counteracted by methionine supplementation in the dam's diet (24). While this suggests that maternal stress lowers methionine levels in milk, which could have important programming effects on brain health, the AA concentrations in milk were not determined in this study (24).

This study aimed to investigate whether maternal stress in the first month postpartum is associated with the fraction of protein-bound AA (BAA), which makes up 90–95% of the AAs, and the free AA (FAA), which comprises a relatively small fraction of the AA in HM (17). We further focused on methionine as a key AA that may play a role in the long-term consequences of early life stress and investigated the associations between maternal stress and this specific AA. A better understanding of potential stress-induced changes in HM AA composition will contribute to our knowledge of how maternal stress can be transferred to the infant.

2. Materials and methods

2.1. Research design and study population

We studied a prospective observational cohort of lactating women who were followed over their first month postpartum, and experienced various amounts of stress. Participants were recruited during pregnancy or within the first 10 days after giving birth, via social media, flyers at midwife practices, or at the maternal or neonatal ward of the Amsterdam University Medical Center (Amsterdam, The Netherlands). Mothers were eligible to participate when they were 18 years of age or older, and if they had the intention to breastfeed their infant for at least the first month after birth. Exclusion criteria were as follows: (1) maternal (gestational) diabetes mellitus, as the glucocorticoid system may be regulated differently (25, 26), (2) maternal use of psychopharmaceuticals or glucocorticoid medication, as this may interfere with questionnaire scoring, glucocorticoid system regulation, and maternal cortisol concentrations (27), and (3) major congenital disease of the neonate and/or a life expectancy of the neonate of <1 month (duration of the study).

To ensure the inclusion of a large enough range of stress levels among the included participants, two groups of women who delivered at term were included: a high-stress group (HS group) and a control group (CTL group). Women were included in the CTL group when they gave birth to a healthy infant at term. Women were included in the HS group when they gave birth to an infant at term who was admitted to the hospital for a minimum of 2 days. Hospitalization of the infant was considered a maternal stressor.

Recruitment took place in The Netherlands between November 2017 and December 2019. Written informed consent was obtained from all participants prior to participation. This study was approved by the Ethics Committee of the Amsterdam University Medical Centre, AMC on 2 May 2017 (METC 2017025, NL59994.018.16) and conducted in accordance with the Declaration of Helsinki.

2.2. Data collection and storage

2.2.1. Study timeline

Figure 1 shows the study timeline. Recruitment took place within the first 10 days postpartum. After the participant's recruitment, a strand of hair was collected for glucocorticoid measurements, and the participants completed a questionnaire about their general health, their pregnancy, and their lifetime stress experiences. The study collected data on 3 collection days: at postpartum (P) days 10, 17, and 24. On these collection days, women collected two saliva samples and three HM samples. After each collection day, the participants filled out a 24-h food recall questionnaire. At the end of the study, participants filled out three questionnaires about their stress experiences during the study period. See the items below for further details on sample collection, measurements, and questionnaires.

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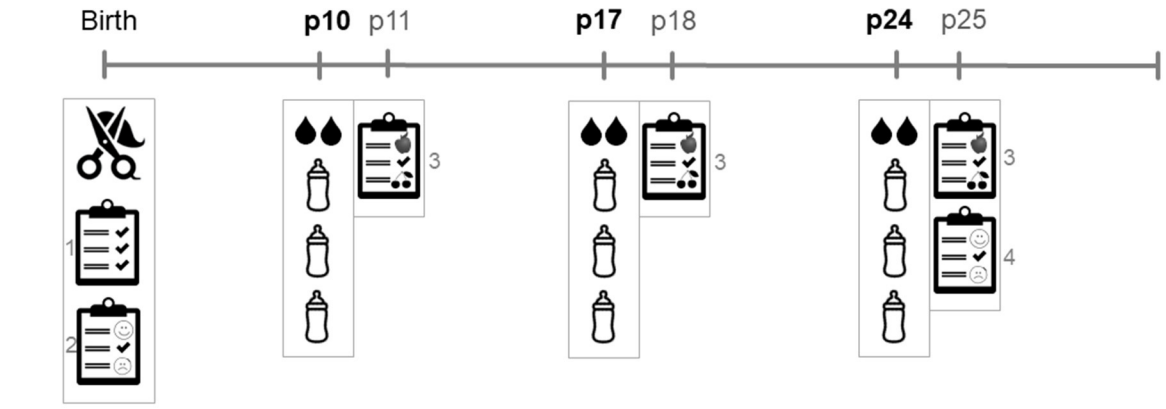

= Maternal hair strand. Collected to measure baseline (cumulative) cortisol concentrations.

= Two saliva samples. Collected in the morning for measurement of cortisol awakening response.

= Human milk sample. Collected in at three times during the day for measurement of amino acids and cortisol.

= Questionnaires.

- 1: General questionnaire about mother-infant characteristics.
- 2: Two questionnaires to measure maternal life time stressors as a baseline of maternal stress: the Life Stressor Checklist- revised and the Youth Trauma Checklist
- 3: 24-hour food intake questionnaire to assess maternal food intake.
- 4: Three questionnaires to measure maternal stress over the study period: the perceived stress scale, the Edinburgh Postnatal Depression Scale and the State-Trait Anxiety Inventory

FIGURE 1
Timeline of study procedures. *p*, postpartum day.

2.2.2. Hair sample collection and storage

A strand of hair (~100 hairs, 3 mm diameter) was cut by the researcher as close to the scalp as possible at the posterior vertex position. The hair was stored in the dark at room temperature until analysis. A short questionnaire was filled out by the participants in order to correct for factors influencing hair glucocorticoid concentrations. Hair samples were analyzed for cortisol and cortisone as a baseline biological stress measurement reflecting the last trimester of pregnancy (28, 29).

2.2.3. Saliva sample collection and storage

Saliva was collected two times in the morning on every collection day to measure the cortisol awakening response. Participants were instructed to chew on a swab (Salivette, Sarstedt, Nümbrecht, Germany) for 1 min. The first sample (S1) was obtained within 0–10 min after waking, and the second sample (S2) was obtained 30–45 min after waking (30). Participants were requested to write down their wake-up time and the time of saliva collection. After collection, saliva samples were sent to the study site, where they were centrifuged, aliquoted, and stored at −20°C until analysis.

2.2.4. Milk sample collection and storage

On every collection day (P10, P17, and P24), participants collected three HM samples in which concentrations of AAs and cortisol/cortisone were measured. HM cortisol/cortisone was measured to be able to directly correlate this with the HM AAs. To take into consideration, the circadian rhythm of HM cortisol and to make sure that circadian variation in HM AAs was represented in the samples (31, 32), participants were instructed to collect one HM sample in the morning, one in the afternoon, and one in the evening on each day that they collected milk. To make sure the HM sample would contain a mixture of both foremilk and hindmilk, participants were requested to fully empty one breast before feeding their infant, mix the milk, and thereafter put 5 ml of HM in a sterile polypropylene container (Sarstedt, Germany) for analysis. Participants were free to choose from which breast the milk was collected. Participants were asked to record the date and time of milk collection, the pumping method used (i.e., manually or with an electric pump), and the total amount of milk collected. Participants stored the milk samples in their freezer (−20°C) up until collection by the researcher. At the study site, HM samples were stored at −20°C until analysis.

2.3. Questionnaires

The questionnaires used in the study are described below. For the analyses, the total questionnaire scores and their ranges were used. To establish the participant's lifetime stress exposure, the participants filled out two questionnaires at the start of the study.

2.3.1. The life stressor checklist-revised

The Dutch version of the LSC-r questionnaire. This checklist is a 26-item scale to identify exposure to traumatic events or other stressful life events (33). Each item questions whether a certain event happened in the participant's life (34).

2.3.2. The youth trauma checklist

The Dutch version of the JTV questionnaire (25 items) is a self-reported inventory that provides a brief and relatively non-invasive retrospective assessment of early life traumatic experiences (35). The JTV discriminates against five domains of abuse/neglect (physical, sexual and emotional abuse, and physical and emotional neglect).

2.3.3. Twenty-four-hour food recall questionnaire

After each collection day, participants received a 24 h-recall using the digital program Compl-eat^{TM} developed by the Department of Human Nutrition at Wageningen University (the Netherlands) (36). This questionnaire assesses the exact food intake (in grams per day) on the collection day. The Compl-eat^{TM} web-based module was specifically designed for the Dutch population and guides participants to accurately report all foods and drinks consumed during the previous 24 h. Information on AA supplement intake was taken into account in the 24 h-recall. The mean intake for protein and all AAs of the three collection days was calculated and used as a measure of maternal intake over the study period.

A measure of psychological stress levels *during* the study period was obtained by three questionnaires that the participants filled out at the end of the study, concerning the levels of stress they experienced during the past month.

2.3.4. Perceived stress scale

The perceived stress scale is a validated 14-item questionnaire. The questionnaire determines the degree to which certain situations are experienced as stressful (37, 38). Each question is scored on a 5-point Likert scale.

2.3.5. Edinburgh postnatal depression scale

The Dutch version of the well-validated EPDS is a 10-item self-inventory to assess symptoms of depression and/or anxiety in women who recently gave birth (39).

2.3.6. State-trait anxiety inventory

The Dutch version of the STAI is a well-established measure of trait and state anxiety and consists of two parts. The first part of this inventory, the STAI-state (STAI-s), contains 20 items to assess anxiety at this moment, rated on a 4-point intensity scale. The second part, the STAI-trait (STAI-t), contains 20 items and assesses anxiety, in general, rated on a 4-point intensity scale (40).

2.4. Laboratory analysis

2.4.1. Hair cortisol/cortisone

For analyses, the proximal 3-cm hair segment was used. Wash and steroid extraction procedures were performed as described by Stalder et al., with some changes being made to allow analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (41). The lower limits of quantification were below 0.1 pg/mg for cortisol and cortisone. The inter- and intra-assay coefficients of variance were between 3.7 and 8.8%.

2.4.2. Saliva cortisol/cortisone

Cortisol and cortisone in saliva were determined using supported liquid extraction (SLE+) followed by LC-MS/MS detection. Quantification was performed using an isotope dilution, with the limit of quantification being 0.3 nmol/L. The mean intra-assay variation was 6 and 7% for cortisol and cortisone, respectively.

2.4.3. HM cortisol/cortisone

For each HM sample, cortisol and cortisone were determined using liquid extraction followed by SLE+ and LC-MS/MS detective as described earlier by van der Voorn et al. (42). Quantification was performed using an isotope dilution.

2.4.4. Protein-bound AA in HM

For the determination of BAA in HM samples, the three milk samples from 1 collection day were mixed to have a good representation of BAA concentrations during the whole day. Thereafter, 0.20 ml of diluted hydrochloric acid containing 0.5% 2-mercaptoethenol was added to the HM sample and mixed. The present oxygen was removed by flushing the headspace of the tube with nitrogen for 60 s. The protein was hydrolyzed by heating the mixture for 20–22 h. When the mixture was cooled down, 0.20 ml of sodium hydroxide solution, 2.0 ml of demi water, and 0.2 ml of internal standard (Norvaline 20 μg) were added and mixed. The mixture was centrifuged, and a small part of the liquid was filtered over a 0.45 mm polyvinylidene difluoride filter. The peak area of each AA was calculated using the LabSolutions software from Shimadzu and compared to the peak area of the internal standard (Sigma).

2.4.5. Free AA in HM

The mixed HM samples were also used to determine the free AA in HM. An ultra-fast liquid chromatography (UFLC)-based protocol was used. Each 50 μl of milk sample was mixed with 1.0 ml of internal standard solution (2.5 mg/ml of L-norvaline). This mixture was centrifuged, and 25 μl of supernatant was transferred into a sample vial. A pre-column derivatization process was carried out by adding 30 μl of o-phthalaldehyde (OPA) reagent to the vial and mixing three times with a mixing volume of 45 μl. One microliter of this OPA-derivatized sample was injected and analyzed in a UFLC system with fluorimetry to detect the signal.

Standard AA solution Sigma AA-S-18 was used for calibration. To prepare the calibration AA solution, asparagine, and tryptophan

were added in to Sigma AA-S-18 stock solution to reach a concentration of 2.5 μM/ml of each AA. Next, 0.50, 1.0, 2.0, and 5.0 ml of this solution were mixed with 1.6 ml of perchloric acid and further diluted to 50 ml. The calibration AA solution was prepared to OPA derivate as described previously and measured in an UFLC system. The calibration curve was constructed from peak areas and AA concentrations. Response factors for each AA were obtained by an extra analysis of a standard AA solution containing internal standards.

2.5. Statistical analysis

Sample characteristics were described as mean with standard deviation (SD), median with 25th and 75th percentiles (Q1–Q3), or frequencies. To test differences in maternal and infant characteristics (including maternal BMI and infant sex), dietary AA intake over the study period, and stress measurements (questionnaires and cortisol) between both study groups, unpaired Student's *t*-tests (for continuous normally distributed data), chi-square tests (for binary categorical data), Mann–Whitney *U*-tests (for continuous not normally distributed data), or linear mixed models (for data that were measured at multiple time points) were used. The mean of the three 24-h recalls was used to compare the dietary intake of AA between groups, as this reflects the overall intake of the participants during the study period.

The HM cortisol area under the curve (AUC) was calculated to provide a value that better reflects HM cortisol throughout the day, which is known to follow a circadian rhythm (31). Therefore, all HM cortisol values were standardized to 7:00 a.m., 14:00, and 22:00 by regressing the time of collection to cortisol values. For each participant, we then calculated the estimated cortisol value at 07:00, 14:00, and 22:00. To do this, the following formula was used: HM cortisol in mmol/L ± unstandardized regression coefficient of all HM cortisol values * [new (standardized)] time point – real-time point) (43). Subsequently, the HM cortisol AUC for each collection day was calculated as described by Pruessner et al. (44). The cortisol value at 7:00 a.m. was considered the HM cortisol morning peak (31). The highest cortisol value of the two morning saliva samples was considered the saliva cortisol morning peak. When the time of S1 collection was >30 min after waking up, saliva values were excluded.

Analyses were performed separately for BAA and FAA. Before analysis, the HM AAs were categorized into different outcome variables: total AA, essential AA, and non-essential AA. Because AA from one precursor family can be converted into other members of this family, AAs were also grouped into precursor groups: the glutamate precursor group (glutamic acid, glutamine, and arginine), the aspartate precursor group (aspartic acid, methionine, isoleucine, threonine, and lysine), the serine precursor group (serine, glycine), the pyruvate precursor group (valine, leucine, and alanine), the aromatic precursor group (phenylalanine, tyrosine, and tryptophan), and the histidine precursor group (histidine). Methionine was analyzed separately (24).

All outcome variables were checked to see whether they were normally distributed. When variables were not distributed normally, a log transformation was performed. When participants completed <1 full day of sample collection, they were excluded from the final analysis.

To answer the research question whether and how maternal stress affects the BAA and FAA concentrations in HM, the AA outcomes as described above were compared between the HS and CTL groups. As all study time points were taken into account in the comparison, linear mixed models were used to analyze the group differences to control for within-person repeated measures. The analysis was corrected for factors differing between study groups. In addition, we tested whether maternal dietary AA intake during the study period differed between study groups. If the maternal dietary intake of a specific AA statistically differed between groups, the comparison of that specific HM AA was corrected for the maternal intake. As HM AA concentrations differ between the different weeks and stages of lactation, the interaction between the study time point and study group was investigated and reported.

To answer the research question of whether HM cortisol concentrations (cortisol AUCs) are related to HM AA concentrations, we performed a secondary analysis. The relationship between HM AA and HM cortisol AUCs was investigated independently of the study group. This relationship was only investigated for total AA, essential AA, non-essential AA, and methionine in HM. As all study time points were taken along in this secondary analysis, linear mixed models were used to control for within-person repeated measures. The analysis was corrected for potential confounding factors that have been shown to influence the AA composition of HM in the previous literature: AA dietary intake and maternal BMI (17, 45–47). Due to the explorative nature of the study, the statistical analyses were not corrected for multiple testing. To reduce the number of statistical tests and the likelihood of a type 1 error, most of the separate AAs were categorized into their precursor families and analyzed as such. Statistical analyses were two-sided. A *p*-value of <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 27. GraphPad Prism 9 for Windows was used to display the results.

3. Results

3.1. Maternal characteristics, food intake, and stress measures

In total, 86 lactating women were included in the CTL group and 30 in the HS group. Nineteen women stopped participating before the end of the study due to various reasons (see Figure 2 for drop-out reasons), 13 women (15%) in the CTL group, and six women (20%) in the HS group (Figure 2). Characteristics and dietary protein and AA intake of the participants are shown in Tables 1, 2, respectively. Maternal baseline characteristics, including maternal age, BMI, ethnicity, education level, alcohol consumption, smoking, dietary habits, and mode of delivery, did not differ between the study groups. Maternal protein/AA intake during the study period was also similar between both study groups. In addition, the HM pumping method used and the storage time of the samples did not differ between study groups. The only difference between study groups was that mothers in the HS group gave birth to a male infant more often than mothers in the CTL

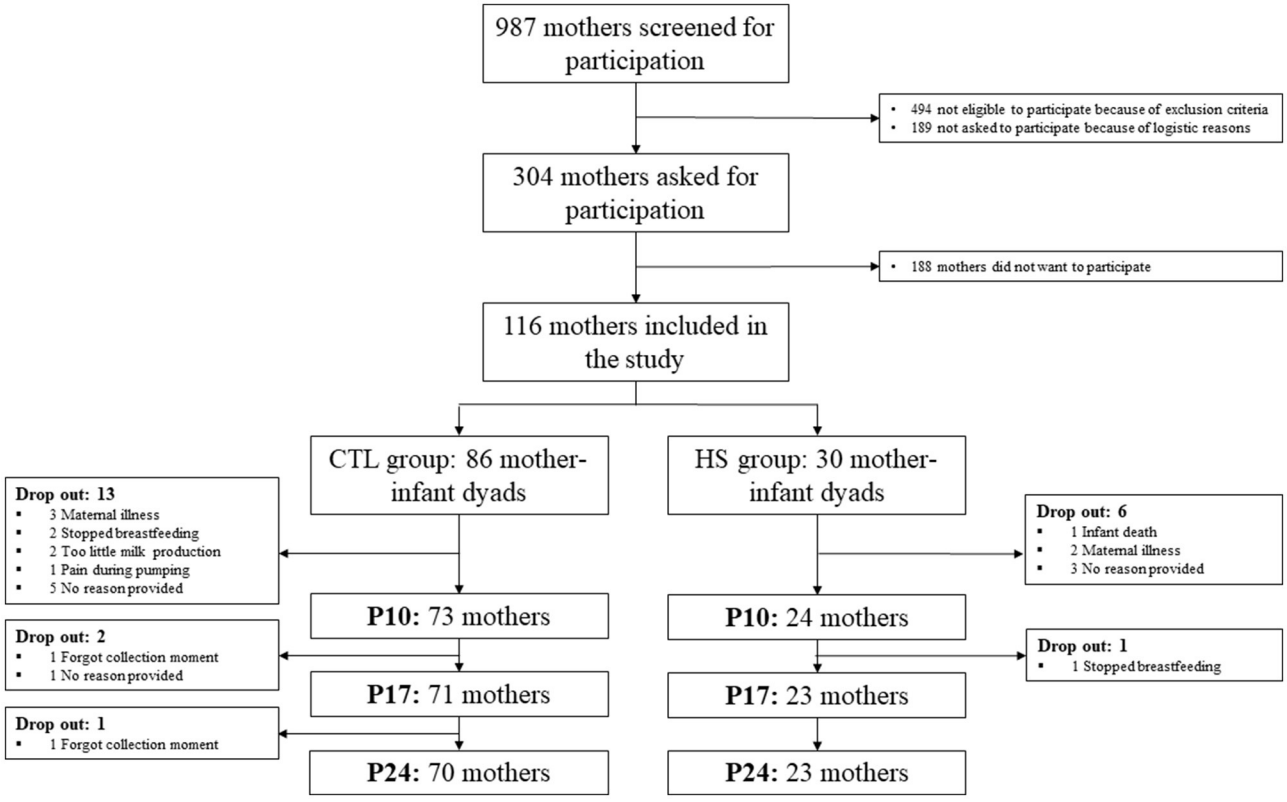

FIGURE 2
Flowchart of the study population. CTL group, control group; HS group, high-stress group; p, postpartum day.

group, 75 and 49%, respectively ($p = 0.048$); the birthweights did not differ between study groups. In the HS group, hospitalization duration ranged between 2 and 12 days, with a median of 7 days. The duration of hospitalization in this group was not associated with maternal stress scores ($p = 0.282$ for PSS score).

Lifetime psychological (JTV and LSC-r questionnaire) and biological stress (hair cortisol) measurements were the same between study groups (Table 3). Perceived stress during the study period was higher in the HS group, and women in the HS groups scored higher on the PSS, EPDS, STAI-s, and STAI-t ($p < 0.01$) (Table 3). There were no differences in the HM cortisol AUCs or the HM/saliva cortisol morning peak concentrations between study groups.

3.2. BAA concentrations in HM are higher in the HS group

Table 4 shows the concentrations of BAAs in HM per study group on all collection days, and Figures 3A, B depicts the BAA dynamics over the study period per study group. Total concentrations of BAAs were higher in the milk of women in the HS group compared to the CTL group [819 (92.4, 1,547); $p = 0.028$]. This was also the case for essential BAAs [476 (55.8, 896); $p = 0.027$] and non-essential BAAs [363 (55.9, 669); $p = 0.021$] and for the concentrations of the BAA precursor groups ($p < 0.035$), except for the glutamate precursor group, which showed the same concentrations in both study groups. There were no interactions between the study group and study time point (Table 3; Figure 3). All statistical analyses were corrected for infant sex.

3.3. FAA concentrations in HM did not differ between study groups

Table 5 shows the concentrations of FAA in HM per study group on all collection days, and Figures 3C, D depicts the FAA dynamics over the study period per study group. Total concentrations of FAA over the entire study group did not differ between the HS and CTL groups, nor did concentrations of essential FAA, non-essential FAA, and the concentrations of the FAA precursor family groups. All statistical analyses were corrected for infant sex.

3.4. Bound methionine does not differ between study groups, free methionine is higher in the HS group

Despite higher concentrations of BAA of the aspartate precursor family in the HS group [255 (46.0, 465); $p = 0.017$], to which protein-bound methionine belongs, protein-bound

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TABLE 1 Maternal and infant baseline characteristics.

	CTL ($n = 75$)	HS ($n = 24$)	p-value
Maternal characteristics			
Age, mean (SD)[a]	32.4 (3.3)	32.5 (3.6)	0.815
BMI (kg/m^2), mean (SD)[a]	23.1 (3.7)	24.3 (5.5)	0.246
Ethnicity %[b]			0.338
Dutch	88.0%	83.3%	
Surinamese	0%	4.2%	
Antillean	1.3%	0%	
Other Western[1]	8.0%	12.5%	
Education %[b]			0.090
Low education[2]	1.3%	0%	
Medium education[2]	8.0%	29.2%	
High education[2]	86.7%	70.8%	
Smoking %[b]			0.333
Never	65.3%	58.3%	
In the past	26.7%	41.8%	
Yes	1.3%	0%	
Alcohol use %[b]			0.281
Never	82.7%	83.3%	
1×/month or less	4.0%	12.5%	
2–4×/month	8.0%	4.2%	
Vegan %[b]	2.7%	0%	0.513
Vegetarian %[b]	8.0%	4.2%	0.576
Mode of delivery[b] % cesarean	29.4%	16.7%	0.453
Infant characteristics			
Birthweight (gr), mean (SD)[a]	3,564.2 (487.5)	3399.7 (584.6)	0.176
Sex, % of male[b]	49.3%	75.0%	0.048

[1]Europe, North America, Oceania, Indonesia, and Japan.
[2]Based on the International Standard Classification of Education (ISCED) 2011.
- Low education (ISCED 2011): levels 0–2.
- Medium education (ISCED 2011): levels 3–4.
- High education (ISCED 2011): levels 5–8.

Statistical difference between groups tested using: [a]Student's t-test, [b]chi-square test.
CTL, control group; HS, high-stress group; SD, standard deviation; BMI, body mass index; gr, grams.

methionine concentrations did not differ between the HS and CTL groups. In contrast, free methionine concentrations were higher in the HS group compared to the control group [0.49 (0.21, 0.76); $p = 0.001$]. All statistical analyses were corrected for infant sex.

TABLE 2 Maternal energy, protein, and amino acid dietary intake.

Intake component mean (SD)	Maternal dietary intake		
	CTL ($n = 73$)	HS ($n = 21$)	p-value
Energy total Kcal	2,163 (572)	1,950 (664)	0.192
Energy total Kilojoule	9,073 (2,396)	8,183 (2,780)	0.194
Total protein (gr)	77.1 (22.9)	70.7 (20.6)	0.227
Plant protein (gr)	36.2 (10.4)	30.9 (11.0)	0.059
Animal protein (gr)	41.1 (20.6)	39.9 (15.2)	0.773
Amino acids (mg)			
Total	74,883 (23,438)	67,295 (19,226)	0.138
Essential	33,033 (11,068)	30,133 (9,001)	0.225
Non-essential	41,850 (12,514)	37,162 (10,522)	0.093
Glutamate family	26,222 (7,599)	22,824 (6,744)	0.056
Aspartate family	18,519 (6,632)	17,125 (5,234)	0.319
Methionine	1,642 (603)	1,513 (450)	0.292
Serine family	7,684 (2,320)	7,030 (2,076)	0.224
Pyruvate family	13,382 (4,406)	12,226 (3,574)	0.224
Aromatic family	7,093 (2,298)	6,298 (1,778)	0.100
Histidine family	1,982 (659)	1,792 (577)	0.207

Food intake values are the mean of the three 24-h recall questionnaires, which reflects the overall intake of the participants during the study period.
Kcal, kilocalories; CTL, control group; HS, high-stress group; SD, standard deviation; gr, gram; mg, milligram.
All statistical differences between groups were tested using Student's t-test. Glutamate family members contain glutamine, arginine, and proline. Aspartate family members contain asparagine, methionine, threonine, isoleucine, and lysine. Serine family members contain serine, glycine, and cysteine. Pyruvate family members contain valine, alanine, and leucine. Aromatic family members contain phenylalanine and tyrosine. Histidine family members contain histidine.

3.5. Dynamics of FAAs in HM over the first month of lactation differ between study groups

In general, the BAA concentrations in HM decreased over the study period ($p < 0.001$), while the FAA concentrations increased ($p = 0.019$). As indicated in Table 5, for total FAA, there was an interaction between the study group and study time point. This was the same for essential and non-essential FAAs, as well as glutamate, pyruvate, and aromatic precursor groups. The different dynamics over the study period between the HS and CTL groups are depicted in Figures 3C, D. All statistical analyses were corrected for infant sex.

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TABLE 3 Maternal perceived stress scores and cortisol values between study groups.

	CTL ($n = 73$)	HS ($n = 24$)	p-value
Questionnaire-based stress scores			
Lifetime stress (test score)			
JTV, median (Q1–Q3)[a]	28.0 (25.0–35.5)	29.5 (27.3–38.8)	0.459
LSC-r, median (Q1–Q3)[a]	6.0 (3–10)	5.5 (2–10)	0.432
Perceived stress during the study period (test score)			
PSS, mean (SD)[b]	16.52 (6.4)	20.48 (6.7)	0.001
EPDS, median (Q1–Q3)[b]	5.0 (2–7)	8.0 (5.8–11)	0.004
STAI-s, median (Q1–Q3)[b]	27.0 (23–34)	36.0 (25–40)	0.005
STAI-t, median (Q1–Q3)[b]	29.0 (26–36)	37.0 (31–40)	0.004
Biological measures of stress: cortisol			
Stress over the last 3 months of pregnancy			
Hair cortisol, median (Q1–Q3)[b]	6.0 (3.1–14.4)	7.1 (5.3–11.6)	0.280
Stress on collection days			
Saliva cortisol (morning peak), median (Q1–Q3)[c]	5.4 (3.4–8.0)	5.7 (3.3–8.9)	0.490
p10[b]	5.5 (3.6–8.3)	5.9 (3.0–10.5)	0.811
p17[b]	5.0 (3.3–8.0)	6.2 (4.4–8.3)	0.274
p24[b]	5.4 (4.0–7.6)	5.2 (2.1–6.1)	0.268
Human milk cortisol AUC, median (Q1–Q3)[c]	52.0 (36.9–72.1)	64.0 (41.0–91.2)	0.074
p10[b]	59.1 (41.2–81.4)	69.3 (40.7–86.4)	0.465
p17[b]	46.2 (35.3–70.9)	57.1 (44.6–93.2)	0.088
p24[b]	51.0 (36.7–70.6)	62.9 (41.0–112.8)	0.282

CTL group, control group; HS group, high-stress group; JTV, Dutch version of the Youth Trauma Questionnaire; LSC-r, life stressor checklist revised; PSS, perceived stress scale; EPDS, Edinburgh Postnatal Depression Scale; STAI-s, State and Trait Anxiety Inventory (state); STAI-t, State and Trait Anxiety Inventory (trait); Q1–Q3, 25th to 75th percentiles; SD, standard deviation; HM, human milk; AUC, area under the curve.
Statistical difference between groups tested using: [a]Mann–Whitney U-test, [b]Student's t-test, [c]linear mixed effects model (correction for within-person repeated measures).

3.6. HM BAAs are positively associated with HM cortisol levels

Total BAA concentrations, as well as essential BAAs and non-essential BAAs, were positively associated with the HM cortisol AUC [5.54 (0.73, 10.35); $p = 0.024$, 2.66 (0.01, 5.30); $p = 0.049$ and 3.07 (0.85, 5.29); $p = 0.007$, respectively]. Protein-bound methionine was not associated with the HM cortisol AUC. Total FAA, essential FAA, non-essential FAA, and free methionine were also not associated with the HM cortisol AUC, at the separate study time points.

4. Discussion

In this study, we explored associations between maternal stress and the AA composition of HM. We demonstrated that perceived and biological maternal stresses in the first month postpartum were positively associated with the concentrations of BAAs in HM. In particular, BAA concentrations were increased in the HM of mothers with high-stress levels, with the exception of bound methionine. Methionine was, contrary to our hypothesis, not associated with maternal stress. However, while the overall concentrations of FAAs in HM did not differ between study groups, free methionine was higher in the HM of mothers with high levels of perceived stress.

Our results are in line with previous animal experimental studies (22, 23). In stressed dams, an increase in some of the milk BAAs was observed, while milk FAAs were overall not affected by stress exposure. To date, human studies on the effect of maternal stress on the AA composition of HM are scarce. One study investigated the influence of maternal postpartum stress on the metabolome of HM, including three AAs, and found that high-stress levels were positively associated with the concentrations of tryptophan and tyrosine. However, after correction for multiple testing, these associations disappeared (16). Another study by Ziomkiewicz et al. found that proteins in HM were not associated with maternal psychological or biological stress (15). This difference may be attributed to the fact that Ziomkiewicz et al. measured whole proteins instead of specific AAs.

The biological mechanism behind the observed associations between maternal stress and HM AA composition is not yet clear. In fact, the process behind the transport of AAs from the maternal bloodstream into the mammary gland and into the HM is complex and not yet fully understood (21). Both BAAs and FAAs in HM can be transported from the maternal circulation, and BAAs can be synthesized out of FAAs by the mammary gland itself (21). It has further been demonstrated, in contrast to our current results in HM, that almost all AAs in the maternal plasma are decreased as a result of maternal stress (18–20). The origin of such a reduction is not fully elucidated. It might be due to the influence of catecholamines produced during stress, which exert an anti-insulin effect on the metabolism of AAs (18–20). As we observed higher concentrations of HM BAAs in the HS group but no association between stress and FAAs in HM, this may indicate an increased active transport of AAs from the maternal circulation into HM and/or an increased synthesis of BAAs in the mammary gland.

Exactly how and to what extent stress influences transport processes also remains to be elucidated. As previous research showed associations between glucocorticoid levels and the expression of certain AA transporters in the mammary gland, e.g., the system L and the system y^+ amino acid transporters, one could hypothesize that cortisol is involved in the mechanisms leading to these stress-induced changes in HM (21, 48). Indeed, we observed a positive association between cortisol levels and BAA levels in HM,

TABLE 4 Protein-bound amino acids in human milk over the study period per study group.

Amino acids in μg/L, mean (SD)	CTL group			HS group			Difference between study groups
	P10 ($n = 73$)	P17 ($n = 71$)	P24 ($n = 70$)	P10 ($n = 24$)	P17 ($n = 23$)	P24 ($n = 23$)	Estimate (CI)
Total	11,539 (1,534)	10,238 (1,158)	9,792 (1,337)	12,442 (2,739)	11,103 (2,917)	10,311 (2,547)	819 (92, 1,547)*
Essential	5,815 (842)	5,247 (649)	4,960 (708)	6,357 (1,632)	5,753 (1,639)	5,234 (1,564)	476 (55, 896)*
Non-essential	5,576 (704)	4,952 (515)	4,779 (636)	5,976 (1,134)	5,309 (1,272)	5,023 (987)	363 (55, 669)*
Glutamate family	2,636 (331)	2,398 (210)	2,357 (293)	2,737 (365)	2,500 (377)	2,412 (287)	92.4 (−15, 200)
Aspartate family	3,142 (432)	2,702 (337)	2,601 (397)	3,387 (806)	2,950 (872)	2,799 (707)	255 (46, 465)*
Methionine	205 (44.9)	180 (28.1)	163 (28.4)	213 (52.1)	200 (58.1)	164 (36.1)	7.07 (−7, 21)
Serine family	1,038 (154)	925 (121)	868 (136)	1,173 (419)	1,044 (414)	951 (399)	124 (23, 225)*
Pyruvate family	3,264 (473)	2,997 (373)	2,797 (389)	3,570 (826)	3,226 (835)	2,893 (811)	235 (17, 454)*
Aromatic family	980 (151)	883 (110)	831 (122)	1,103 (340)	992 (334)	899 (329)	110 (26, 194)*
Histidine family	331 (49.8)	293 (37.9)	284 (42.6)	363 (93.2)	320 (93.5)	303 (85.4)	27.6 (3, 52)*

Protein-bound amino acids in human milk per study group at all collection time points in μg/L.

Statistics: Group comparisons are corrected for infant sex.

*Significantly higher in the HS group ($p < 0.05$). No study group—time point interactions were found. Group differences and interaction were tested using linear mixed effects models to correct for within-person repeated measures.

Glutamate family members contain glutamine, arginine, and proline. Aspartate family members contain asparagine, methionine, threonine, isoleucine, and lysine. Serine family members contain serine, glycine, and cysteine. Pyruvate family members contain valine, alanine, and leucine. Aromatic family members contain phenylalanine and tyrosine. Histidine family members contain histidine.

CTL, control; HS, high stress; p, postpartum day; SD, standard deviation.

which was also found in previous animal experimental research (22). A possible explanation for the fact that cortisol values were not elevated in the HS group but were positively associated with HM BAAs might be that the sample size of the HS group was not sufficient to detect a statistically significant difference. In fact, there was a "trend" toward a higher HM cortisol AUC in the HS group ($p = 0.074$).

We further focused specifically on methionine, as in mice, maternal stress has led to reduced methionine in the brain and plasma of the offspring, which was associated with later-life cognitive deficits (24). Contrary to our hypothesis, bound methionine levels were not decreased in the HM samples in our HS group, and free methionine levels were even higher in mothers with high perceived stress levels. This was an unexpected result, but it can be speculated that the absence of an increase in protein-bound methionine is the result of stress-induced breakdown into free methionine, which would result in an increase in the free form of this essential AA. In addition, another possible explanation for the absence of an increase in protein-bound methionine is that while some transporters of AAs in the mammary gland seem to be upregulated under the influence of cortisol, one of the three transporters that facilitate the transport of methionine into HM is rather downregulated by glucocorticoids (21, 48). Unfortunately, the influence of cortisol on the other two methionine transporters remains so far unknown (21).

Increased concentrations of BAA in the HM of mothers in the HS group may be due to the fact that mothers with high levels of stress produce less milk, in general, compared to mothers with lower levels of stress (49–51). When AA transport into milk is maintained at the same level, the concentrations of BAA in HM will then increase. This might lead to the appropriate transmission of these important nutrients to the infant. On the other hand, this would also mean that the infant might be at risk of receiving inappropriate amounts of methionine, which was not increased in HM under stressful circumstances, as was shown in our results. As we did not measure the total milk volume production during the day or AA concentrations in the infant, this remains speculation and awaits future research.

During lactation, milk-specific proteases break down HM proteins into FAAs. HM further contains protease activators and protease inhibitors (52). Over the first month of lactation, protease inhibitors decrease, which leads to an increase in total FAAs, but subsequently, this also contributes to a decrease in HM BAAs over time (52). Indeed, a decrease in BAA and an increase in total FAA over time were observed for the mothers in our study, except for the concentrations of HM FAAs of the mothers in the HS group, where the total FAA levels decreased over time. The different dynamics of FAA observed in the HS group might be due to a different regulation or production of proteases under the influence of stress. Another explanation for the decrease in total HM FAA under stressful circumstances is that FAAs are more often bound in response to stress, resulting in the increase in HM BAA observed in the HS group.

The strengths of our current study are its longitudinal design and the timing and frequency of HM sample collection. The first month postpartum is a sensitive time window that has been frequently missed in earlier HM research, but since breastfed infants depend on HM during this period as their only source of nutrition these first weeks after birth represent a very important period. Furthermore, the fact that mothers were exposed to

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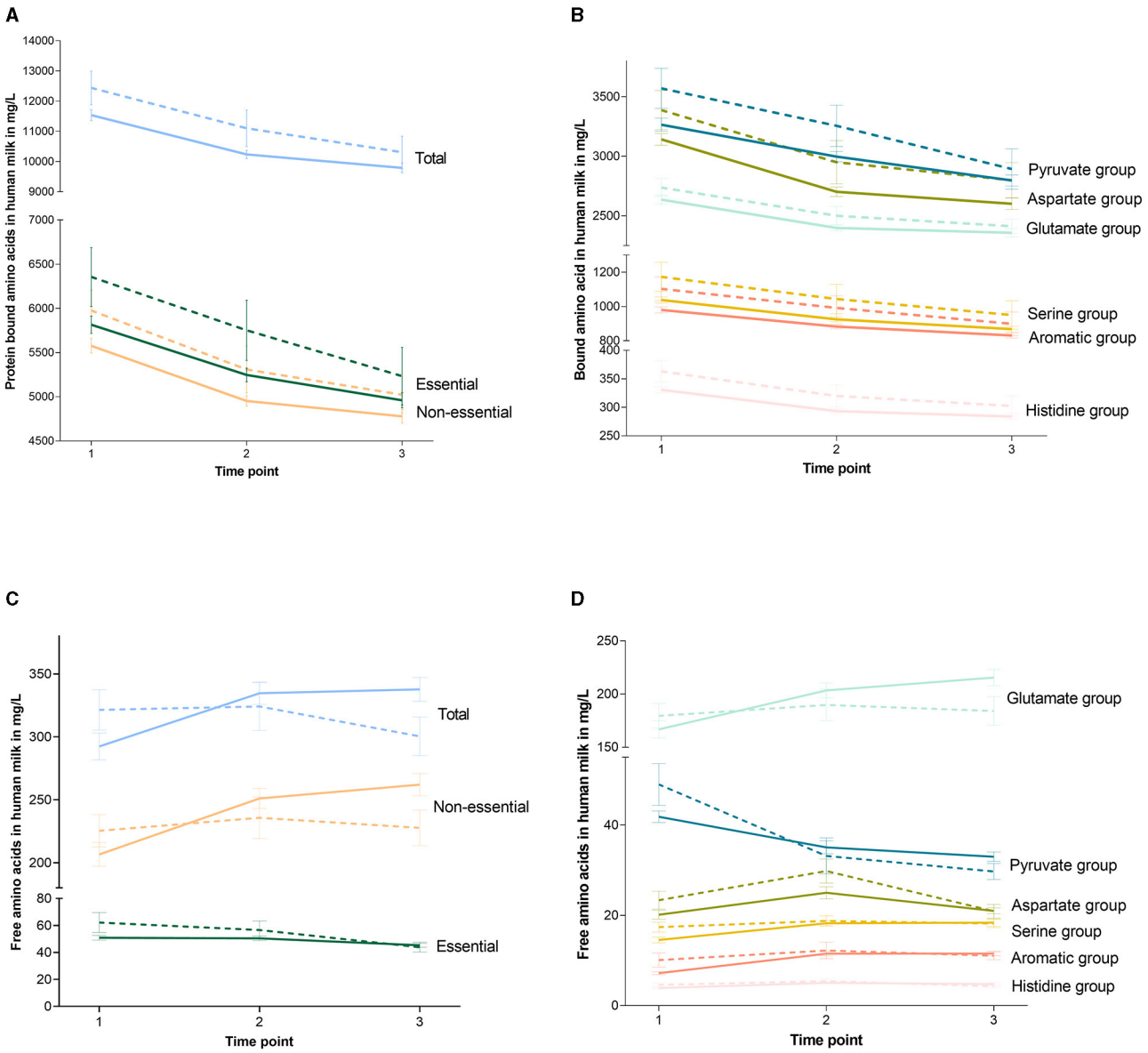

FIGURE 3
Dynamics of amino acids over the study period per study group. Graphs show the concentrations of the protein-bound amino acids **(A, B)** and the free amino acids **(C, D)** at the different collection moments. The continued line indicates the control group and the dotted line indicates the high-stress group. Error bars indicate the standard error of the mean. Glutamate family members contain glutamine, arginine, and proline. Aspartate family members contain asparagine, methionine, threonine, isoleucine, and lysine. Serine family members contain serine, glycine, and cysteine. Pyruvate family members contain valine, alanine, and leucine. Aromatic family members contain phenylalanine and tyrosine. Histidine family members contain histidine.

a stressor (i.e., infant hospitalization) ensured that the study population contained participants with established high levels of perceived stress. Additionally, extensive information on both maternal psychological and biological stress was collected, and the HS and CTL groups were similar at baseline with regard to lifetime stress levels or other important characteristics that could have influenced the relationship between stress and HM AA levels. The limitations of this study are, first, the relatively small sample size, especially in the HS group. Second, current perceived stress scores were only measured once, i.e., at the end of the study. Therefore, we were not able to investigate whether the different dynamics of FAA in the HS group were related to changes in the amount of stress experienced at that exact moment. Moreover, because of the exploratory nature of this study, we decided not to correct for multiple testing, even though we did perform a relatively large number of statistical tests, which increases the likelihood of a type 1 error. Therefore, our results should be interpreted with caution, and future studies should demonstrate whether these findings can be replicated. In addition, no reliable information was collected about infant feeding modes and additional feeding practices. The relatively high loss to follow-up and the fact that this was higher in the HS group compared to the CTL group (19 vs. 23%, respectively)

TABLE 5 Free amino acids in human milk over the study period per study group.

Amino acid in μg/L, mean (SD)	CTL group			HS group			Difference between study groups
	P10 ($n = 73$)	P17 ($n = 71$)	P24 ($n = 70$)	P10 ($n = 24$)	P17 ($n = 23$)	P24 ($n = 23$)	Estimate (CI)
Total	292 (91.2)	334 (74.3)	338 (79.2)	321 (78.8)	324 (91.4)	300 (73.8)	−16.1 (−48.4, 16.3)[a]
Essential	50.8 (15.4)	50.4 (13.9)	45.3 (12.9)	62.2 (36.1)	56.6 (32.8)	43.8 (17.4)	2.1 (−4.0, 8.3)[a]
Non-essential	207 (80.5)	251 (66.5)	262 (73.1)	225 (62.9)	235 (79.0)	227 (68.1)	−16.5 (−46.0, 13.1)[a]
Glutamate family	167 (68.8)	203 (59.0)	215 (64.9)	179 (57.1)	190 (71.5)	184 (64.9)	−13.8 (−40.2, 12.6)[a]
Aspartate family	20.1 (8.5)	25.0 (11.2)	21.0 (5.7)	23.4 (9.8)	29.8 (12.9)	20.9 (7.2)	1.6 (−1.1, 4.3)
Methionine	1.1 (0.4)	4.1 (5.5)	1.2 (0.8)	1.5 (1.3)	6.6 (7.9)	1.5 (2.1)	0.5 (0.2, 0.8)*
Serine family	14.6 (5.6)	18.3 (4.9)	18.4 (7.2)	17.4 (5.4)	18.8 (5.4)	18.2 (4.3)	1.0 (−1.0, 3.0)
Pyruvate family	41.8 (11.0)	35.1 (12.1)	33.0 (8.9)	49.0 (22.7)	33.2 (19.1)	29.7 (8.5)	−2.2 (−6.3, 1.9)[a]
Aromatic family	7.2 (2.8)	11.5 (4.0)	11.5 (4.0)	10.1 (7.7)	12.2 (8.7)	11.1 (4.2)	0.9 (−0.7, 2.5)[a]
Histidine family	3.9 (1.7)	5.1 (1.3)	4.8 (2.4)	4.6 (1.7)	5.4 (2.6)	4.3 (1.4)	0.2 (−0.4, 0.9)

Free amino acids in human milk per study group at all collection time points in μg/L.

*Significantly higher in the HS group ($p < 0.05$). Group comparisons are corrected for infant sex.

[a]Significant study group—time point interaction ($p < 0.05$).

Statistics: Group differences and interactions were tested using linear mixed effects models to correct for within-person repeated measures.

Glutamate family members contain glutamine, arginine, and proline. Aspartate family members contain asparagine, methionine, threonine, isoleucine, and lysine. Serine family members contain serine, glycine, and cysteine. Pyruvate family members contain valine, alanine, and leucine. Aromatic family members contain phenylalanine and tyrosine. Histidine family members contain histidine.

CTL, control; HS, high stress; p, postpartum day; SD, standard deviation.

may have caused selection bias. Dropout of women who may have been more stressed, especially in the HS group, may have led to an underestimation of associations between maternal stress and AA. Finally, selection bias might have contributed to the fact that our cohort mostly consisted of healthy and highly educated women. Furthermore, the majority of our participants were of Western ethnic background. This may limit the generalizability of the results.

The results of our study suggest that mothers with high-stress levels have increased concentrations of BAA in their milk. The increased concentrations of BAA that we found may partially compensate for the higher nutritional requirements of infants under stressful circumstances. However, as impaired growth and development have been observed in children of mothers with high amounts of stress in the postnatal period, this increase in AA in the HM might not be sufficient to maintain optimal growth and development. Moreover, the increase was not observed for protein-bound methionine or total HM FAA.

In conclusion, findings from this unique prospective cohort study suggest that there is a relationship between maternal stress in the first month postpartum and the AA concentrations of HM during this time. Our findings emphasize the importance of the maternal psychological state during lactation. In the last few years, attention to the prevention of the detrimental consequences of stressful experiences in early life has increased. Stress reduction programs for children and parents have been developed, such as family-integrated care and relaxation therapy using relaxing music (53–59), with one study demonstrating effects on milk composition (59). Future research should replicate our findings, investigate the entire period of lactation, and focus on to what extent these stress-induced changes in human milk composition are of clinical importance for short- and long-term infant development and health.

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 Ethics Committee of the Amsterdam University Medical Centre, AMC. The patients/participants provided their written informed consent to participate in this study.

Author contributions

EN, JG, SR, LS, and AK designed the research. HJ and EN conducted the research. LS, JG, and AK provided essential materials. HJ, JH, and SR analyzed the data or performed statistical analysis. JH, HJ, BK, SR, and AK accessed, verified, and interpreted the data. HJ, SR, and AK wrote the first version of the manuscript. AK had primary responsibility for the final content. All authors critically read and contributed to finalizing the manuscript, had full access to all the

data in the study, and accept responsibility to submit it for publication.

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Conflict of interest

JG is the founder and director of the Dutch National Human Milk Bank and a member of the National Health Council. JG has been a member of the National Breastfeeding Council from March 2010 to March 2020. LS was employed by Danone Nutricia Research.

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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Psychometric testing of the breastfeeding self-efficacy scale to measure exclusive breastfeeding in African American women: a cross-sectional study

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Background: In United States, African American women are the least likely group to breastfeed exclusively compared with Hispanic and non-Hispanic White women. It is crucial to examine the perceived confidence of African American women towards practicing exclusive breastfeeding. Previous studies have examined breastfeeding self-efficacy and other factors influencing exclusive breastfeeding. However, there is no research on exclusive breastfeeding self-efficacy of this population. The purpose of this study was to examine the validity and reliability of the breastfeeding self-efficacy scale to measure exclusive breastfeeding, and the relationship between exclusive breastfeeding self-efficacy and general self-efficacy and demographic variables in African American women.

Methods: Descriptive cross-sectional design was used. A convenience sample of 53 pregnant African American women completed an online survey. Construct and criterion-related validity were assessed and reliability of the breastfeeding self-efficacy scale to measure exclusive breastfeeding (BSES-EBF) was examined using Cronbach's reliability. The general self-efficacy scale measured general self-efficacy. Descriptive statistics, bivariate correlation and non-parametric analyses were performed using statistical package for social sciences (v.28).

Results: The breastfeeding self-efficacy to measure exclusive breastfeeding scale had a Cronbach's alpha score of 0.907. One principal component was extracted from the BSES-EBF scale, with an Eigenvalue of 5.271 and which explained 58.57% of the variance in the instrument. The mean prenatal exclusive breastfeeding self-efficacy of participants was 35.15 ($\pm$7.41) from a range of 9 to 45. Exclusive breastfeeding was significantly associated with general self-efficacy ($r = 0.503$, $p \leq 0.001$) and exclusive breastfeeding intention ($p = 0.034$).

Conclusion: Breastfeeding self-efficacy scale to measure exclusive breastfeeding is a valid and reliable tool to measure exclusive breastfeeding self-efficacy in African American women. African American women had high exclusive breastfeeding self-efficacy (internal motivation). Hence, there is a need to address breastfeeding barriers and provide access to culturally sensitive support (external motivation) to increase exclusive breastfeeding in African American women.

KEYWORDS

African American, cross-sectional, exclusive breastfeeding, self-efficacy, validation, women, prenatal breastfeeding self-efficacy

1. Introduction

Infant nutrition in the first 1,000 days is important, as it is a crucial period of development (1). Failure to provide adequate nutrition during this period may result in adverse health outcomes including diarrhoea, pneumonia, decreased vaccine efficacy (2), and stunting, leading to poor cognitive performance (3). To this end, the World Health Organization recommended exclusive breastfeeding (EBF) for 6 months of life to promote the health of infants (4). In many countries, women who followed this recommendation reported health benefits not just for their infants but for them as well; these include adequate weight gain and absence of hospitalization for infants, weight maintenance, prevention of conception and hormonal imbalance, cardiac disease and cancer, for women (5, 6).

Racial disparities exist in EBF rates in the United States. African American (AA) women are the least likely group to intend (57%), initiate (61%) and maintain breastfeeding (6.4 weeks) compared with Spanish-speaking Hispanic (92, 91%, 17.1 weeks) and non-Hispanic White (77, 78%, 16.5 weeks) women, respectively, Mckinney et al. (7). Indeed, among women who gave birth in 2019, only 19.1% of AA women breastfed exclusively for 6 months compared with 23.5 and 26.9% of Hispanic and non-Hispanic White women, respectively, Centers for Disease Control and Prevention (8). This low rate of EBF among AA women may be attributed to maternal (attitude, breastfeeding self-efficacy) and contextual (socioeconomic status, generational trauma of wet nursing) factors (9, 10).

Breastfeeding self-efficacy refers to a woman's confidence in her ability to breastfeed her infant (11). The role of breastfeeding self-efficacy towards achieving and sustaining both breastfeeding (12, 13) and EBF (14–17) has been established in many studies. While the available instruments for measuring breastfeeding self-efficacy measure all or part of the breastfeeding self-efficacy construct (18, 19), these instruments may not be the most appropriate to predict EBF, especially because Bandura had argued that self-efficacy should be examined using a behaviour-specific approach (20). As such, an instrument that specifically measures EBF self-efficacy may more precisely measure the relationship between EBF self-efficacy and EBF. Prenatal breastfeeding intention has been identified as a strong predictor of breastfeeding and EBF because it reflects maternal sociodemographic characteristics, maternal knowledge, and attitude towards breastfeeding and social norms (21, 22). In addition, learning about breastfeeding (knowledge) may promote a woman's breastfeeding intention, which can turn into a behaviour later (practice) (23). There is a strong relationship between prenatal breastfeeding intention and prenatal breastfeeding self-efficacy. Both variables were found to mediate breastfeeding and EBF duration in first and second child (24). In addition, the prenatal rating of efficacy in preparation to breastfeed scale was highly correlated with breastfeeding intention (19). Similarly, women who planned to breastfeed had higher prenatal breastfeeding self-efficacy scores compared to women who planned to formula feed their infants (25).

Given the low rate of EBF in AA women, it is important to examine EBF self-efficacy and identify its predictors in this population. Several studies reported that AA women have lower prenatal breastfeeding self-efficacy compared to non-Hispanic White women (26). One study also reported that AA women had low postnatal breastfeeding self-efficacy (27). Boateng and colleagues developed a new tool, the breastfeeding self-efficacy scale to measure exclusive breastfeeding (BSES-EBF) (28). The tool, originally validated using a longitudinal design among women in Uganda (28), was adapted and validated using a cross-sectional design among women in Egypt (29). Women in Uganda had a mean BSES-EBF score of 30.65 whereas, about 50.2% of women had high BSES-EBF scores in Egypt (29). For African women, breastfeeding is considered a norm (30). On the other hand, early supplementation is common among AA women because of the generational trauma of wet nursing (9, 31), and the belief that formula is more quality than breast milk (32). This may be a plausible reason for the lower breastfeeding self-efficacy among AA women compared to African women (27). Boateng and colleagues recommended that future studies should test their BSES-EBF tool in a new population (with low EBF rates) and examine the construct validity of the instrument by assessing the true correlation between the BSES-EBF scale scores and related constructs. No study has validated this tool among AA women in the United States; hence this study will fill the research gap. The aim of this study was to assess psychometric properties of the BSES-EBF tool, and the relationship between EBF self-efficacy and general self-efficacy, and demographic characteristics among pregnant AA women. The research questions are: (1) what is the relationship between exclusive breastfeeding intention and exclusive breastfeeding self-efficacy? (2) what is the relationship between parity and exclusive breastfeeding self-efficacy? and (3) what is the relationship between general self-efficacy and exclusive breastfeeding self-efficacy?

Dennis' breastfeeding self-efficacy theory, one of the two most used theories that supported interventions to promote EBF, guided this study (11, 33). The breastfeeding self-efficacy theory originated from Bandura's social cognitive theory (34). Self-efficacy, according to Bandura, is the belief in one's ability to organize and accomplish tasks required to manage prospective situations (20). Self-efficacy comprises outcome expectancy (the perception that a behaviour will produce a specific outcome) and self-efficacy expectancy (belief that one can perform a behaviour that will result in a desired outcome) (20). Thus, to be identified as having self-efficacy, a person must believe that performing a behaviour will result in a desired outcome and be confident in one's ability to perform the behaviour. Dennis proposed that breastfeeding self-efficacy plays an important role in breastfeeding duration and emphasized that it also predicts (a) a woman's decision to breastfeed, (b) the intensity of effort she will expend, (c) probability that she will persevere in her efforts until mastery is achieved, (d) whether she will have self-enhancing or self-defeating thought patterns, and (e) how she will respond emotionally to difficulties (11, 35). In AA women, it is necessary to examine predictors of EBF self-efficacy, considering their low socioeconomic status, generational trauma associated with wet nursing, and other challenges they may face while attempting to breastfeed exclusively.

2. Methods

2.1. Design and setting

Descriptive cross-sectional design was used in the study to collect data from July 8, 2021, to February 13, 2022 (36). Research setting was

Abbreviations: AA, African American; BSES-EBF, breastfeeding self-efficacy scale to measure exclusive breastfeeding; EBF, Exclusive breastfeeding; SD, standard deviation.

the United States, and data were collected online due to Covid-19 pandemic. Research advertisement was posted on RL's website and social media platforms including Facebook, LinkedIn, Instagram, and Twitter. Most of the participants (90%) were recruited from Facebook using ads targeted at the research population.

2.2. Sample

The target population for the study were AA women living in the United States. AA women population were chosen because they have lower EBF rates compared with other minority ethnic groups in United States (37). Inclusion criteria were as follows: (a) English language comprehension: questionnaires were written in English, (b) access to internet: survey was delivered online through UConn Qualtrics, (c) currently pregnant: prenatal exclusive breastfeeding self-efficacy is the outcome variable, and (d) age range 18–50 years: eligible age for provision of informed consent for participation in research in United States is 18 years (38). Sample size for the study was calculated using G*power software (39). The correlation coefficient method used for sample size calculation in a previous validation study was adopted in this study (40). Hence, the correlation between exclusive breastfeeding self-efficacy, measured using BSES-EBF, and exclusive breastfeeding social support (range 0.23–0.47) served as a reference for the sample size calculation in the present study (28). The midpoint of the correlation range is 0.35, hence, to detect a correlation of 0.35 from a two-tailed test, power (1−β) of 0.8 and alpha of 0.05 yielded a sample size of 59.

2.3. Measurements

Independent variables were general self-efficacy and demographic characteristics while EBF self-efficacy was the dependent variable.

2.3.1. Demographic characteristics and infant feeding method

Demographic information in the survey include age, marital status, parity, highest level of education, employment, and intention to breastfeed exclusively.

2.3.2. Exclusive breastfeeding self-efficacy

Exclusive breastfeeding self-efficacy, defined as a woman's confidence in her ability to breastfeed exclusively was the main outcome variable in this study. The breastfeeding self-efficacy scale to measure exclusive breastfeeding (BSES-EBF) (28), which originated from the short form of Dennis' breastfeeding self-efficacy scale (35), was used to measure EBF self-efficacy. BSES-EBF is valid and reliable, with Cronbach's alpha coefficients of 0.82 and 0.85, and 0.77 and 0.79 at 3 months for the Cognitive and Functional sub-scales of the BSES-EBF, respectively, Boateng et al. (28). The instrument contains 9 items on a 5-point Likert scale ranging from 1 (not at all confident) to 5 (very confident) with higher scores indicating greater confidence to practice exclusive breastfeeding. The minimum and maximum scale scores are 9 and 45, respectively. BSES-EBF was positively correlated with exclusive breastfeeding social support ($r = 0.28$, $p = 0.001$) and negatively correlated with depression ($r = -0.14$, $p = 0.05$) (28). In the present study, EBF self-efficacy scores were grouped into three categories: low (0–15), medium (16–30), and high (31–45) scores for descriptive analysis.

We reviewed items on the BSES-EBF to appraise the tool's appropriateness for AA women since the tool was developed in Uganda. As recommended by Boateng and colleagues, the BSES-EBF is suitable for population with low EBF rates (28). AA women have lower rate of exclusive breastfeeding compared to any type of breastfeeding (8). In addition, items on the instrument were written in a simple language that is easily comprehensible for people with formal education. More than half of AA women have at least high school education (41). Therefore, we determined that items on the BSES-EBF are culturally appropriate for AA women.

2.3.3. General self-efficacy

General self-efficacy is the belief in one's ability to cope with stressful situations (42). This variable was measured using the General self-efficacy scale developed by Schwarzer and Jerusalem (43). The general self-efficacy scale is valid and reliable, positively correlated with optimism, negatively correlated with stress and depression, with Cronbach's alpha scores between 0.76–0.9 (43). The instrument contains 10 items on a 4-point Likert scale ranging from 1 (not at all true) to 4 (very true). The minimum and maximum scale scores are 10 and 40, respectively. In the present study, general self-efficacy scores were grouped into two categories: low (0–20) and high (21–40) for descriptive analysis.

2.4. Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (v.28). Seventy-six women responded to the survey however, only 55 women met eligibility criteria, and two women did not provide any response to the questionnaire. One of these two women did not provide a response to the question about provision of informed consent for the study and the other, who provided consent did not answer any question in the survey. Therefore, 53 participants provided data for the study nonetheless, one of the 53 participants had missing responses to five items in the general self-efficacy instrument. Hence data analysis involving general self-efficacy was conducted with 52 complete responses. EBF self-efficacy and general self-efficacy were not normally distributed in our sample (skewed to the left). The Shapiro–Wilk test further revealed significant p-values for both variables, affirming their skewness: $p = 0.03$ and $p = 0.014$ for EBF self-efficacy and general self-efficacy, respectively. Therefore, the relationship between EBF self-efficacy and general self-efficacy was assessed using correlation analysis (Spearman's correlation) while the relationships between EBF self-efficacy and other demographic characteristics were assessed using Kruskal-Wallis' test. Both tests use rank of rather than value of observations in the analyses. Spearman's rank-order correlation is the preferred test when Pearson's correlation test is unsuitable due to non-normality of data (44). Similarly, Kruskal-Wallis' test is the preferred test when one-way ANOVA is unsuitable due to non-normality of data (45). For descriptive statistics, means [standard deviations (SD)] and frequencies (percentages) of variables were computed.

2.5. Ethics approval

The study was approved by the Institutional Review Board at University of Connecticut in May 2021 (approval number: X21-0090). The survey included the information sheet which also contained a question on informed consent. Only participants who provided informed consent were granted access to the survey.

3. Results

3.1. Participant characteristics

The majority of participants were within the age group 18–30 years, had given birth to one or two children (60.4%), and planned to breastfeed exclusively after birth (81.1%) (Table 1). Only 28.4% of participants had a college degree. Early in the study, a comment posted on the Facebook ad warned women not to participate in our study and making reference to the Tuskegee study which may have limited responses to the study.

TABLE 1 Descriptive statistics ($n = 53$).

Characteristics	Frequency (*N*)	Percentage (%)
Age (in years)		
18–30	27	50.9
31–40	25	47.2
41–50	1	1.9
Marital status		
Single	27	50.9
Married	16	30.2
Separated	3	5.7
Divorced	4	7.5
Prefer not to answer	3	5.7
Parity		
0	5	9.4
1–2	32	60.4
3–4	13	24.5
5 or more	3	5.7
Education		
Grades 1–11	4	7.5
High school	1	1.9
High school diploma or GED	10	18.9
Some college	17	32.1
Graduated 2-year college	6	11.3
Graduated 4-year college	10	18.9
Masters	3	5.7
PhD	2	3.8
Employment		
Full-time	18	34.0
Part-time	17	32.1
Unemployed	14	26.4
Student	4	7.5
Exclusive breastfeeding intention		
Formula feed only	2	3.8
Breastfeed only	43	81.1
Formula feed and breastfeed	7	13.2
Undecided	1	1.9
General self-efficacy, mean ± SD	33.56 (4.67)	
Exclusive breastfeeding self-efficacy, mean ± SD	35.15 (7.41)	

3.2. Construct (factorial) and criterion-related validity

Principal factor analysis was conducted to identify latent variable(s) underlying the BSES-EBF scale. Results from the principal component extraction showed that the instrument had only one component that met Kaiser's criterion (Eigenvalue>1) (46, 47). The principal component had an Eigenvalue of 5.271 and explained 58.57% of the variance (Figure 1). All the nine items in the BSES-EBF instrument loaded strongly and positively on the principal component (range: 0.571–0.898) (Table 2). All factor loadings were greater than 0.4, suggesting that all items in the instrument are stable, as such, it was not necessary to repeat reliability analysis (which is required only in cases where items with loadings <0.4 were removed) (48). EBF self-efficacy was significantly associated with general self-efficacy and intention to breastfeed exclusively in this study, implying that the instrument has construct and criterion-related validity, respectively.

3.3. Internal consistency reliability

In the present study, Cronbach's alpha coefficient was used to assess reliability of instruments. BSES-EBF scale and general self-efficacy scale had Cronbach's alpha scores of 0.907 and 0.888, respectively. Cronbach's alpha of 0.7 and above is generally considered acceptable (49, 50). BSES-EBF and general self-efficacy scales are reliable to measure EBF self-efficacy and general self-efficacy in AA women. Because the BSES-EBF items were relatively small, we examined items as a whole, not the sub-scales as in Boateng et al. (28).

3.4. Predictors of exclusive breastfeeding self-efficacy

About 1.9, 20.8, and 77.4% of participants had low, medium, and high EBF self-efficacy scores, respectively. The mean EBF self-efficacy score of participants was 35.16 (SD = 7.41; range 9–45) and the mean general self-efficacy score was 33.56 (SD = 4.67; range 22–40). All independent variables were categorical variables except general self-efficacy. EBF self-efficacy, the dependent variable was a continuous variable. Data were assessed to ensure that the assumptions of one-way ANOVA (51) and bivariate

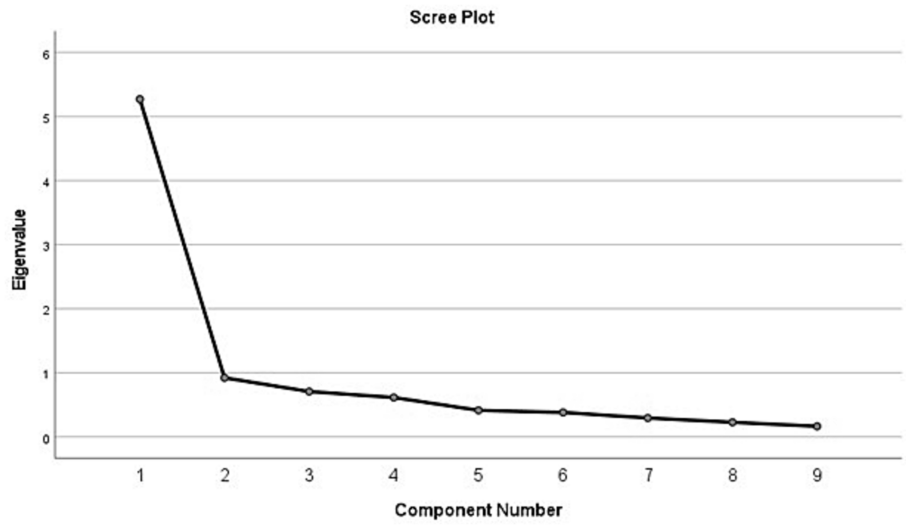

FIGURE 1
Scree plot of the 9-item BSES-EBF scale.

TABLE 2 BSES-EBF items and their principal component factor loadings.

Items	Loadings
I will always know whether my baby is getting enough milk.	0.787
I will always be able to give my baby breast milk without using animal milk, formula, or other liquids or foods as a supplement.	0.771
I will be able to continue exclusive breastfeeding for as long as I want.	0.742
I will always be satisfied with my exclusive breastfeeding experience.	0.757
I will always be able to deal with the fact that breastfeeding can be time consuming.	0.724
I will continue to breastfeed my baby for every feeding.	0.841
I will always be able to keep up with my baby's breastfeeding demands.	0.898
I will always exclusively breastfeed without my baby receiving even a drop of water or any liquid.	0.755
I will always stop someone from trying to feed my baby liquids or foods other than breast milk, including purchased baby foods (e.g., infant formula, milk, porridge, juice, and tea [whatever is given]), before 6 months of age.	0.571

correlation (52) test were met thereafter, these tests were performed in Statistical Package for the Social Sciences (v.28). Exclusive breastfeeding self-efficacy was significantly associated with EBF intention ($p = 0.034$) (Figure 2), and general self-efficacy ($r = 0.387$, $p = p \leq 0.001$) (Table 3), but not associated with parity (Figure 3).

4. Discussion

The present study examined the validity and reliability of the BSES-EBF tool, and the relationship between exclusive breastfeeding self-efficacy and demographic variables. Findings revealed that the BSES-EBF instrument is valid and reliable to measure EBF self-efficacy in AA women. The positive association between EBF self-efficacy and general self-efficacy suggests that the BSES-EBF tool has construct validity. In addition, intention to breastfeed exclusively was positively associated with EBF self-efficacy in AA women, also suggesting that the BSES-EBF has criterion-related validity. At 1 month, Boateng et al. (28) reported Cronbach's alpha coefficients of 0.82 and 0.85, and 0.77 and 0.79 at 3 months for the Cognitive and Functional sub-scales of the BSES-EBF, respectively. Similarly, the adapted BSES-EBF tool also had a Cronbach's alpha coefficient of 0.86 among women in Egypt (29). In the present study, Cronbach's alpha coefficient of 0.907 was reported, suggesting that BSES-EBF is a reliable tool to measure EBF self-efficacy, as a Cronbach's alpha coefficient of 0.7 and above is generally considered acceptable (49, 50).

AA women had high prenatal EBF self-efficacy and general self-efficacy with means of 35.15 and 33.56, respectively. Similar finding was reported in previous studies that examined prenatal breastfeeding self-efficacy in AA women in the United States (12, 25). Conversely, in McCarter-Spaulding and Gore's (27) study, AA women had the lowest postpartum breastfeeding self-efficacy scores compared with other women who identified as Black (African, Cape Verdean, Caribbean) (27). Similarly, compared with non-Hispanic White women, AA women had lower general self-efficacy scores (53). Assari (53) argued that the lower level of education and income of AA population compared with non-Hispanic White population explained the difference in general self-efficacy scores (53). In the present study, many participants (71.7%) had at least some college education, which

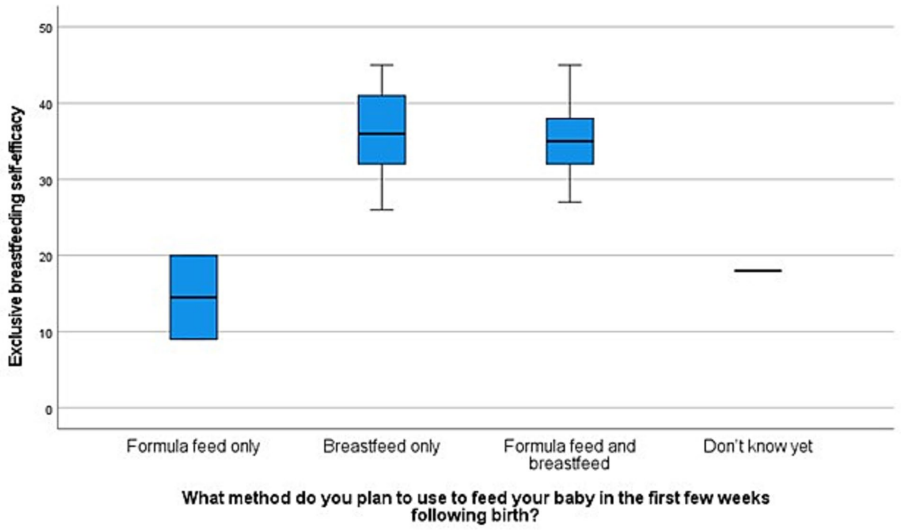

FIGURE 2
Box plot of exclusive breastfeeding self-efficacy scores according to planned feeding method.

TABLE 3 Predictors of exclusive breastfeeding self-efficacy ($n = 53$).

Characteristic	*p*-value
Age	0.374
Marital status	0.377
Parity	0.470
Education	0.912
Employment	0.600
Exclusive breastfeeding intention	0.034*
General self-efficacy	0.001*

*Significant at $p \leq 0.05$.

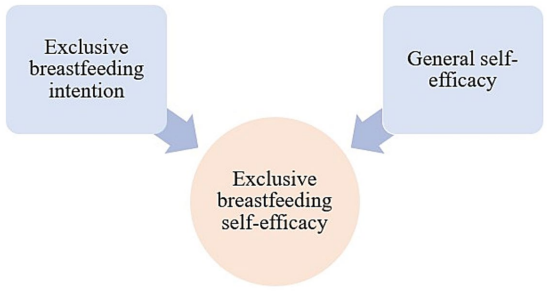

FIGURE 3
Conceptual model of prenatal exclusive breastfeeding self-efficacy predictors in AA women.

may explain the different findings reported in this study compared with Assari's study. Self-efficacy predicts self-esteem and persistence (54, 55); therefore, we may infer that AA women have a high self-esteem which is reflected in their strong determination to breastfeed exclusively. Indeed, in two studies – Ahmed and Rojjanasrirat (56) and Aderibigbe and Lucas (9), women who breastfed exclusively were reported to have strong determination and high breastfeeding self-efficacy (9, 56). The high EBF self-efficacy of AA women may reflect interventions to reduce breastfeeding disparities (57). Most participants (71.8%) had at least some college education, suggesting that more AA women are acquiring college education, similar to non-Hispanic White women, and that women with college education are more likely to participate in research compared with those with lower level of education (58). The level of education of AA women may also explain their high EBF self-efficacy as reported in a previous study (59). Finally, more than half of participants (50.9%) were relatively young, being within the age range of 18–30. The high EBF self-efficacy and general self-efficacy scores may also be attributed to the women's age as younger women were reported to have higher self-efficacy compared to older women (60). The low sample size in the present study should be considered when interpreting inferences from this study.

Most women (81.1%) in this study planned to breastfeed exclusively. Conversely, McKinley and colleagues observed a significantly lower breastfeeding intention in AA women compared with non-Hispanic White women (26). As expected, EBF self-efficacy was significantly associated with general self-efficacy and exclusive breastfeeding intention, however, it was not associated with age, marital status, parity, education, and employment. Conversely, Ahmed et al. reported that EBF self-efficacy was significantly associated with age, education, and employment (29). Further exploration of the association between EBF self-efficacy and intention to breastfeed exclusively revealed that women who were undecided about infant feeding method and those who planned to feed their infants with formula only in the first weeks after birth had the lowest EBF self-efficacy scores.

4.1. Limitations

Preliminary literature review showed that no study has examined EBF self-efficacy and its predictors in AA women. Data collection over 8 months recruited 53 participants thus the sample size limits generalization. Previous studies reported that online

surveys have low response rate compared with telephone or paper-based surveys (61). In addition, we received a comment posted on the Facebook ad warning women not to participate in our study and making reference to the Tuskegee study. Hence, the low sample size supports the assertion that AA persons may be wary of participating in research due to mistrust (62). Data were collected via an online survey which may have introduced a self-selection bias (63). However, to increase credibility, inclusion criteria were included in the survey to ensure that only participants who met the criteria had access to the survey. The cross-sectional design of the study may not have provided a robust assessment of the validity (especially predictive validity) and reliability of the BSES-EBF, compared to a longitudinal design as in Boateng et al. (28) where data were collected at 1 and 3 months postpartum. Further, the present study assessed all BSES-EBF items, providing no information about the Cognitive and Functional sub-scales of the tool.

4.2. Implications

Findings from this study have implications for research and clinical practice. We examined EBF self-efficacy nonetheless more information is required about the validity of the BSES- EBF scale to predict EBF (predictive validity). Therefore, future longitudinal studies should assess the relationship between EBF self-efficacy and EBF practice after giving birth to their infants in AA women and in other population with low EBF rates using a larger sample size. Additionally, researchers should strive to maintain transparency and earn the trust of participants, especially AA population to facilitate increased research participation. Most items in the BSES- EBF and general self-efficacy scale focused on women's ability to overcome difficulties. Previous studies reported that AA population have higher physical and psychological resilience compared with non-Hispanic White population (64, 65). Hoffman et al. (66) also found that half of White medical students and residents believed that "black people's skin is thicker than White people's skin" (p. 4296). Thus, they reported lower pain ratings for a black person compared to a White person (66) therefore, caution should be exercised when applying findings from this study such that interpretations of the high EBF-self efficacy and general self-efficacy of AA women do not suggest that AA women are monolithic, particularly because of the low sample size for this study. Lastly, intention to breastfeed exclusively was one of the predictors of EBF self-efficacy. Prenatal breastfeeding education increased breastfeeding self-efficacy postpartum among women (23). Hence, nurses and midwives should continue to emphasize the importance of feeding infants with only breast milk (education) for the first 6 months during antenatal classes. It is expected that this intervention might encourage more women to decide to breastfeed their infants exclusively for 6 months while leveraging on the current formula shortage in the United States.

5. Conclusion

The exclusive breastfeeding self-efficacy scale used in this study is valid and reliable to measure EBF self-efficacy in AA women. AA women had high exclusive breastfeeding self-efficacy, predicted by intention to breastfeed exclusively and general self-efficacy. Women who did not intend to breastfeed had the lowest EBF self-efficacy scores. Hence, the BSES-EBF tool is indeed valid to identify women with low confidence to breastfeed their infants exclusively after birth. Finally, only one component was extracted from the factor analysis, suggesting that there is only one latent variable (confidence to practice exclusive breastfeeding) underlying the BSES-EBF tool.

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 humans were approved by University of Connecticut Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

TA: conceptualization, methodology, literature review, and data collection. TA and SW: preliminary and final data analysis. TA: writing – original draft preparation. SW, WH, and RL: supervision, and writing – review and editing. 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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Maternal capital predicts investment in infant growth and development through lactation

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Introduction: Maternal capital (MC) is a broad term from evolutionary biology, referring to any aspects of maternal phenotype that represent resources available for investment in offspring. We investigated MC in breastfeeding mothers of late preterm and early term infants, examining its relationship with infant and breastfeeding outcomes. We also determined whether MC modified the effect of the relaxation intervention on these outcomes.

Methods: The data was collected as part of a randomized controlled trial investigating breastfeeding relaxation in 72 mothers of late preterm and early term infants. Indicators of MC (socioeconomic, social, somatic, reproductive, psychological, and cognitive) were collected at baseline at 2–3 weeks post-delivery. Principal Component Analysis was conducted for the MC measures and two components were identified: 1."Subjective" maternal capital which included stress and depression scores, and 2."Objective" maternal capital which included height, infant birth weight, and verbal memory. Univariate linear regression was conducted to assess the relationship between objective and subjective MC (predictors) and infant growth, infant behavior, maternal behavior, and infant feeding variables (outcomes) at 6–8 weeks. The interaction of MC and intervention assignment with outcomes was assessed.

Results: Higher objective MC was significantly associated with higher infant weight (0.43; 95%CI 0.21,0.66) and length z-scores (0.47; 95%CI 0.19,0.76), shorter duration of crying (–17.5; 95%CI –33.2,–1.9), and lower food (–0.28; 95%CI –0.48,–0.08) and satiety responsiveness (–0.17; 95%CI –0.31,–0.02) at 6–8 weeks. It was also associated with greater maternal responsiveness to infant cues (–0.05, 95%CI –0.09,–0.02 for non-responsiveness). Greater subjective maternal capital was significantly associated with higher breastfeeding frequency (2.3; 95%CI 0.8,3.8) and infant appetite (0.30; 95%CI 0.07,0.54). There was a significant interaction between the intervention assignment and objective MC for infant length, with trends for infant weight and crying, which indicated that the intervention had greater effects among mothers with lower capital.

Conclusion: Higher MC was associated with better infant growth and shorter crying duration. This was possibly mediated through more frequent breastfeeding and prompt responsiveness to infant cues, reflecting greater maternal investment. The findings also suggest that a relaxation intervention was most effective among those with low MC, suggesting some reduction in social inequalities in health.

KEYWORDS

maternal capital, life history theory, maternal stress, lactation, infant growth, maternal investment

1. Introduction

Lactation is an ancient reproductive feature with a long evolutionary history that is thought to predate the origin of mammals, more than 200 million years ago (1, 2). In modern mammals, the primary role of breast milk is to provide a complete source of nutrition. However, it also provides a medium through which 'signaling' or communication between the mother and offspring can occur. From an evolutionary perspective such signaling is expected on the grounds that the magnitude of nutritional investment that maximizes maternal Darwinian fitness will not be identical to that which maximizes the Darwinian fitness of each offspring, as the two parties share only 50% of their alleles (3). Signaling through lactation might occur by altering milk production, milk energy transfer, milk composition, and/or the duration of lactation; all of which are aspects of lactation strategy and vary between and within species. Some examples of these lactation strategies include arctic hooded seals feeding for a short period milk of very high energy and fat concentration in response to unstable and cold climates, or bats delivering small volumes of concentrated milk to avoid impairing the ability to fly (4, 5). Therefore, signaling enables the mother to alter investment in the offspring and regulate their growth and behavior according to her phenotype and to changing environmental circumstances (6).

There is compelling evidence that the magnitude of maternal investment, including lactation, has long-term implications for the health and Darwinian fitness of the offspring (7–12). The classic 'thrifty phenotype' hypothesis of Hales and Barker (13) proposed that low levels of maternal investment, as indicated by low birth weight, increase the risk of cardiometabolic risk and diabetes in adulthood, especially if overweight develops. The thrifty phenotype hypothesis prompts the question, why should variability in maternal investment have such long-term effects on the health of the offspring?

One evolutionary perspective on this developmental association was the predictive adaptive response (PAR) hypothesis, which assumes that maternal investment in early life provides a signal of the likely ecological conditions that will be encountered by the offspring in adulthood, which therefore calibrates its phenotype to this signal (14). If the adult environment turns out to be different ('mismatch'), then the risk of non-communicable disease is assumed to increase. However, the PAR hypothesis has been criticized on both empirical and theoretical grounds. Empirical tests fail to show that survival and fitness in adult environments of famine are improved among those who experienced famine in early life (15). From a theoretical perspective, the notion that environments will remain constant over long time periods has also been questioned (16, 17).

An alternative evolutionary perspective is that the signals provided through maternal investment in early life, including those transferred through breast milk, do not reveal information directly about the external environment, but rather about maternal phenotype itself (17). Within any given setting, mothers may vary in their capacity to invest in offspring, examples being variability in maternal energy stores, parity and socioeconomic status. Some maternal traits may be determined in the mother's own early life (e.g., height and resting metabolic rate), while others may vary on shorter timescales (body fat stores, psychological state) (17). Even when a stimulus emerges from the environment (e.g., stress), maternal phenotype (the stress response) may still mediate the exposure that is actually experienced by the offspring.

Building on the embodied capital approach of Kaplan and colleagues (18), aspects of maternal phenotype that enable differential investment in the offspring have been defined as "maternal capital" (17). Maternal capital is an umbrella term that encompasses several broad categories of traits, including somatic capital (e.g., body weight, height and composition), social capital (e.g., support networks), cognitive capital (e.g., knowledge and skills acquired from formal education, or informally), psychological capital (e.g., resilience to psychological distress) and material capital (e.g., financial income and savings, housing quality). Overall, maternal capital represents the environment to which the offspring is directly exposed in early-life (17), and thus different levels of maternal capital may produce varying levels of investment in the offspring, thereby shaping offspring phenotype and development.

The magnitude of maternal capital is influenced by maternal life-history trajectory (19). Life-history theory is based on the principle of thermodynamics, and holds that energy used for one purpose cannot be used for another (20). Organisms harvest energy from their environment, and invest this energy in competing biological functions, resulting in trade-offs between them. The four main functions competing for energy are maintenance (including repair of cells and tissues), growth, immunity or defense against predators, and reproduction (20, 21). The strategies by which energy is allocated can be described along a continuum of the pace of the life-course (22). 'Slow' life history patterns are favored when mortality risk is low, and are characterized by slower growth, delayed maturation, producing fewer offspring but investing more in each, and reduced risk taking. Conversely, 'fast' patterns are favored when mortality risk is high, and are characterized by early maturation, rapid growth, producing many offspring but investing little in each, and more risky behaviors. In high-risk situations, organisms generally favor rapid growth and reproduction over maintenance and defense in order to maximize the chances of reproducing before mortality occurs (21). Maternal life-history trajectories can further influence those of their offspring, indicating intergenerational dynamics. For example, many studies have demonstrated that age at menarche in mothers is strongly associated with age at menarche in daughters (23–26), and with timing of pubertal development in sons and daughters (23, 27). Additionally, earlier age at menarche for mothers has been shown to be associated with higher offspring BMI (28, 29) and more rapid weight gain in infancy (29), suggesting that a 'faster' maternal life-history trajectory might predict a 'faster' offspring trajectory. Overall, we can expect that mothers with slower life-history trajectories would produce fewer, larger offspring and have greater capital available for investment in each (Figure 1).

Studies have consistently shown that individual aspects of maternal capital are associated with infant and childhood outcomes. For example, data from India, Peru and Vietnam showed that maternal social capital, specifically group membership, was associated with higher infant birth weight across the three countries (30). Humans are cooperative breeders and group members, or alloparents, can provide protection, care and provisioning to the offspring (31). This in turn lowers the direct costs of parental care to the mother, and allows her to invest more in her own maintenance and her future reproduction. This is especially evident in many traditional societies that practice a confinement/rest/quarantine

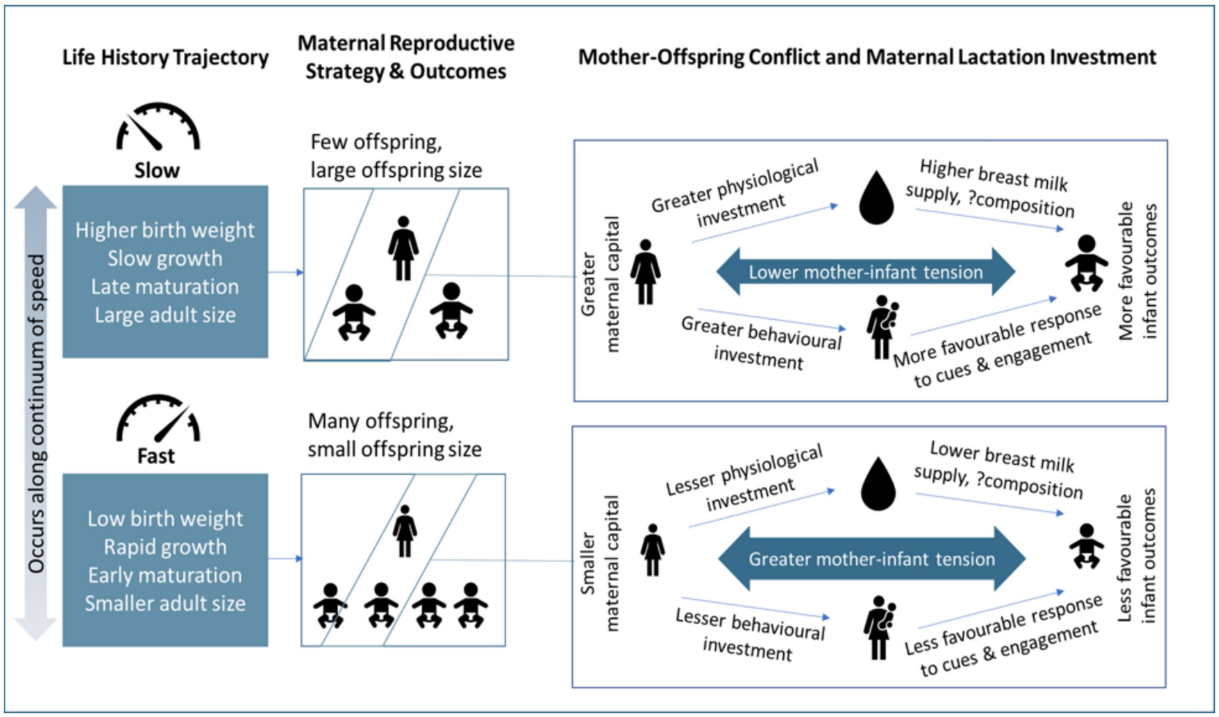

FIGURE 1
The relationship between life history theory, maternal reproductive strategy, mother-offspring conflict and maternal capital on lactation investment and infant outcomes. The figure shows that slower life history trajectories often favor reproducing fewer offspring and investing more in each offspring. In a more detailed perspective, slower life history trajectories often translate to greater maternal capital and thus lower mother-infant tension over resources and greater investment in the offspring through physiological (breast milk) and/or behavioral pathways.

period postpartum, where the mother is provided with help with household chores, her health, infant care and breastfeeding/infant feeding by family members (32–34). Other studies have shown that maternal anthropometry (somatic capital) was positively associated with infant birth weight (35, 36) and childhood educational attainment (37). Psychological capital is also important to consider. In wild olive baboons, mothers who experienced more early life adversity had higher concentrations of glucocorticoids, and subsequently spent more time nursing their offspring (as a possible reflection of more dilute or lower volumes of milk produced) (38). This study also found that high social status buffered against some forms of early life adversity, where some aspects of early life adversity had larger effects on mortality, nursing, and carrying among lower ranking mothers.

In some cases, it may be helpful to evaluate maternal capital as a composite trait, recognizing that mothers with better social circumstances may also have more favorable somatic capital. For instance, an analysis of a birth cohort in Brazil combined data on maternal height, pre-pregnancy BMI, income and education to create a score reflecting overall maternal capital (39). The results indicated that low maternal capital was associated with clustering of adverse outcomes in the daughters, such as lower birth weight, early reproduction, school dropout, shorter adult height and more central fat distribution. It is already acknowledged in biomedical research that many aspects of maternal characteristics are associated with infant growth and development. However, it may also be informative to evaluate different aspects of maternal capital simultaneously, and to examine these relationships from an additional anthropological lens.

From a public health perspective, strategies that enhance maternal capital could potentially improve children's health and development by improving the environment the offspring is exposed to during critical windows of growth (40). Ideally, interventions should target women pre-conception, as maternal capital is a reflection of the mother's own development and past and current experience. However, strategies during pregnancy and lactation are also essential, especially for high-risk women to mitigate the effects of capital penalties.

Breast-feeding is clearly a key component of maternal investment, where the mother supplies not only the nutrients required for metabolism and growth, but also primes the offspring's immune system and thus supports 'defense'. Reflecting the discussion above, the exact nature of this investment is expected to vary in association with components of maternal capital. To date, most attention on this association has focused on somatic traits, such as maternal BMI (41), or socio-economic traits such as household wealth and maternal education (42–44). However, there is growing evidence that maternal psychological state is associated with the volume and composition of breastmilk that the infant consumes (45–48). We previously showed in a randomized controlled trial that reducing psychosocial stress, by asking breastfeeding mothers of late preterm and early term infants to listen to a breastfeeding meditation audio, resulted in higher infant weight gain (49). Not only did the intervention yield greater investment in the offspring but also in the mother herself, as evidenced by better verbal memory scores of mothers in the intervention group.

This paper investigates maternal capital in the same sample of breastfeeding mothers of late preterm and early term infants, and examines its relationship with infant, maternal, and breastfeeding

outcomes. It also determines whether the relaxation intervention is able to modify or moderate the relationship between maternal capital and infant, maternal and breastfeeding outcomes.

2. Methods

2.1. Study design

The data was collected as part of a randomized controlled trial investigating a breastfeeding relaxation in mothers of late preterm and early term infants in London, United Kingdom. The main trial results were published elsewhere (50). Briefly, mothers of healthy infants of $34^{0/7}$ to $38^{6/7}$ gestational weeks were identified and screened before discharge from three hospitals in London. Mothers were eligible if they had a singleton pregnancy, intended to breastfeed for at least 6 weeks, spoke and understood English, did not smoke, were free of serious illness, and did not have a prior breast surgery that could interfere with breastfeeding. Seventy-two participants provided informed consent and were randomized to the relaxation group, where the audio was provided, or to the control group where no intervention was given. The study was ethically approved by the Health and Research Authority in the United Kingdom (IRAS:252031) and registered with ClinicalTrials.gov (NCT03791749).

2.2. Data collection

2.2.1. Maternal capital (predictors)

Data that was deemed indicative of maternal capital, i.e., reflective of maternal capital budget was included for this analysis (Figure 2). Detailed description of data collection is mentioned elsewhere (49, 50). In summary, indicators of maternal capital (socioeconomic, social, somatic, reproductive, psychological, and cognitive) were collected at 2–3 weeks post-delivery, prior to providing the relaxation intervention for those in the intervention group. Participants were asked to report their income, education level, amount of weight gained during pregnancy, height, parity and the infant's birth weight (as an indication of nutritional investment during pregnancy). When measuring the participant's weight at 2–3 weeks was not feasible, mothers were asked to report their current weight, if known. Maternal psychological state at baseline was assessed using the Perceived Stress Scale (51) and Edinburgh Postnatal Depression Scale (52), for stress and depression, respectively. Maternal investment in helping others in the community was assessed at baseline using the Helping Attitude Scale, which is a 20-item measure of the participant's beliefs, feelings, and behaviors associated with helping (53). Each item is answered on a 5-point Likert scale, ranging from 1 (strongly disagree) to 5 (strongly agree). Higher scores indicate "better" helping attitude.

Verbal memory, is one measure of cognitive function which could be influenced by various short- and long-term factors such as hormones, psychological state, education and skills. Therefore, verbal memory can serve as an indicator of cognitive capital which might differentially influence investment in the offspring. We assessed verbal memory using Rey's Auditory Verbal Learning Test (RAVLT) (54) at 2–3 weeks. RAVLT consists of 5 recall trials (Trial 1–5) using List A (15 noun word list) and one recall after an interference period (Trial 6) using List B (another 15 noun word list). During each of the first 5 trials, 15 words (from List A) were presented to the participant (with a 1-s interval between each word) after which they were asked to recall as many words as possible. The recall after each trial was recorded. An interference list was then read to the participants consisting of 15 other words (List B) and they were again asked to recall as many nouns as possible. Immediately after, the participants were asked to recall words from List A (Trial 7). The outcomes of the test include the following based on previously described criteria: 1. Immediate recall: sum of correct responses after first 5 trials (Trial 1–5), 2. Verbal learning: difference in number of correct responses after Trial 5 and Trial 1, and 3. Verbal forgetting: difference between correct responses after Trial 7 and Trial 5. Higher immediate recall, higher verbal memory and lower verbal forgetting are indicative of better verbal

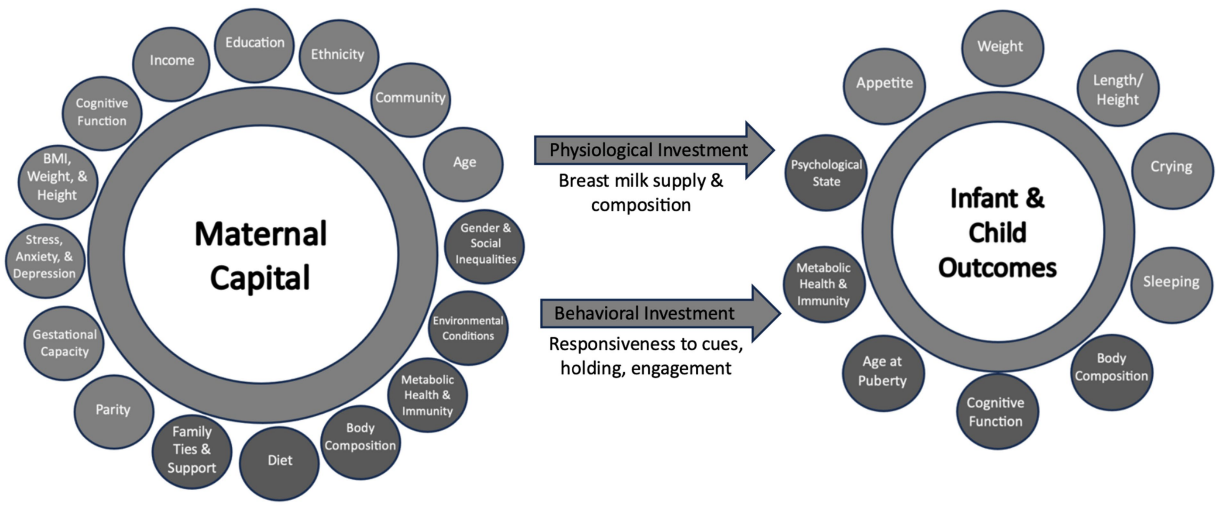

FIGURE 2
Proposed indicators of maternal capital, maternal investment and infant outcomes. Light circles represent the variable collected and considered in the principal component analysis (PCA), while dark circles represent variables not collected in this study.

memory. It is expected that mothers who have higher maternal capital budget would have better verbal memory.

2.2.2. Infant and maternal investment outcomes

Data on infant and breastfeeding variables were collected at 6–8 weeks post-delivery. In summary, infant variables were weight-for-age and length-for-age z-scores, appetite assessed using the Baby Eating Behavior Questionnaire (55) and duration of crying and sleeping assessed using a 3-day baby behavior diary (56). Appetite included four eating traits: 'enjoyment of food', 'food responsiveness', 'slowness in eating' and 'satiety responsiveness'. Enjoyment of food and food responsiveness indicate a greater appetite or interest in food as perceived by the parent, whereas the other two categories indicate a better appetite control or lower interest in milk.

Outcomes related to maternal investment in breastfeeding were breast milk macronutrient composition, breast milk volume supplied at the breast (estimated using a 24-h breastfeeding diary- method described in (49)), the number of expressed breast milk bottles and formula bottles provided in the previous 7 days, exclusive breastfeeding status, and the frequency of direct breastfeeding in a day. Outcomes related to behavioral investment in the infant were attachment to the infant (using Maternal Attachment Index (57)) and the extent to how prompt the mother responds to infant cues [using Maternal Responsiveness Questionnaire (58)].

2.3. Relaxation intervention

As part of the randomized controlled trial described in (49, 50), a breastfeeding meditation audio was offered to breastfeeding mothers of late preterm and early term infants who belonged to the intervention group. Mothers were asked to listen to the 11-min guided-imagery meditation audio that contained breastfeeding messages, at least once a day for at least 2 weeks between 2–3 weeks and 6–8 weeks post-delivery. The main target of the intervention was to reduce mother-infant tension over resources, by reducing maternal stress, and consequently improve infant weight gain.

2.4. Statistical analysis

Principal Component Analysis was conducted for the maternal capital measures (mentioned in Section 2.2 and in Table 1) to create composite scores. Kaiser-Meyer-Olkin Measure of Sampling Adequacy and the Bartlett test of sphericity were used to assess whether PCA is appropriate. Eigenvalues (of 1), scree plots, and parallel analysis were used to identify the relevant components of maternal capital. It was found that PCA was appropriate (KMO = 0.563) for a few measures: maternal height, infant birth weight, perceived stress score, depression score, and verbal learning. Two components were identified: 1. "Subjective" maternal capital which included stress and depression scores, and 2. "Objective" maternal capital which included height, infant birth weight, and verbal learning. Regression factors were saved and objective and subjective maternal capital were also each transformed into two categories: low maternal capital which included participants in the bottom third percentile, and high capital which included the top two-thirds of participants. Descriptive statistics were conducted for maternal capital measures in the whole sample but also for low and high capital groups to ascertain the characteristics of the participants that belong to these categories. Univariate linear regression was conducted to assess the relationship between objective and subjective maternal capital at baseline (predictors) and infant growth, infant behavior, maternal behavior and attitude, and infant feeding variables (outcomes) at 6–8 weeks (unadjusted model). The regression model was then adjusted for intervention assignment. General linear model was used to assess the interaction between objective/subjective maternal capital (as a continuous variable and as low/high categories) and intervention assignment on infant, maternal and breastfeeding outcomes. p-values <0.05 were considered statistically significant.

3. Results

3.1. Descriptive

The different aspects of maternal capital (socioeconomic, social, somatic, reproductive, psychological and cognitive) are described in Table 1. The differences in individual maternal capital measures between those in the low ($n = 17$) vs. high/moderate maternal capital composite scores ($n = 36$) were compared as shown in Table 1. There were trends toward lower maternal age in the low subjective maternal capital group, and toward higher frequency of participants who identified as Black or Asian and/or who had degrees of a lower level than bachelor's degree in the low objective maternal capital group.

3.2. Maternal capital and maternal investment

Higher objective maternal capital was significantly associated with lower non-responsiveness to infant cues at 6–8 weeks (OR = −0.05, 95% CI −0.09, −0.02; Table 2) while higher subjective maternal capital (lower stress and depression scores) was significantly associated with a higher breastfeeding frequency (OR = 2.3; 95% CI 0.8, 3.8).

3.3. Maternal capital and infant growth and behavior

Higher objective maternal capital at 2–3 weeks significantly predicted higher weight (OR = 0.43; 95% CI 0.21, 0.66) and length z-scores (OR = 0.47; 95% CI 0.19, 0.76) and lower infant food responsiveness (OR = −0.28; 95% CI −0.48, −0.08) and satiety responsiveness (OR = −0.17; 95% CI -0.31, −0.02) at 6–8 weeks. Higher objective maternal capital also predicted a lower duration of crying at 6–8 weeks (OR = −17.5; 95% CI −33.2, −1.9), whereas higher subjective maternal capital was significantly associated with greater infant appetite (OR = 0.30; 95% CI 0.07, 0.54).

3.4. Adjustment for relaxation intervention effects

Since this data was collected as part of a randomized controlled trial, the relationship between each predictor and outcome was

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TABLE 1 descriptive maternal capital measures in the whole sample and in low/high objective and subjective maternal capital groups.

Socioeconomic capital	Overall		Low objective capital		High objective capital		Low subjective capital		High subjective capital	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Income										
< £20 K - 30 K	11	22	3	21	8	22	5	29	6	18
< £45 K - 75 K	12	24	2	14	10	28	4	24	8	24
> £75 K	27	54	9	64	18	50	8	47	19	58
Ethnicity										
White	32	60	7	41[c]	25	69[c]	12	71	20	56
Asian	8	15	5	29[c]	3	8[c]	2	12	6	17
Black	7	13	4	24[c]	3	8[c]	2	12	5	14
Other	6	11	1	6[c]	5	14[c]	1	6	5	14
Social capital	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Helping attitude	52	81.9 (7.9)	14	82.6 (8.1)	33	81.9 (8.3)	16	82.3 (8.5)	31	82.1 (8.2)
Somatic capital	n	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Pregnancy weight gain (kg)	64	11.2 (5.7)	16	10.0 (7.8)	34	12.1 (4.9)	17	12.4 (6.7)	33	11.0 (5.7)
Infant birth weight (kg)	72	2.6 (0.4)	17	2.4 (0.2)[b]	36	2.7 (0.4)[b]	17	2.7 (0.3)	36	2.6 (0.4)
Height (cm)	71	163.3 (6.9)	17	158.6 (5.3)[b]	36	164.9 (5.6)[b]	17	161.2 (6.0)	36	163.7 (6.2)
Weight at 2–3 weeks (kg)	60	68.9 (11.3)	16	69.4 (11.5)	31	67.9 (10.6)	15	68.0 (11.4)	32	68.6 (10.7)
Reproductive scheduling	n	Mean (SD)	*n*	Mean (SD)	n	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Primiparous[a]	37	70	10	59	27	75	13	76	24	67
Gestational age	72	36.5 (1.0)	17	36.6 (1.1)	36	36.2 (1.0)	17	36.2 (1.0)	36	36.4 (1.1)
Maternal age	72	33.1 (4.9)	15	33.2 (4.7)	36	33.6 (4.4)	17	31.9 (4.8)[c]	34	34.2 (4.1)[c]
Psychological capital	n	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Stress	64	15.0 (5.5)	17	14.6 (3.8)	36	14.4 (6.2)	17	20.3 (3.7)[b]	36	11.8 (3.8) [b]
Depression	58	7.7 (4.5)	17	8.4 (4.4)	36	7.3 (4.6)	17	12.1 (4.0)[b]	36	5.5 (2.9) [b]
Cognitive capital	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Immediate recall	61	55.2 (6.3)	17	55.2 (7.1)	36	55.1 (6.3)	17	53.8 (7.2)	36	55.7 (6.1)
Verbal learning	61	5.9 (1.9)	17	3.9 (1.4)[b]	36	6.7 (1.4)[b]	17	5.9 (2.0)	36	5.8 (1.9)
Verbal forgetting	61	1.5 (1.7)	17	−1.5 (1.7)	36	−1.5 (1.7)	17	−1.1 (1.7)	36	−1.7 (1.7)
Education[a]										
A-level, GCSE, or less	10	19	6	35[c]	4	11[c]	4	24	6	17
Bachelor's degree or equivalent	20	39	6	35	14	40	6	35	14	40
Postgraduate degree	22	42	5	29[c]	17	49[c]	7	41	15	43

[a]Frequency- Percentage, [b]$p < 0.001$; [c]$p = 0.1$.

adjusted for the intervention assignment. The data shows higher objective capital still predicted higher weight z-score, length z-score and maternal responsiveness to her infant's cues and lower food and satiety responsiveness. There was still a trend toward lower infant crying duration with higher objective maternal capital, but it is likely that the small sample size meant that this analysis was underpowered. A higher subjective maternal capital predicted a higher breastfeeding frequency and greater infant appetite, even after controlling for the intervention. The trend toward higher breast milk volume and lower protein concentration remained after adjustment.

3.5. Does the relaxation intervention moderate the association between maternal capital and investment?

The association between maternal capital and some outcomes varied according to whether the mothers received the relaxation intervention or not. Figure 3 suggests that the intervention was an 'equalizer' for the association between objective maternal capital and weight z-score, length z-score, infant crying, and maternal non-responsiveness, where those of lower maternal capital benefitted the most from the intervention. However, the degree and direction of correlation between maternal capital

TABLE 2 The relationship between subjective and objective maternal capital at 2–3 weeks with infant growth, infant behavior, maternal engagement with the infant, and infant feeding at 6–8 weeks, using simple linear regression.

Outcomes at 6–8 weeks	Predictors at 2–3 weeks post-delivery							
	Subjective capital (HV1)	*p*-value	Adjusted β[1]	Adjusted *p*-value[1]	Objective capital (HV1)	*p*-value	Adjusted β[1]	Adjusted *p*-value[1]
Infant growth								
Infant weight Z-score	0.04 [−0.22, 0.32]	0.74	0.04 [−0.22, 0.30]	0.77	**0.43 [0.21, 0.66]**	**0.000**	**0.39 [0.16, 0.62]**	**0.001**
Weight Z-score gain	−0.01 [−0.20, 0.18]	0.94	0.00 [−0.19, 0.18]	0.96	0.06 [−0.13, 0.25]	0.52	0.02 [−0.17, 0.20]	0.85
Infant length Z-score	0.12 [−0.22, 0.47]	0.49	0.13 [−0.22, 0.47]	0.47	**0.47 [0.19, 0.76]**	**0.002**	**0.46 [0.17, 0.75]**	**0.003**
Length Z-score gain	0.04 [−0.22, 0.30]	0.75	0.04 [−0.22, 0.31]	0.76	0.03 [−0.23, 0.29]	0.81	0.05 [−0.22, 0.32]	0.71
Infant behavior								
Crying/colic	−7.8 [−25.8, 10.8]	0.38	−3.2 [−21.9, 15.6]	0.73	**−17.5 [−33.2, −1.9]**	**0.03**	−14.4 [−31.2, 2.4]	0.09
Fussiness	−3.0 [−26.8, 20.7]	0.79	−5.2 [−31.4, 21.0]	0.69	−11.7 [−22.8, 10.4]	0.29	−14.9 [−38.9, 9.0]	0.21
Sleeping	1.7 [−51.7, 55.1]	0.95	3.9 [−52.5, 60.3]	0.89	7.0 [−44.5, 58.5]	0.78	15.4 [−30.9, 61.8]	0.50
General appetite	**0.30 [0.07, 0.54]**	**0.01**	**0.32 [0.09, 0.55]**	**0.009**	0.01 [−0.23, 0.26]	0.90	0.02 [−0.21, 0.26]	0.84
Satiety responsiveness	−0.03 [−0.19, 0.13]	0.72	−0.02 [−0.19, 0.14]	0.78	**−0.17 [−0.31, −0.02]**	**0.02**	**−0.16 [−0.31,-0.02]**	**0.03**
Food responsiveness	−0.04 [−0.27, 0.19]	0.75	−0.03 [−0.26, 0.20]	0.79	**−0.28 [−0.48, −0.08]**	**0.008**	**−0.27 [−0.48,-0.07]**	**0.009**
Slowness in eating	0.00 [−0.22, 0.22]	0.98	−0.01 [−0.24, 0.21]	0.90	−0.05 [−0.25, 0.16]	0.65	−0.07 [−0.28, 0.14]	0.51
Enjoyment of food	0.05 [−0.10, 0.20]	0.49	0.05 [−0.10, 0.20]	0.51	−0.01 [−0.14, 0.13]	0.94	−0.01 [−0.15, 0.13]	0.93
Maternal engagement with infant								
Attachment	0.34 [−1.15, 1.83]	0.64	0.31 [−1.2, 1.8]	0.68	−0.15 [−1.50, 1.17]	0.82	−0.19 [−1.5, 1.2]	0.78
Responsiveness to cues	0.07 [−0.06, 0.20]	0.28	0.07 [−0.07, 0.20]	0.31	0.09 [−0.02, 0.21]	0.11	0.09 [−0.03, 0.21]	0.13
Delayed responsiveness	0.03 [−0.23, 0.28]	0.83	0.04 [−0.22, 0.30]	0.74	**−0.22 [−0.45, 0.00]**	**0.047**	−0.22 [−0.44, 0.01]	0.06
Non-responsiveness	−0.02 [−0.06, 0.02]	0.29	−0.02 [−0.06, 0.02]	0.38	**−0.05 [−0.09, −0.02]**	**0.004**	**−0.05 [−0.08, −0.01]**	**0.007**
Infant feeding								
Exclusive breastfeeding[a]	1.28 [0.66, 2.47]	0.46	1.28 [0.66, 2.47]	0.46	1.09 [0.59, 2.00]	0.79	1.08 [0.58, 2.03]	0.80
Formula feeds	−0.9 [−6.4, 4.6]	0.74	−0.8 [−6.3, 4.7]	0.77	0.0 [−5.0, 5.0]	0.99	0.4 [−4.7, 5.6]	0.86
Expressed milk feeds	2.1 [−2.1, 6.3]	0.34	2.1 [−2.2, 6.3]	0.33	0.2 [−3.7,4.1]	0.92	0.5 [−3.5, 4.6]	0.79
Breastfeeding frequency	**2.3 [0.8, 3.8]**	**0.006**	**4.1 [2.0, 6.1]**	**0.001**	0.7 [−1.3, 2.7]	0.44	0.3 [−1.5, 2.1]	0.71
BM volume (24 h Recall)	105.8 [−7.0, 218.7]	0.06	106.9 [−4.0, 217.8]	0.06	13.5 [−114.4, 141.4]	0.82	−0.6 [−131.3, 131.2]	0.99
Fat g/100 mL	−0.07 [−0.43, 0.31]	0.72	−0.07 [−0.43, 0.31]	0.73	−0.07 [−0.42, 0.27]	0.67	−0.07 [−0.42, 0.29]	0.71
True Protein g/100 mL	−0.06 [−0.12, 0.01]	0.11	−0.06 [−0.12, 0.01]	0.10	0.02 [−0.04, 0.09]	0.68	0.02 [−0.04, 0.09]	0.50
Carbohydrates g/100 mL	0.00 [−0.11, 0.11]	0.99	0.00 [−0.11, 0.11]	0.99	−0.06 [−0.17, 0.04]	0.22	−0.08 [−0.18, 0.03]	0.14
Energy Kcal/100 mL	−0.96 [−4.31, 2.40]	0.56	−0.95 [−4.35, 2.44]	0.57	−0.90 [−4.06, 2.26]	0.57	−0.84 [−4.10, 2.42]	0.61

[1]The relationship was adjusted for the intervention assignment. Significant results are highlighted in bold, and trends are highlighted in blue.

and the appetite traits and breastfeeding frequency did not change with the relaxation intervention. There was a significant interaction between the intervention assignment and objective maternal capital on length z-score ($p=0.006$), and trends toward an interaction for weight z-score ($p=0.07$), infant crying ($p=0.06$) and maternal non-responsiveness ($p=0.06$). This is further illustrated in Figure 4 where the effects of low maternal capital are mostly costly to infants whose mothers did not receive a relaxation intervention.

4. Discussion

Maternal capital represents the only environment that the offspring is exposed to during pregnancy, and largely the nutritional one they are exposed to postnatally if breastfeeding (17). Therefore, combining several maternal traits when studying the relationship between maternal capital and infant outcomes might be more meaningful than investigating individual traits. For instance, while we know that earlier menarche is usually associated with lower infant birth weight (mostly an indicator of lower parental investment) (59, 60), that is not always the case (61, 62). Psychosocial stress has been reported to be a moderator of the relationship between the two, where the presence of stressful life events as well as earlier menarche predicted lower birth weight, but earlier menarche alone did not (63). This suggests that the mother is able to provide a buffer for the offspring against some/few capital 'insults.' Therefore, it is important to account for how many insults on maternal capital are present to better predict differential investment in the offspring.

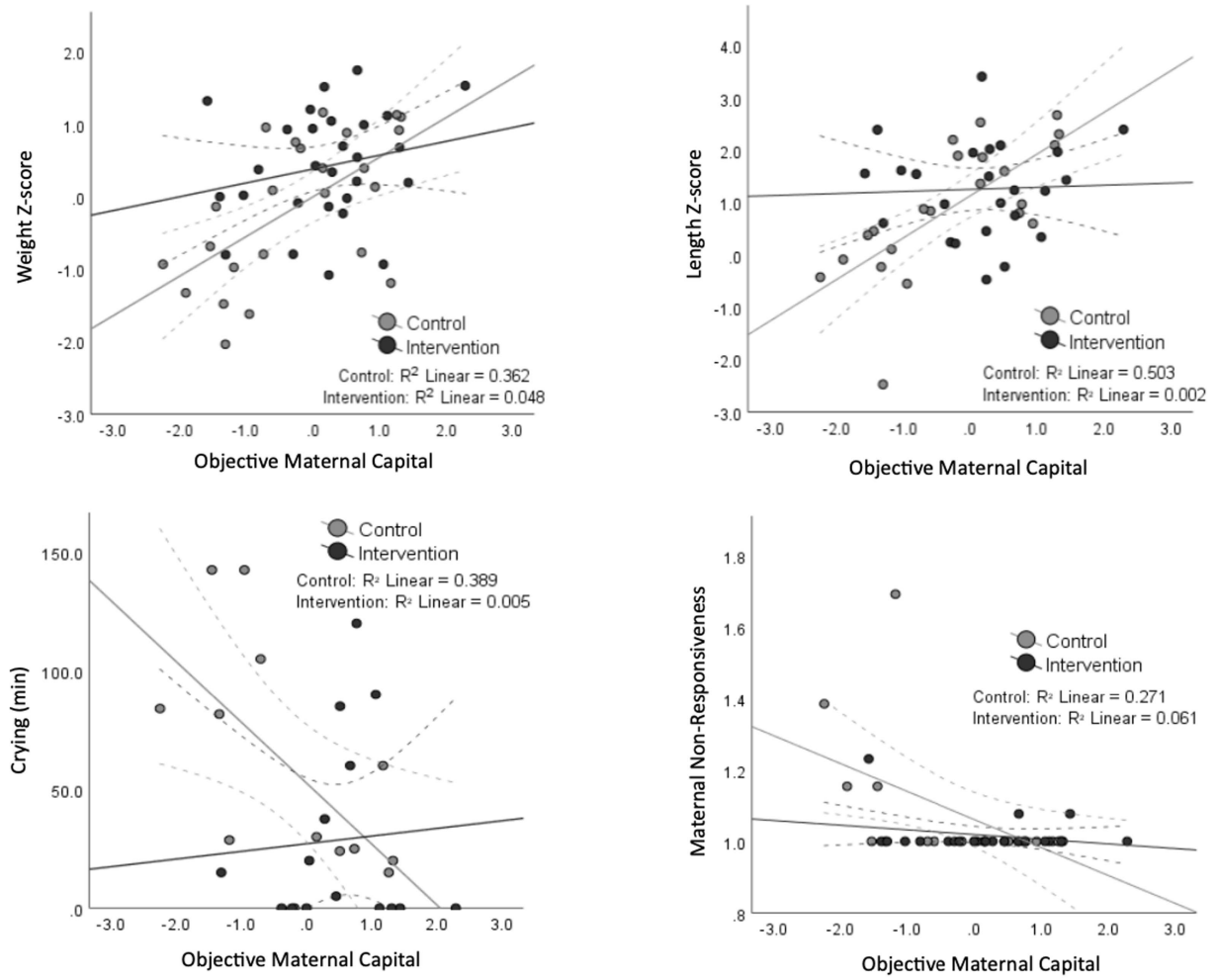

FIGURE 3
Scatter plots demonstrating the significant association (and trends) between maternal capital and infant, maternal and breastfeeding outcomes separated by the intervention assignment. The black dots and lines represent the intervention group, while the gray ones represent the control group. 95% confidence interval lines are shown as dashed lines. A significant interaction between the intervention assignment and maternal capital is present for length z-score, whereas a borderline significant interaction is shown for crying, weight and responsiveness.

The results show that objective maternal capital at baseline (2–3 weeks post-delivery) was positively associated with weight and length z-scores at 6–8 weeks, but not with infant growth rate between these two time points. This suggests that mothers with higher energy available for investment in the offspring invested more during their intervention period, hence the larger size of their infant at 6–8 weeks. However, the lack of association between maternal capital and growth rate might mean that these infants allocate enough energy for growth without favoring it over other competing life functions such as metabolic homeostasis or immunity (we did not collect data on these markers to examine this). The results are in line with a previous study that showed that daughters of mothers of lower maternal capital (or with higher maternal capital penalties) were lighter and shorter at 1 year than those of higher capital mothers (39). However, by adulthood these daughters remained shorter but had higher BMI and markers of adiposity, indicating catch-up in weight rather than in linear growth. It is possible that long-term follow up data in our study may show similar accelerated growth trajectories post-infancy in the children of lower capital mothers.

Our study shows that maternal capital can also shape infant behavior. For instance, we found that higher objective maternal capital was associated with lower infant satiety responsiveness (i.e., higher drive to eat) and with somewhat shorter duration of crying. It is possible that maternal capital regulates infant appetite and behavior through bioactive components in breast milk acting as signals. During pregnancy, signals are transmitted from maternal blood to the fetus, and through breast milk postnatally. These signals reflect maternal condition and how the mother perceives her environment which in turn are strongly influenced by maternal development and early life experiences (17). Some of these signals are hormones associated with maternal psychological state and have the potential to regulate infant growth and development. For instance, studies, mostly in animals, have shown that maternal stress is associated with increased fearfulness, anxiety, crying and sleeping problems and attenuated growth in the offspring (64–70). We found that lower subjective maternal capital (i.e., higher stress and depression) was associated with lower infant appetite; however, we did not find any association

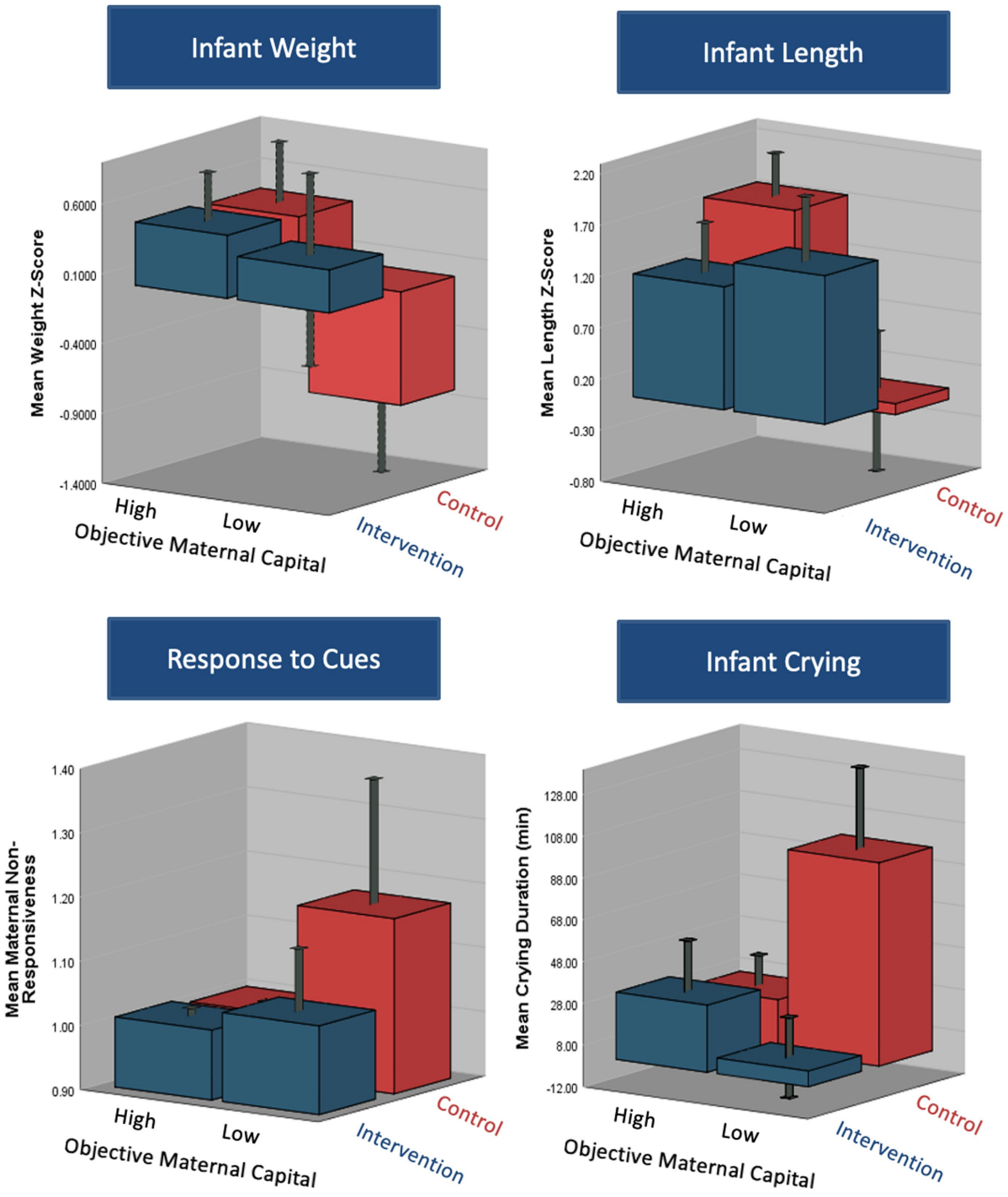

FIGURE 4
3D plots demonstrating the differences in infant, maternal and breastfeeding outcomes according to maternal capital group (high/moderate vs. low objective capital) and intervention group (intervention vs. control). The plots demonstrate a significant interaction between the capital and intervention assignment on infant weight, length and crying duration. There was a borderline significant interaction between the two on maternal responsiveness to infant cues.

with infant growth, crying, or sleeping. The correlations between maternal psychological state and infant appetite could be, at least partially, explained by some confounding factors. For example, gestational age could influence both psychological state (mothers of infants who are more premature are likely to be more stressed) and infant appetite (infants who are more premature are more likely to be feeding for shorter durations, more frequently). It is likely that maternal capital would serve as a buffer against short-term stressors and thus the infant is more likely to be influenced by the mother's response to stressors rather than to short-term stress signals. That is why long-term markers of stress are needed to better predict infant response to stress exposure.

A potential pathway by which maternal capital can influence infant growth and behavior is through maternal behavioral and physiological investment. For example, we found that higher maternal capital predicted better maternal responsiveness to infant cues and higher breastfeeding frequency with a trend toward increased breast milk volume. From an evolutionary perspective, in uncertain environments characterized by higher stress levels reduced breastfeeding could be a method that has evolved to limit maternal investment (71). It could be explained from an energy trade-off point of view, where in distressed mothers, energy is being diverted from investment in the offspring toward mounting a stress response and preserving energy for the mother's survival or future reproduction. This constraint can be in the form of reduced production of breast milk, or through reducing certain constituents of breast milk (macronutrients or hormones) that might be costly to supply. Distressed mothers may also have less available energy for nurturing behaviors such as holding, bonding, or responding promptly to cues.

Many of the maternal capital measures discussed above are already acknowledged to be important predictors of child health in conventional biomedical research. However, public health interventions aimed at improving breastfeeding or exclusive breastfeeding rates, and consequently infant outcomes, by targeting the mother are not always successful in achieving what they intended. This might be because from a medical perspective, milk is seen primarily as a source of nutrition, and lactation as a largely one-way process in which the mother provides whatever her infant needs (72). This perspective does not consider the social, cultural, historical and environmental basis of variability in lactation and the complex role the mother plays in regulating her offspring's growth and behavior. Therefore, incorporating evolutionary concepts into the design of public health interventions could improve the success of these interventions. For instance, we previously combined medical and anthropological concepts into the design of three randomized controlled trials using relaxation therapy to reduce maternal stress and improve infant outcomes in breastfeeding women of term and preterm infants in the United Kingdom (current study (49)), Malaysia (73) and China (74). All three studies were successful in improving infant weight gain, with some finding differences in infant behavior, breast milk composition, and/or infant gut microbiome (Yu et al. unpublished data).

The results from this study show that the simple relaxation intervention could manipulate the association between maternal capital and outcomes. This could be interpreted in two ways: (i) capital influences responsiveness to the intervention, and/or (ii) the intervention directly influences capital by increasing investment in the offspring regardless. Based on our results, both interpretations are valid. We found that the intervention increased investment in the infant, but mainly in the low capital group, where it buffered against the consequences of low maternal capital, i.e., lower weight z-score, lower length z-score, prolonged infant crying and higher maternal non-responsiveness (although the relationship was significant for length z-score only). As such, infants whose mothers have low maternal capital and belonged in the control group were the most affected by maternal capital 'penalties'. It is possible that the capacity of mothers with certain developmental stresses to invest in their offspring may become more severely impaired, if they experience psychosocial stress during lactation. On this basis, a stress reduction therapy during lactation might be especially beneficial. Additionally, parent-offspring theory explains that the mother and infant compete and negotiate over how much maternal resources will be invested in the offspring (3). How much the mother invests is the point at which maximum benefit is given to the infant without incurring maximal cost on the mother. It is possible that for mothers of low capital the surplus energy gained as a result of the intervention shifts this point of balance upwards to a point where the infant benefits more without incurring more cost for the mother. This might be different for mothers of high capital where the surplus of energy would not be invested in the infant, as the infant is already receiving maximal benefit, but is rather invested in the mother herself for maintenance, immunity and/or future reproduction.

Wells (17) differentiated between two different broad types of maternal capital: liquid and illiquid. The liquidity of capital refers to how quickly these resources could be gained and lost. For example, maternal height is a stable trait and thus is illiquid, whereas fat or vitamin stores could be accumulated or used through the reproductive career, and so are liquid capital. This theoretical approach could be extended to explore psychological aspects of maternal capital. For example, we could think of maternal temperament as a stable, relatively illiquid component of psychological capital, whereas short-term changes in mood, reflecting changing environments and circumstances, would indicate liquidity in psychological capacity and resilience. Temperament, reflecting genetic constitution and developmental experience in early life, might also shape fluctuations in short-term moods, just as linear growth reflecting the same influences may shape adult adiposity. Liquid psychological capital might also be assayed indirectly by assessing cortisol, a biomarker of activation of the stress response. Under the same adult environmental circumstances, some mothers may become stressed more easily than others, potentially due to illiquid traits such as temperament. Therefore, it is possible that the relaxation intervention might beneficially impact the liquid aspect of maternal psychological capital, by reducing maternal stress and anxiety.

Lasty, it is worth considering that participants in this study were mostly highly educated, most had high household incomes, all were above 20 years old (and most above 30 years), and most had only one child. Therefore, mothers in the low maternal capital group arguably did not truly have low capital, even though it was low relative to others in the study. It is unclear whether the intervention (or other similar interventions) would be more beneficial to mothers with truly low maternal capital and future studies should investigate this.

The main limitation of this analysis is the small sample size. We were unable to examine the differences between female and male infants who might exhibit different trade-off patterns. The small sample size also reduced the possibility of including more variables for Principal Component Analysis to represent maternal capital. The other limitation is that we did not collect data on other maternal capital traits pre-pregnancy such as age at menarche and maternal birth weight which could have provided more details on maternal capital and better predicted investment. Additionally, because this was a sample of mothers living in Greater London and intending to breastfeeding their late preterm

or early term infant, the generalizability of the findings could be limited. However, one of the strengths of this study is that it demonstrated a novel analytical approach that could be applied to more representative samples of the general population. Another strength is that we used an experimental design to manipulate maternal capital. Principal Component Analysis was also used to identify composite scores of maternal capital which might provide a better indicator of maternal capital than assessing individual traits. Lastly, we collected a large number of maternal investment indicators such as maternal behavior, breast milk volume and composition, infant growth, and infant behavior and appetite.

In conclusion, we have used a novel approach to examine how mothers differentially invest in their offspring and regulate their growth and behavior according to maternal phenotype and environmental conditions. The findings show that higher maternal capital was associated with better infant growth and shorter infant crying duration, possibly mediated through more frequent breastfeeding and more prompt responsiveness to infant cues. The findings also suggest that a simple relaxation intervention could buffer against maternal capital insults, as the effects of low maternal capital appeared most detrimental to infants whose mothers did not receive the intervention. Overall, understanding and applying evolutionary concepts such as maternal capital to health-related studies could improve understanding of how the environment interacts with the mother, milk and infant. It could also help to better predict the outcome of interventional studies and achieve the desired results such as improved infant growth and promotion of breastfeeding.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the the corresponding author, upon reasonable request.

Ethics statement

The studies involving humans were approved by Health Research Authority (United Kingdom). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SD: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. MF: Conceptualization, Supervision, Writing – review & editing. JW: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

MF has received an unrestricted donation for research on infant nutrition from Philips (not related to the current manuscript).

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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Lactation physiokinetics—using advances in technology for a fresh perspective on human milk transfer

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Introduction: Though the nature of breastfeeding is critical, scant information is available on how the action of the milk transfer from mother to infant is regulated in humans, where the points of dysfunction are, and what can be done to optimize breastfeeding outcomes. While better therapeutic strategies are needed, before they can be devised, a basic scientific understanding of the biomechanical mechanisms that regulate human milk transfer from breast to stomach must first be identified, defined, and understood.
Methods: Combining systems biology and systems medicine into a conceptual framework, using engineering design principles, this work investigates the use of biosensors to characterize human milk flow from the breast to the infant's stomach to identify points of regulation. This exploratory study used this framework to characterize Maternal/Infant Lactation physioKinetics (MILK) utilizing a Biosensor ARray (BAR) as a data collection method.
Results: Participants tolerated the MILKBAR well during data collection. Changes in breast turgor and temperature were significant and related to the volume of milk transferred from the breast. The total milk volume transferred was evaluated in relation to contact force, oral pressure, and jaw movement. Contact force was correlated with milk flow. Oral pressure appears to be a redundant measure and reflective of jaw movements.
Discussion: Nipple and breast turgor, jaw movement, and swallowing were associated with the mass of milk transferred to the infant's stomach. More investigation is needed to better quantify the mass of milk transferred in relation to each variable and understand how each variable regulates milk transfer.

KEYWORDS
breastfeeding, physiokinetics, human milk transfer, oral pressure, biosensor

Introduction

The importance of breastfeeding is well recognized, and the success of breastfeeding promotion in the United States is seen in the increase in initiation rates from 33% in 1975 (1) to 88% in 2019 (2). In many low- and middle-income countries, the weighted prevalence of early initiation of breastfeeding was 52% (3). In European countries, while 56%–97% of infants receive human milk at birth, exclusive breastfeeding rates are

declining (4). According to the 2020 Breastfeeding Report Card, (Centers for Disease Control and Prevention (5) there needs to be more progress in developing evidence-based interventions that lead to increased breastfeeding duration (6). The most commonly stated reason for stopping breastfeeding is the maternal perception of inadequate milk supply (7), grounded in low volumes of milk transferred to infants (8). However, the perception of insufficient milk (9) is not a moment in time but a cascade of events. Milk supply is determined by the amount removed from the breast after the initial hormone-driven period of approximately 72 h post-delivery. Breastfeeding is a learned behavior, and for over 50 years in the last century, breastfeeding rates were meager (10), with much of the practical knowledge about breastfeeding lost in the United States as that knowledge was not passed on to many of the young medical practitioners or to new mothers desiring to breastfeed as breastfeeding initiation rates dropped below 33% (11).

Unlike other biologic systems and the fundamental nature of breastfeeding to human existence, scant information is available on how milk flow is established between mother and infant, its paired regulation, and the potential points of dysfunction. Much of the practical information about breastfeeding, such as nuances of positioning and recognition of resolutions, was lost and needed to be recovered. The use of new technology could lead to improved breastfeeding outcomes.

The current understanding of milk transfer does not have a sufficient evidentiary basis for planning interventions and supporting breastfeeding dyads when milk transfer fails. Indeed Lee and Kelleher point out that "*understanding factors that impact lactation and developing methods to assess lactation outcomes before a breastfed infant becomes ill accurately will directly inform the development of therapeutic strategies to improve poor lactation performance*" (12). The technology to resolve this problem is not available as very little is known about the relationships between the maternal and infant inputs that make up this "living" biological secretion of human milk (13). We know that current support techniques do not ensure breastfeeding success (14–16), especially in areas of underserved populations and where lactation professionals are scarce.

The actions exhibited by infants at the breast differ significantly from the skills they use to feed from a bottle. A feeding bottle equipped with a small video recorder and a pressure sensor was used to evaluate tongue movements and oral pressure, with the researchers concluding that jaw motion was correlated with oral pressure (17). Ultrasound imaging has been used extensively to compare different types of artificial nipples in bottle-feeding infants (18–20). Ultrasound has also been used to further understand the infant oral mechanics of the breastfeeding infant, mainly in research settings. Geddes et al., supporting the premise that tongue movements are associated with milk flow into the mouth (21). We theorize that infants who are feeding well, to a great extent, control the flow and volume of milk in some way rather than simply a function of oral pressure changes. Indications of this premise can be seen in numerous ultrasound studies on the importance of the lower portion of the infant's face in contact with the breast to optimize milk flow (22). The mandible movement of the tongue corresponds with milk flow in breastfeeding infants (23). In another ultrasound study, vacuum strengths were not associated with milk intake but were related to the time spent actively feeding (24). It has been suggested that the absence of milk alters tongue movement (25). More recently, research on tongue kinematics has shown differences in movements during breast- and bottle-feeding using *in vivo* submental ultrasound video clips (26). Douglas and Geddes noted that physiologic approaches are insufficient to ensure successful breastfeeding for many women in the weeks and months post-birth (14).

There has been considerable controversy regarding how infants remove milk from the breast. Despite claims to resolve the dispute through ultrasound imaging and computational simulations (27), an accurate understanding of how the infant facilitates milk removal from the breast remains elusive.

Much biomechanical research on infant feeding has been based on what infants do when bottle feeding. Lang et al., using a specially designed bottle apparatus, found that the feeding patterns of normal infants were more diverse than expected but did suggest that quantification of infant feeding patterns was possible (28). Additional work did allow for the characterization of differences in the maturation of feeding between healthy preterm and full-term infants using the Orometer feeding device (29). However, this line of inquiry has limited application outside the laboratory setting, and the information gathered on oral pressure only provides one measure when attempting to remedy lactation failure in clinical practice.

While the inefficiencies in the system can be complex and perplexing to pinpoint (7, 30), system dysregulation is manifested via malnutrition (31), irrecoverable immune deficiency (32), short- and long-term morbidities (33), and poor health outcomes (34). The problems should be recognized sooner for timely interventions to correct the issues of inadequate milk transfer. Before developing better therapeutic strategies, the physiologic and biomechanics that regulate human milk transfer from breast to stomach must be identified, defined, and understood as interrelated systems. There are hidden interactions between mother and infant, regulated concomitantly, which need to be mapped and characterized to better understand the regulatory points before developing therapeutic strategies. Systems Biology focuses on the complex interactions within biological microcosms aimed at understanding complex biologic processes. Breastfeeding is a biological system that needs scientific investigation to elucidate the biomechanical mechanisms that regulate milk movement from mother to infant. At the same time, it has been suggested that human milk should be considered a biological system (13). Clearly, a new perspective is needed to advance the current understanding of human milk transfer in a way that can lead to an increased number of families meeting their breastfeeding goals. A more comprehensive approach is needed to facilitate the development of strategies to minimize the dysfunction of the human milk transfer system.

Systems biology provides a framework for understanding biology as an interconnected and dynamic system, enabling

researchers to explore and manipulate biological processes more comprehensively and interactively. Systems Biology uses four fundamental properties to characterize biologic systems: **system structures** which include physiologic, biochemical, and mechanical components of the system; **system dynamics** of how the process behaves over time and under varying conditions; **control methods** which methodically regulate those processes; and **design methods** which can be used to modulate those processes. Systems medicine aims to transform healthcare by incorporating a holistic understanding of an individual's biology and environmental factors. Systems Medicine is the application of Systems Biology for **predicting function**, **preventing malfunction**, **personalizing interventions**, and **resolving the problem** with participatory engagement (35, 36). Combining a Systems Biology (37) and Systems Medicine (38) approach to create a new conceptual framework for milk transfer can provide a better understanding of the regulatory mechanisms of human milk transfer from mammary ducts in the breast to the infant's stomach. Using this framework, pairing biology and medicine creates a multidisciplinary approach that may bring better understanding to the function of breastfeeding physiology and biomechanics by integrating large-scale data. Utilizing engineering design principles (to recognize and define the need, seek systemic causes, and establish baseline parameters to create iterative solutions) can support this new conceptual framework.

While some features of human milk transfer, such as milk flow (39) (maternal side) and swallowing (40) (infant side), have been identified, little is known about regulatory points throughout the Maternal/Infant Lactation physioKinetics (MILK) system. This research study explores the scientific questions about potential regulatory points for milk transfer in the MILK system.

Materials and methods

The Institutional Review Boards for the University of Texas approved the research protocol. The study population comprised a convenience sample. Mother/infant dyads were recruited from the community through flyers, email list announcements, breastfeeding support groups, and ongoing maternal health studies. Mother/infant dyads were screened for eligibility through a telephone interview with the study team. Inclusion criteria for mothers are (1) an age range of 18–50 years; (2) intention to breastfeed for at least six weeks; and (3) intention to feed directly from the breast. Inclusion criteria for infants are those who (1) were born at 38–42 weeks gestational age, and (2) returned to birth weight by two weeks after delivery with breastfeeding. Exclusion criteria for mothers include (1) maternal age < 18 years; (2) presence of inverted nipples; (3) tape allergy; and (4) history of smoking, which may decrease maternal milk supply. Exclusion criteria for infants are those who were (1) <38 weeks gestational age and (2) diagnosed with ankyloglossia or other congenital anomalies that affect feeding. If the infant cried for 60 s during the sensors' placement, the session ended, and the sensors were removed as this was considered infant dissent per the research protocol.

The research team completed a health history and demographics form at the telephone interview. Each participant electronically signed a written consent form for herself and her infant. This study collected data from the mother-infant dyad in three fragments: initial data before breastfeeding, active data during the breastfeeding session, and data after breastfeeding. The primary aim of this study was to understand better and explain the complex physiological and anatomical properties of mammary tissue during milk transfer and infant orofacial muscle and bone movement during feeding. Maternal side data included the variables of breast skin temperature, breast turgor, nipple turgor, and maternal weight. Infant variables included temporomandibular joint movement, intra-oral pressure, infant temperature, contact force, and swallowing.

At each observation session prior to feeding, the mother was weighed, nipple diameter and length were measured, and breast turgor and breast temperature were recorded. The nipples were measured in millimeters using digital calipers. Breast turgor was evaluated using a durometer. The breast temperature was measured in degrees Celsius using an infrared thermometer. The mother was settled into a comfortable position. Sensors were applied to the breast for contact force, intraoral pressure, and infants' nasal airflow. Nasal temperature was measured by a thermistor type (10 kΩ @ 25°C) nasal temperature probe (ADInstruments) connected to a bridge amplifier with noise filtering for fast temperature transient monitoring with an output voltage of 50 mV/°C and a response time of about 200 milliseconds. The probe was placed approximately 1 cm from the infant's nasal passage during breastfeeding and secured to the mother's breast using surgical tape.

The infant was measured in centimeters for length and weighed in a clean diaper. Infant weight was measured twice (before and after feeding) in the session, using a Tanita BD-815 U Neonatal / Lactation Baby Scale. The sensors for jaw movement and swallowing were placed on the infant, and the infant was then placed at the mother's breast. We incorporated the nasal probe with the intention of determining nasal temperature, which served to identify the patterns of inhalation and exhalation. While exhalation leads to a temperature increase, inhalation results in decreased temperature. Data collection commenced as the infant was placed at the breast. Clinical notations were made for infants unlatching from the breast, fussiness, and sensor disruptions. When the infant signaled the completion of the feeding, the sensors continued collecting data for 60 s. The sensors were then removed from the infant. The infant was weighed for the second time. The mother's after-feeding breast temperature, turgor, and weight were recorded.

Descriptive statistics were used to describe the basic features of the data. Central tendency and dispersion measures were calculated, including mean, median, minimum, maximum, and standard deviation. Multiple logistic regression was used for suggestions about which independent variables influenced the volume of milk transferred. ANOVA statistical tests were performed using Jupiter and SPSS.

Results

In total, fourteen breastfeeding sessions were recorded. The mean age of the adult participants was 31 years (SD ± 1.8) Overall, 66% of the participants identified as Hispanic with the remaining participants identified as White. All of the participants had a college degree.

The mean infant age was 49 days (SD + 25) of age. The mean mass of milk transferred was 120 g (SD ± 57) per feeding session. The range of the mass of milk transferred was 34 g–222 g.

For this exploratory study, we focused on evaluating the nipple and breast turgor contact force, jaw movement, and oral pressure. There were additional measurements collected of infant respiration (using a respiratory belt or nasal temperature) and swallowing (using hyoid movement).

Nipple turgor was evaluated using the change in diameter and length between pre- and after-feeding measurements. The average nipple diameter before feeding was 16.4 mm and 15.7 mm after feeding. The average nipple length before feeding was 7.6 mm and 8.2 mm after feeding. Nipple diameter was typically slightly less after feeding, while nipple length was typically longer after providing no statistical difference, as shown in **Figure 1**. The mean change in nipple diameter was −0.74 (SD ± 1.75). The mean change in nipple length was 0.67 mm (SD ± 1.19).

Breast turgor was measured using a type "OO" durometer, which is recognized as an accurate and reliable tool to quantify hardness on various parts of the human body (41). This type of durometer is also recommended for applications involving human skin. tissue (42). The durometer was placed at the area that was 3 cm from the nipples in the 6 o'clock position. Higher measurements represented increased turgor. The mean for breast turgor before breastfeeding was 19.9 pounds per square inch (psi) (SD ± 7.02), ranging from 12 to 38. The mean after breastfeeding was 11.6 psi (SD ± 6.82), with a range from 4 to 29. The before and after differences shown in **Figure 2** were statistically significant using paired t-tests with $p < 0.001$.

The volume of milk transferred to infants during feeding was calculated such that mass/density Mass = W(after)-W(before) in grams. Mass was divided by 1.25 g/ml (density of human milk) to get the volume in milliliters. The mean mass of milk transferred to the infant stomach was 119.5 g (SD ± 53) per feeding, ranging from 34 g–222 g. The average time of feeding was 10.5 minutes. The average amount of milk transferred during feeding was 117 ml, ranging from 34 ml to 224 ml. The mean rate of milk transfer was 10.6 ml/minute.

Descriptive statistics were calculated for contact force, Oral Pressure, and jaw movement. Contact force was measured using a Millar pressure sensor, adapted to measure the contact pressure of the infant's chin against the mother's breast by converting mechanical force into a voltage signal. Intra-oral pressure was measured using a Millar catheter positioned and attached to the breast of the mother, with the tip of the catheter protruding approximately 3 mm past the tip of the nipple before placing the infant at the breast. Jaw movement was measured using a piezoelectric film and a data acquisition device. Six feeding sessions had complete data needed for the descriptive statistics, as shown in **Table 1**.

Coherence was demonstrated between the time period of biomechanical action of oral pressure and contact force with jaw movement. ANOVA was performed for the biomechanicl time period between oral pressure and jaw movement, as well as between contact force and jaw movement. Results from ANOVA showed no statistical differrence between all sensor channels (p-value < 0.05. This significance indicates a synchronicity of the actions. A rhythmic contact force of approximately 0.1 milliNewtons is maintained between the infant's chin and the breast. In the representative example seen in **Figure 3**, the pressure within the infant's oral cavity fluctuates between the latching pressure of −15 mmHg and −190 mmHg. During active feeding, the jaw movement sensor records regular oscillations of ±8 mV. The nasal temperature sensor channel is an indirect measure of infant respiration. The respiratory belt measures the expansion and contraction of the infant's diaphragm. Swallows were denoted by the clinician observing the infant during feeding. A correlation was seen with the magnitude and changes in amplitude to the mass of milk transferred to the infant.

The Pharynx is a shared anatomic pathway for both swallowing and breathing; however, these two activities are mutually exclusive. How infant's co-ordinate the continuous reconfiguration of swallowing and breathing at the same time is still unknown. We incorporated the nasal probe with the intention of determining nasal temperature, which served to identify the patterns of inhalation and exhalation. While exhalation leads to a temperature increase, inhalation results in decreased temperature. The mechanism of nasal temperature regulation which is associated with respiration is exclusive to contact force generation or oral pressure.

To evaluate correlations between Contact Force, Oral Pressure, and Jaw Movements, we estimated the phase of the contact force, oral pressure, and jaw movement using Hilbert transform (43) and explored any phase locking between the signals by calculating its phase difference (44) with unwrapped phase.

With the infant's age variation, we observed a 1:1 phase-locking phenomenon between contact force and oral pressure. Additionally, a clear indication of 2:1 phase locking between jaw movement with oral pressure and contact force was seen and is shown in **Figure 4**. The peak-to-peak difference in an infant's jaw movement is twice the oscillation in context to oral pressure or contact pressure. However, older infants exhibit 1:1 phase locking between time series.

Discussion

While the sample was small, the percentage of Hispanics in the study is representative of the local population, as 67% of the population is identified as Hispanic with 23% identified as White. Additional racial diversity will be included in future study.

The volume of milk intake was appropriate at each feeding session considering the length of time from the prior feeding session, the size, and age of the infant.

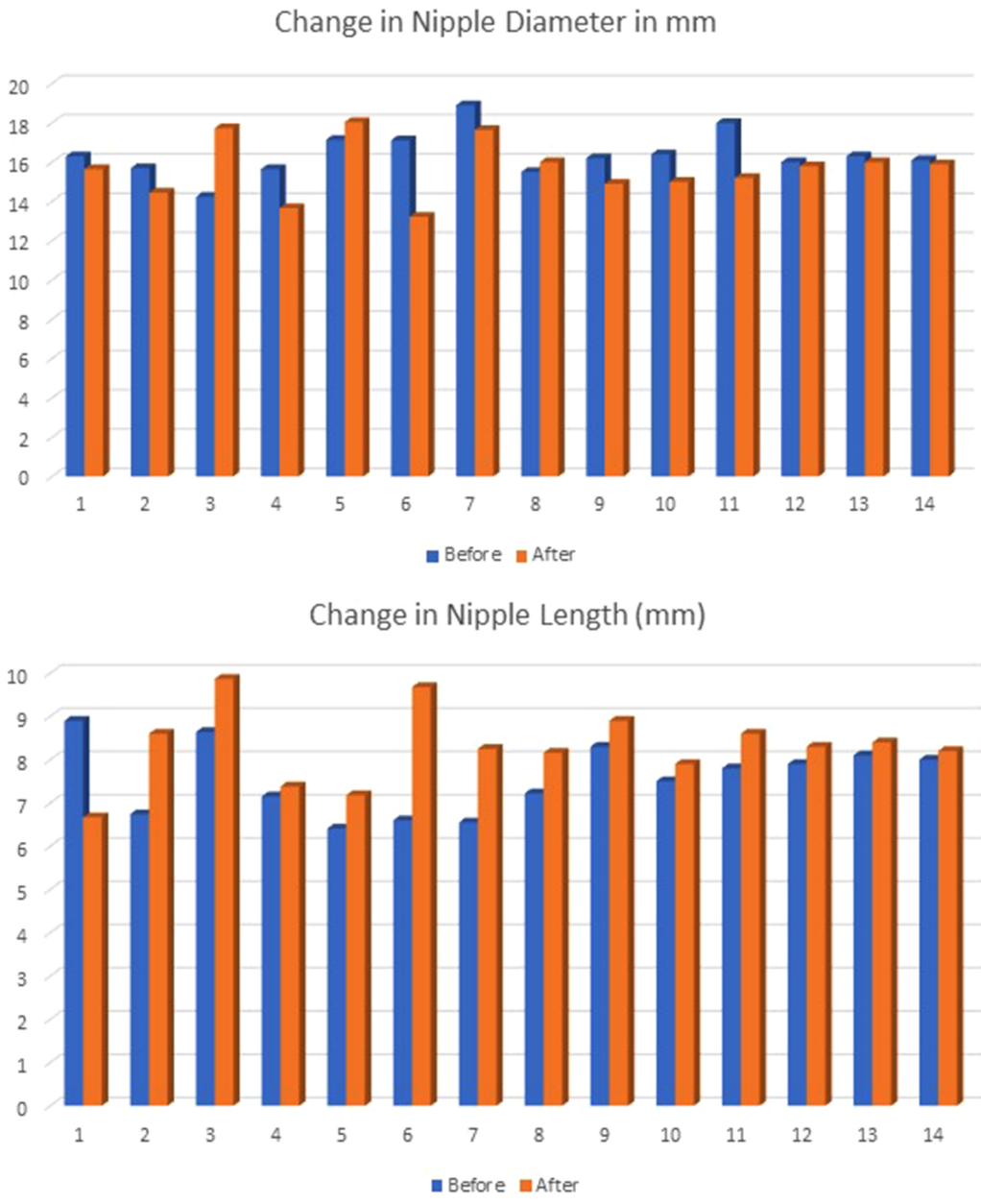

FIGURE 1
The values for nipple diameter and length were measured in millimeters before and after feeding and were not statistically significant in paired *t*-tests (diameter $p = 0.171$; length $p = 0.055$).

Nipple turgor, as measured by nipple length and diameter, was similar to those values that have been previously reported (45). The values, in this study, for before and after breastfeeding were not statistically significant and were not visually different after the baby disconnected from the nipple. Any change in nipple lengthening or diameter increases of the nipple were transitory and may be explained by the contraction of the muscle cells surrounding the nipple itself as occurs upon nipple stimulation, such as when the nipples are exposed to cold, becoming evert. The relationship to breast turgor, if any, needs further evaluation.

Breast turgor refers to the elasticity and firmness of the breast tissue and was used in this study to assess changes in fluid balance. Before the breastfeeding sessions, the breast tissue appeared firm yet elastic. The change seen in breast turgor indicates a fluid shift. Further analysis is needed to evaluate any relationship between change in breast turgor, maternal weight, and mass of milk transferred.

To initiate feeding at the breast, the infant's mouth is open wide, encompassing a significant portion of the areola and the nipple with the chin in firm contact (contact force) with the breast. This study's initial contact force is more robust and lessens with each successive let-down reflex. Contact force variation drops to zero by the end of the feeding. In this study, we observed that contact force was essential to milk ejection, particularly at the beginning of active feeding.

Breastfeeding includes active feeding and quiescent attachment. There are several muscles involved during both active feeding and

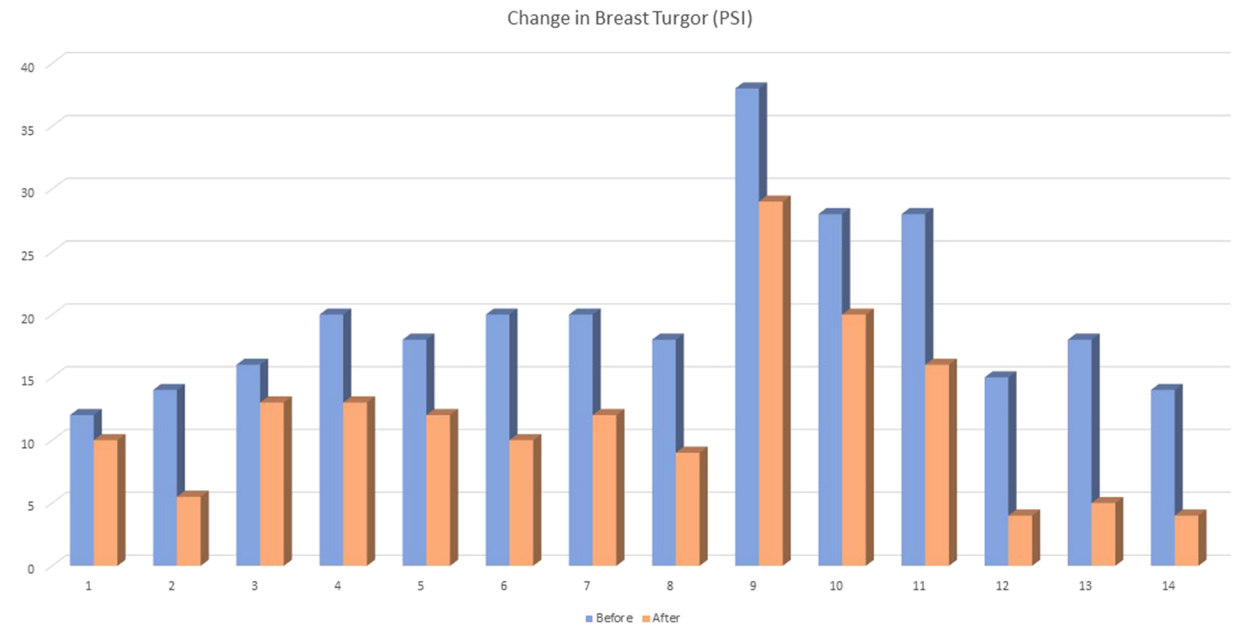

FIGURE 2
Breast turgor is measured in pounds per square inch before and after breastfeeding.

TABLE 1 Summary of the maximum, minimum, and range for contact force, oral pressure, and jaw movement.

	Contact Force (mN)			Oral pressure (mmHg)			Jaw Movement (mV)		
Session	Max.	Min.	Range	Max.	Min.	Range	Max.	Min.	Range
1	80.14	78.31	1.83	−12.68	−188.73	176.04	−6.1	−11.1	5.0
2	79.36	79.32	0.04	−9.5	−333.07	323.56	3.8	−4.4	8.1
3	79.22	79.17	0.05	−16.42	−217.71	201.29	6.5	−7.8	14.3
4	85.92	81.1	4.83	14.74	−162.52	177.25	9.3	−17.8	27.1
5	82.4	78.81	3.59	−19.59	−216.81	197.22	−0.1	−17.3	17.2
6	79.39	79.16	0.23	−18.75	−197.27	178.52	14.8	−14.3	29.1

quiescent attachment. The tongue plays a crucial role in breastfeeding. The infant's tongue moves in a wave-like motion, pressing against the breast and creating the movement necessary to elicit milk flow into the mouth. The coordinated movement of the tongue helps in moving milk from the breast to the mouth. The lips and chin location facilitate the maintenance the latch, while the jaw movement enables the anterior tongue to move as a lever to compress the teat formed by the nipple/areolar complex to control volume of the flow. The jaw and neck muscles are responsible for the undulating movement of the mid-tongue, enabling the baby to create negative pressure to keep the milk flowing. The jaw muscles coordinate with the tongue to create a rhythmic feeding motion. Various facial muscles are engaged during breastfeeding as well. The muscles around the mouth, including the orbicularis oris muscle, help form a secure seal around the breast to maintain suction. These muscles work together to ensure a proper latch and prevent milk leakage. The muscles in the cheeks, such as the buccinator muscles, play a supportive role during breastfeeding to hold the milk in the mouth until the airway is protected.

Fifty pairs of muscles and six cranial nerves working together for human beings to swallow (46). The buccal phase of swallowing is voluntary. The tip of the tongue encircles the nipple/areolar complex, compressing it against the alveolar ridge of the hard palate, while the posterior tongue drops to create a space for milk to be held until the air way is protected. The tongue surface moves upward, gradually expanding the area and squeezing the liquid bolus back along the palate and into the pharynx. It is important to note that the coordination and strength of these muscles develop and improve as the infant grows and gains feeding experience (47). Also, breastfeeding helps develop oral motor skills, muscles used in speech and swallowing later in life. Neurological maturation associated with experiential learning facilitates the transformation in feeding patterns of infants (48).

With respiration, on inspiration our findings demonstrated lower voltage reading, and on expiration, a higher voltage was demonstrated. The respiration rate was approximately 60 breaths per min using the nasal temperature sensor and the respiratory belt. Compared to the quiescent attachment, we see truncated

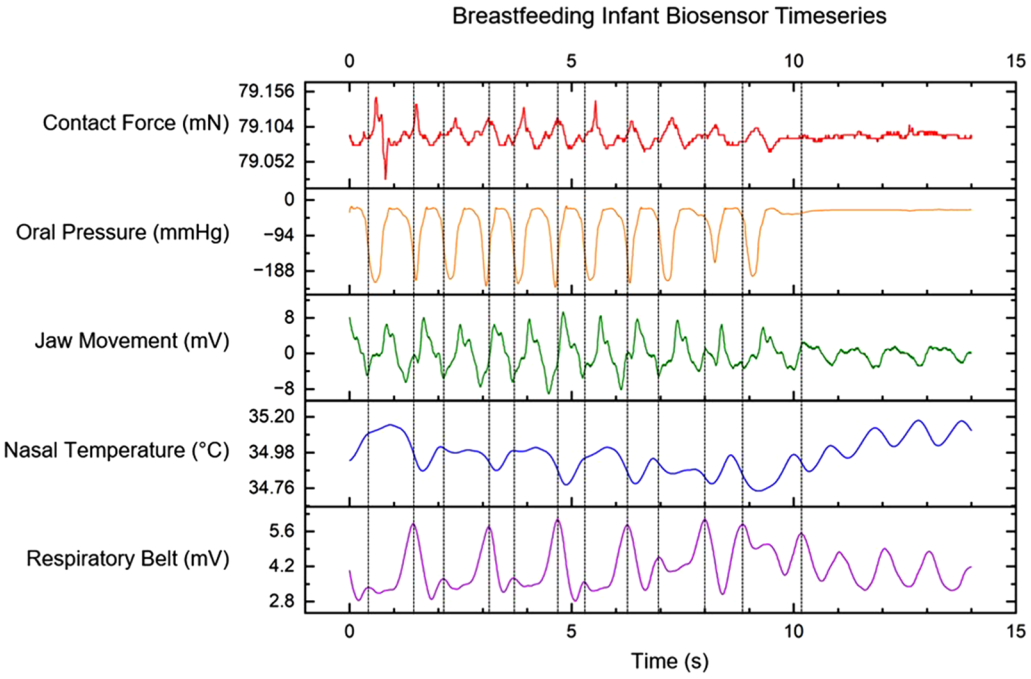

FIGURE 3
Multi-sensor time-series data for contact force between infant chin and breast, intraoral pressure from MEMs catheter, the signal from PVDF film for sensing jaw movement, air temperature measurements using a nasal cannula, and a signal from the respiratory belt. Vertical dashed lines indicate infant swallows observed by the clinician.

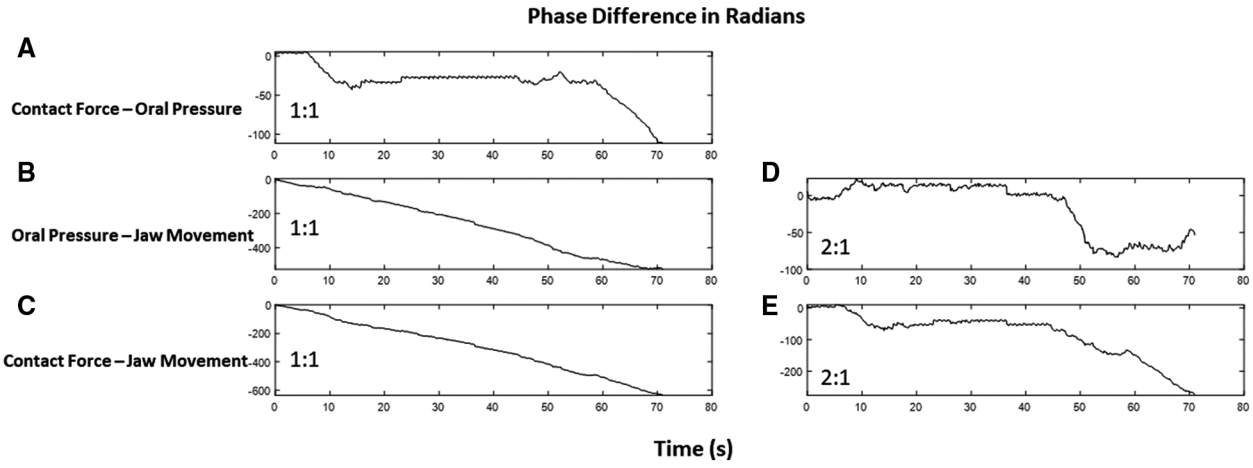

FIGURE 4
The phase difference between time series in radians with respect to time in seconds. The slope of line zero is an indication of locking. Contact force and oral pressure have a locking of 1:1 as the slope of the jittery phase difference remains zero at several time points (A), whereas phase difference linearly changes for oral pressure and jaw movement (B) as well as for contact force and jaw movement (C). Interestingly, these time series exhibit a 2:1 phase locking, as depicted in (D) and (E).

peaks, evidence of the infant's breath being held to swallow. There is an apparent synchronization between diaphragm expansion and observed swallows.

In this study, we observed that once the infant latched to the nipple-areolar complex, they use contact force to elicit milk ejection. They use their tongue, jaw, and facial muscles to manage milk flow. In active feeding, the tongue, palate, and cheeks trap the milk flowing into the mouth to create a bolus of milk, while the epiglottis closes over the larynx to protect the airway. The milk is then swallowed in coordinated pauses of breathing. Breastfeeding is a coordination of milk flow and infants' control of positive and negative pressure.

During active feeding, oral pressure is directly correlated with the movement of the jaw. During quiescent attachment, the oral pressure returns to a steady negative value near the latching pressure. Our findings show that negative oral pressure peaked

when the jaw was most extended, indicating that changes in oral pressure can be seen with the movement of the jaw. The negative oral pressure decreased as the jaw moved toward a neutral position, while maintaining latch pressure. Peak vacuum (−152 ± 38 mmHg) occurred when the jaw was in the lowest position. Positive pressure occurs when the jaw elevates, lips are sealed, and the mid-blade of the tongue elevates to the hard palate. Negative pressure occurs when the jaw drops, moving the tongue away from the hard palate, and the lips remain sealed. The amplitude of the jaw movement sensor drops by more than half to less than ±4 mV. The frequency of jaw motion is also reduced during active feeding.

Analysis of the pilot study data set for connections between the physiologic parameters found and the actual quantity/volume of milk transferred suggest relationships between some measurables and mass of milk transferred. For example, there appears to be a positive trend between the percent change of breast turgor and mass of milk transferred. Additionally, we can observe a similar positive trend between the 'active feeding/nutritive suckling" time and the mass of milk transferred. The "active feeding" time can be easily calculated by analyzing the contact force, oral pressure, and jaw movement sensor channels. By setting threshold values for frequency and amplitude that correspond to active latch and feeding as observed by a clinician, an estimate for the length of time of active milk transfer. These initial findings are suggestive of correlations between the amount of milk transferred and specific measures from biosensors, but the limited size of the pilot study dataset prevents a final conclusion on these observations.

Utilizing Systems Medicine to apply Systems Biology to the human milk transfer systems has enabled the identification and characterization of aspects of the biomechanical and physiologic components of the Maternal/Infant Lactation physioKinetics (MILK) system. With this framework, we can begin to systematically evaluate the dynamics of milk movement from the lactating breast to the infant stomach for predicting function, preventing malfunction, personalizing interventions, and resolving the problem with participatory engagement of the mother and infant.

Despite the small sample size, we can discern and quantify that nipple and breast turgor, jaw movement and swallowing are associated with the mass of milk transferred to the infant stomach. More investigation is needed to better quantify mass of milk transferred and understand how the process behaves over time and under varying conditions such as infants who are not gaining weight appropriately or pain for the mother when breastfeeding.

Based on our observations and data analysis, we conclude that the negative change in intraoral pressure is a function of jaw movement rather than the infant applying negative pressure to remove milk. Both active and quiescent feeding movements are a coordination of mandibular protrusion and retrusion. This jaw motion appears to be a regulatory or driving mechanism behind both the contact force and oral pressure readings. Both analysis methods strongly support a quantitative coherence between jaw movement with oral pressure and contact force. Additional research is needed to make further conclusions about the regulatory mechanisms of the Maternal/Infant Lactation physioKinetics (MILK) system.

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 humans were approved by University of Texas at San Antonio Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JF: Conceptualization, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. PF: Investigation, Methodology, Writing – review & editing. MN: Investigation, Methodology, Writing – review & editing. PI: Formal Analysis, Methodology, Writing – review & editing. DD: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

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The protective associations of breastfeeding with infant overweight and asthma are not dependent on maternal FUT2 secretor status

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Breastfeeding supplies infant gut bacteria with human milk oligosaccharides (HMOs) as a nutrient source. HMO profiles are influenced by the FUT2 gene, which encodes an enzyme affecting the fucosylation of milk sugars. 20 to 40% of individuals have a "non-secretor" polymorphism that inactivates the FUT2 gene, resulting in variable HMO proportions in milk. This has engendered a concerning, yet unfounded, perception that non-secretor milk is "inferior." To address this untested hypothesis, we re-analyzed two datasets in which we previously showed that breastfeeding was protective against early life asthma and excessive infant weight gain in the Canadian CHILD Cohort Study. Using stratified regression models, we found that the protective association of exclusive breastfeeding and infant asthma was not modified by maternal secretor status (secretors aOR: 0.53, 95% CI 0.31 to 0.92; non-secretors aOR: 0.36, 95% CI 0.12 to 1.04; *p* for interaction = 0.50, N = 2086 children). Similarly, the association of breastfeeding with lower infant BMI and weight gain velocity did not vary by maternal secretor status (infant BMI: secretors aβ −0.47, 95% CI −0.66 to −0.29; non-secretors aβ −0.46, 95% CI −0.78 to −0.13; *p* for interaction = 0.60; N = 1971 infants). Our results indicate that secretor and non-secretor mothers can equally promote infant growth and respiratory health through breastfeeding. These findings run contrary to the idea that non-secretor milk is an inferior food source, and instead reify the importance of breastfeeding for all infants. The results of this study can inform feeding recommendations that are applicable to all infants, regardless of maternal secretor status.

KEYWORDS

infant feeding, human milk, maternal secretor status, infant asthma, infant weight gain

Introduction

Breastfeeding provides myriad benefits to infants, including supplying infant gut bacteria with human milk oligosaccharides (HMOs) as a nutrient source (1). HMO profiles are strongly influenced by the FUT2 gene, which encodes an enzyme that affects the fucosylation of milk sugars (2). Globally, 20% to 40% of individuals (3) have a "non-secretor" polymorphism that inactivates the FUT2 gene. These individuals display

altered enzymatic activity and a distinct milk profile that is enriched with certain HMOs, yet contains lower proportions of others (4). One HMO in particular, 2′-fucosyllactose (2'FL), is virtually absent in the milk of non-secretors (4). In contrast, 2'FL is typically the most abundant HMO in secretor milk.

Alongside growing interest in HMOs and their role in infant development, there is a concerning and largely unfounded perception that non-secretor milk is somehow "inferior (5)." In one study, the consumption of non-secretor milk was associated with delayed infant gut colonization by HMO-utilizing bacteria that are critical for immune system development (6), though other studies have failed to find associations between maternal secretor status and infant gut bacterial communities (7). Alongside variation in the gut microbiome based on maternal secretor status, evidence of a relationship between 2'FL exposure and infant cognitive development (8) has led to the speculation that non-secretor milk is "deficient." However, the hypothesis that non-secretor milk is an inferior food source for infants has not been fully tested. These data are essential to informing public health messaging and research priorities related to maternal secretor status, infant feeding, and health. To address this gap, we re-analyzed two datasets in which we previously showed that breastfeeding was protective against early life asthma and excessive infant weight gain. The current analysis was motivated by the central question: *are the benefits of breastfeeding dependent on maternal secretor status?*

Methods

Study population

We leveraged data from two previously published studies that explored associations between infant feeding mode and asthma at 3 years (9), as well as BMI z score and weight gain velocity at 1 year (10) in the Canadian CHILD Cohort Study. This research was approved by the Human Research Ethics Boards at McMaster University and the Universities of Manitoba, Alberta, Toronto, and British Columbia.

Secretor status, asthma diagnosis, and infant body mass index

Secretor status was defined using the methods in Moossavi et al. (11), for genotyping of the rs601338 and rs1047781 single nucleotide polymorphism in the *fucosyl-transferase* 2 (FUT2) gene. Asthma at 3 years of age was diagnosed (as possible or probable) by a healthcare professional, using medical history and physical examination (9). The diagnosis of "possible or probable asthma" reflects the fact that a firm asthma diagnosis is generally not possible until later in childhood. Body mass index was determined from weight and length measured by CHILD Study staff at the 12-month clinical assessment (10). Since measures of underweight, including stunting and wasting, are not common in this infant population (as evidence by the positive median z-scores), lower z-scores (i.e., closer to zero) in the current study indicate healthier body composition and weight gain trajectories. Weight gain velocity was calculated as the change in weight for age z-score during the period from birth to 12 months.

Statistical analysis

We used base packages in R to clean and subset the data, allowing model stratification by maternal secretor status. The glm function within the *stats* package (12) was used to stratify regression models that included infant feeding mode as the exposure and either asthma diagnosis, BMI z score, or weight gain velocity as the outcome. We also formally tested for interactions between maternal secretor status and infant feeding mode for asthma at 3 years (logistic regression) and BMI z score and weight gain velocity at 1 year (linear regression). All models were adjusted for the same covariates used in the original studies (9, 10). Unadjusted model results are presented in Supplementary Figures S1, S2. All plots were generated using the *ggplot2* package (13). The figures display frequency data within the CHILD study, where participants represent a sample of the Canadian population. Since error bars help to visualize the (un)certainty in this estimate, they were calculated and included in the figures as a representation of the confidence intervals associated with sub-sampling from a larger population (9).

Results

We found no evidence of an interaction between infant feeding mode and maternal secretor status for any of the health outcomes. The protective association of exclusive breastfeeding with asthma was not modified by maternal secretor status (p for interaction = 0.50; N = 2086 children) (Figure 1). Adjusting for covariates, infants exclusively breastfed by secretor mothers showed a 47% reduction in the risk of asthma at 3 years (aOR: 0.53, 95% CI 0.31 to 0.92) compared to infants who received only formula. Similarly, exclusively breastfed infants of non-secretor mothers displayed a 64% reduction in the risk of asthma at 3 years (aOR: 0.36, 95% CI 0.12 to 1.04). The unadjusted models produced similar results (secretor mothers: OR: 0.48, 95% CI 0.29 to 0.77; non-secretor mothers: OR: 0.56, 95% CI 0.23 to 1.40) (Supplementary Figure S1).

Similarly, the association of breastfeeding with healthier (lower) infant BMI and weight gain velocity did not vary by maternal secretor status (p for interaction = 0.60 and 0.81; N = 1971 and 1955 infants, respectively) (Figure 2). For example, in covariate-adjusted models, infants exclusively breastfed by a secretor mother had a 0.47 SD lower BMI z-score (aβ −0.47, 95% CI −0.66 to −0.29) compared to formula fed infants; virtually identical to the effect estimate for those exclusively breastfed by a non-secretor mother (aβ −0.46, 95% CI −0.78 to −0.13). The unadjusted models showed similar results (secretor mothers: β −0.59, 95% CI −0.77 to −0.42; non-secretor mothers: β −0.51, 95% CI −0.83 to −0.19) (Supplementary Figure S2).

Discussion

We provide new evidence that the benefits of breastfeeding related to infant weight gain, body composition, and early childhood

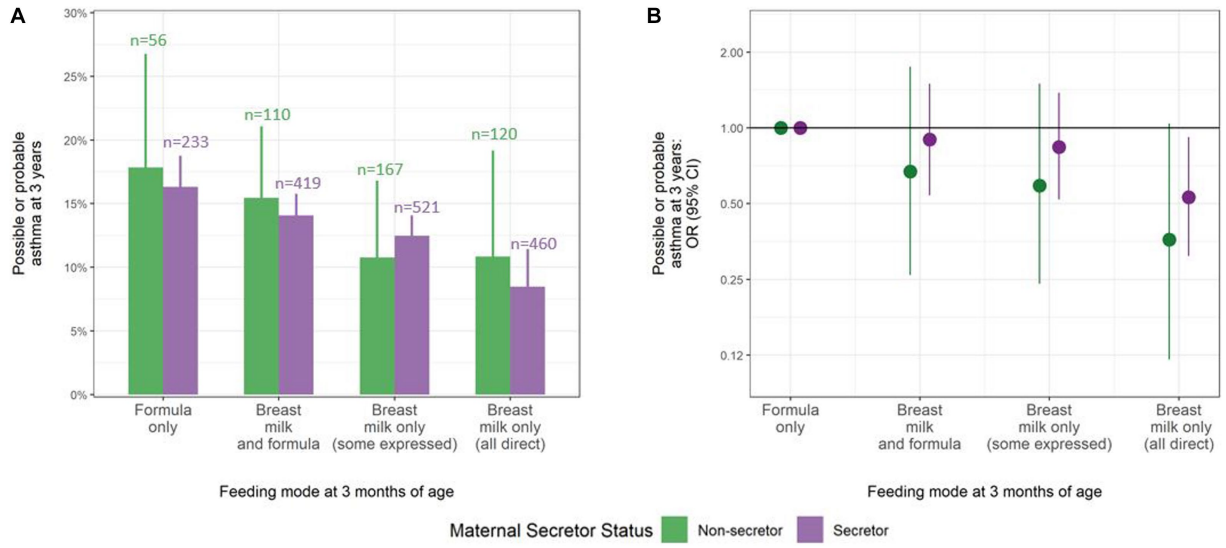

FIGURE 1
Consistent protective association between breastfeeding at 3 months and possible or probable asthma at 3 years, regardless of maternal secretor status. **(A)** Frequency of possible or probable asthma at 3 years by feeding mode at 3 months (total N = 2086). The values above the bars indicate the number of children in each feeding group, stratified by maternal secretor status. **(B)** Adjusted odds ratios (ORs) of possible or probable asthma at 3 years by feeding mode at 3 months. ORs adjusted for infant sex, maternal asthma, ethnicity, method of birth, daycare attendance, and gestational age, with multiple imputation of missing data. Lines represent 95% confidence intervals that display the (un)certainty in sub-sampling study participants from a larger population.

asthma are not dependent on maternal secretor status. Compared to receiving secretor milk, the consumption of non-secretor milk appears to provide a similar degree of dose-dependent protection against asthma and infant overweight. These data run contrary to the idea that non-secretor milk is an inferior food source for infants, and instead reify the importance of breastfeeding, regardless of maternal secretor status.

Further research should explore these associations in relation to other health outcomes and feeding scenarios. For example, data from the UK ALSPAC cohort suggest potentially enhanced protective effects of non-secretor milk against infant diarrhea (14). It will be particularly important to explore this relationship in contexts where infant mortality due to infectious disease remains high, as infections may provide selection pressure on the non-secretor polymorphism in these settings. Additionally, studies should further explore the connections between specific HMOs, the infant gut microbiome, and health outcomes in early life. Key bacterial colonizers, such as *Bifidobacterium*, may be able to utilize a range of HMOs beyond 2'FL (11). If so, then the benefits of breastfeeding that are likely mediated by the infant gut microbiome— including those explored in the current study (15, 16)— may extend to all breastfeeding infants, regardless of maternal secretor status and the associated abundance of 2'FL in milk.

Maternal secretor status may also be relevant to human donor milk, where the HMO profile of the pregnant person may not match the HMO profile of donor milk consumed postnatally. With evidence of fetal exposure to HMOs, including 2'FL, in amniotic fluid (17), infants may be primed to receive a specific amount or profile of HMOs *in utero*. While additional research is needed to understand the mechanisms and consequences of fetal exposure to HMOs, evidence of this phenomenon is in line with the Developmental Origins of Health and Disease literature, which posits that intrauterine exposure to bioactive molecules has a profound impact on birth outcomes and infant physiology (18). In the context of maternal secretor status, it may be that HMOs in amniotic fluid provide a "signal" of the composition of milk to which the infant will be exposed postnatally. Additional research on potential discordance between HMO exposure before and after birth is warranted. Future research should also consider the influence of *infant* secretor status on health outcomes (19), including the potential biological impacts of "mismatched" maternal–infant secretor status.

Overall, our current results indicate that secretor and non-secretor mothers can equally promote infant growth and protect against asthma through breastfeeding. These findings can inform feeding recommendations that are applicable to all infants, independent of maternal secretor status.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: data will be made available upon request from the CHILD Cohort Study as described at https://childstudy.ca/for-researchers/data-access/. Requests to access these datasets should be directed to child@mcmaster.ca.

Ethics statement

The studies involving humans were approved by The Human Research Ethics Boards at McMaster University and the

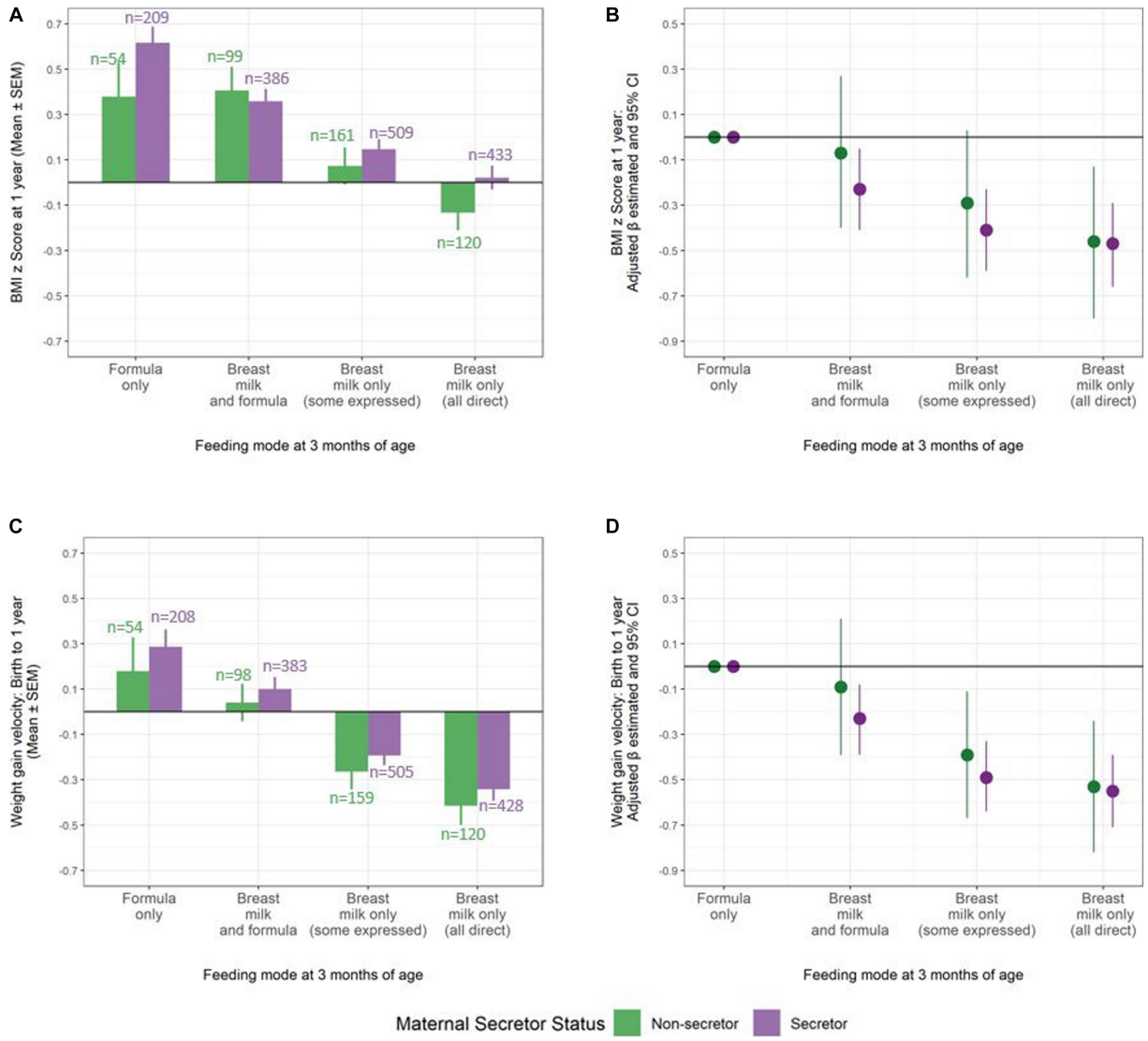

FIGURE 2
Consistent beneficial association of breastfeeding at 3 months with BMI z score and infant weight gain velocity at 1 year, regardless of maternal secretor status. **(A)** Mean BMI z score and **(B)** adjusted β estimates for BMI z score by infant feeding mode at 3 months (N = 1971 dyads). **(C)** Mean weight gain velocity and **(D)** adjusted β estimates for weight gain velocity by infant feeding mode at 3 months (N = 1955 dyads). Models adjusted for study site, maternal age, pre-pregnancy BMI, ethnicity, education, marital status, smoking during pregnancy, mode of delivery, parity, infant sex, gestational age, and birth weight. Lines represent 95% confidence intervals that display the (un)certainty in sub-sampling study participants from a larger population.

Universities of Manitoba, Alberta, Toronto, and British Columbia. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MA conceptualized and designed the study, and critically reviewed and revised the manuscript. MM drafted the initial manuscript and critically reviewed and revised the manuscript. SG carried out the initial analyses and critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MA receives research funding from the Canadian Institutes of Health Research, Research Manitoba, the Canada Foundation for Innovation, the Bill and Melinda Gates Foundation, the Manitoba Children's Hospital Foundation, Prolacta Biosciences, Mitacs, CIFAR and the Garfield G. Weston Foundation. She regularly speaks at conferences and workshops on infant nutrition, some sponsored by Medela, the Institute for the Advancement of Breastfeeding & Lactation Education and Prolacta Biosciences. She has contributed without remuneration to online courses on breast milk and the infant microbiome produced by Microbiome Courses.

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.2023.1203552/full#supplementary-material

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An analysis of actors participating in the design and implementation of workplace breastfeeding interventions in Mexico using the NetMap analysis approach

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Introduction: While breastfeeding is recognized as providing optimal nutrition for infants and toddlers, maternal employment is a commonly mentioned barrier to breastfeeding. The goal was to (a) identify key actors participating in the design and implementation of workplace breastfeeding interventions in Mexico, (b) understand the complexity of interactions between the actors, and (c) map the connections and influence between the actors when looking into networks of Advice, Command, Funding, and Information.

Method: Following the NetMap methodology, a total of 11 semi-structured interviews with 12 interview partners from 10 organizations were conducted. Interview data were analyzed, and networks were analyzed and visualized, using a social network mapping software.

Results: A total of 83 actors from five different actor groups were identified. Four networks were constructed along the four types of connections: Advice, Command, Funding, and Information. The actors were connected by 580 connections with 446 unique links. Based on various network statistics, the Mexican Institute of Social Security, the Mexican Secretary of Labor and Social Welfare, UNICEF, and the Mexican Secretary of Health were identified to be key actors.

Conclusion: To increase the likelihood of success of workplace breastfeeding interventions, the role of the actors "Employers" and "Women" needs to expand. They should be actively involved in the decision-making process, together with the identified key actors. It is further recommended to re-introduce a national breastfeeding strategy for Mexico that includes policies for workplace breastfeeding interventions.

KEYWORDS

breastfeeding support, lactation support, lactation program, working mothers, workplace, social network analysis, NetMap analysis, Mexico

1. Introduction

Breastfeeding is recognized by authoritative bodies as providing optimal nutrition for infants and children for at least the first 2 years of age. The World Health Organization (WHO) and UNICEF recommend the initiation of breastfeeding within 1 h after birth, exclusive breastfeeding for the first 6 months and continued breastfeeding accompanied with the introduction of complementary food for at least the first 2 years of life (1). Breastfeeding provides short- and long-term health benefits to the breastfeeding mother as well as to the breastfed infant and child. Breastfeeding protects mothers against the risk for breast cancer, ovarian cancer, and type 2 diabetes, while breastfed children have a lower risk for morbidity and mortality from infectious diseases, increased intelligence scores and a risk reduction for overweight and potentially for type 2 diabetes later in life (2–4). As such, it is important for governments to invest in improving breastfeeding outcomes among their populations.

Maternal employment and return-to-work after childbirth are commonly mentioned barriers to breastfeeding (5, 6). At the time of writing, 649 million women in the world who work in the formal or informal economy do not have access to adequate maternity benefits (7). In Mexico, according to data from the 2018–2019 National Health and Nutrition Survey (ENSANUT), the prevalence of exclusive breastfeeding in children under 6 months was 28.3%, while women who reported to have a paid job showed an even lower prevalence of 23.2% for the same indicator (8). While the provision of lactation rooms and nursing breaks are considered to be low-cost interventions (9), workplace lactation interventions have been shown to be positively associated with higher breastfeeding rates and longer breastfeeding duration (10) as well as with reduced absenteeism and improved workplace performance, commitment and retention (11, 12). An increasing number of governments and employers are introducing measures to support working mothers in reaching their breastfeeding goals (13) which enables families to better combine their work responsibilities with their infant feeding goals. In order to support the compatibility of family and work responsibilities for more families and to support governments to reach the Sustainable Development Goals by 2030 (14), there is a need for strengthening current workplace interventions and for countries to introduce more robust guidelines and incentives to stimulate more employers to offer more workplace accommodations and support for nursing women.

To guide the scaling up of effective workplace breastfeeding interventions across different contexts, evidence-based policies are needed. One barrier in creating evidence-based policies that are tailored to different contexts is the involvement of local actors in the decision-making process. While there are different types of policy instruments (15, 16), there is a lack of evidence specifically informing decision making on different options across different contexts. To bring the right people to the table around such discussions, it is important to understand the key actors who are currently participating or who have the potential to engage in the design and implementation of sustainable workplace breastfeeding interventions in Mexico.

Breastfeeding interventions are complex service interventions (17) in which multiple actors are involved. Social network analysis methodologies can help to reach the goal of having a holistic understanding of the involved key actors and how they are connected and influence each other. This will be key for (a) selecting, co-designing and successfully implementing workplace breastfeeding support policies and interventions, (b) understanding the roles that different actors should have individually and as a complex network involving multidirectional interactions between actors, and (c) integrating multiple views into an effective consensus process to eventually agree on the choice of policies and corresponding interventions that are collectively endorsed by the network of actors.

The goals of this study were to (a) identify key actors participating in the design and implementation of successful workplace breastfeeding interventions in Mexico, (b) understand the complexity of interactions between the various actors in this area, and (c) map in detail how the actors are connected and influence each other across different networking dimensions including Advice, Command, Funding, and Information. The analysis of the actor networks will be conducted following the NetMap methodology as it allows to aggregate the expertise of multiple key informant partners into a common understanding of the field.

2. Methods

2.1. Ethical disclosure

The study was approved by the Ethics Committee from Universidad Iberoamericana Mexico City and received IRB exemption from the Institutional Review Board of Yale University. A signed written privacy statement was obtained from all interview participants.

2.2. Study design and approach

The main goals of the study were to identify key actors for designing and implementing successful workplace breastfeeding interventions for the Mexican context and to gain a holistic understanding of the interactions between the actors. To complete these goals, we conducted a stakeholder analysis using the NetMap methodology developed by the International Food and Policy Research Institute (IFPRI) (18, 19). It combines social network analysis and power mapping activity in a participatory interview technique. NetMap helps to understand and visualize the actor network at play and to identify key actors that are involved in a given area (19).

The NetMap interviews were structured in three steps: (1) *Actor mapping* – the goal of this step was to identify and visualize all possible key actors who are involved in successful workplace breastfeeding interventions in Mexico. This step was designed to answer the primary research question of "Who is involved in the successful design and implementation of workplace breastfeeding interventions in Mexico?" During this step, interview partners were asked to name all the actors that they could think of and assign them to one of the following four actor groups: government, non-governmental organization (NGO), academic or research institution, or other. During the Zoom interviews, actors were written in color-coded text boxes (according to the assigned actor group) or on color-coded self-adhesive paper notes for the in-person interviews. (2) *Linking actor networks* – the goal of this step was to understand and visualize the ways in which the actors are connected to one another. The interview partners were asked to connect the actors to one another, indicating the flow of power/influence between them. The flow could be identified as either

TABLE 1 Types of connections between actors used to identify networks of actors participating in the design and implementation of workplace breastfeeding interventions in Mexico.

Type of connection	Description
Advice	Actors are linked by giving or receiving advice (e.g., one actor explains another actor how to do something best)
Command	Actors are linked by giving or receiving commands (e.g., one actor tells the other what to do)
Funding	Actors are linked by giving or receiving money or financial incentives (e.g., one actor funds a project of another actor)
Information	Actors are linked by giving or receiving information (e.g., one actor gives out information about something to another actor)

TABLE 2 Organizations represented by interviewees during the NetMap interviews to identify key actors participating in the design and implementation of workplace breastfeeding interventions in Mexico.

Actor group	Number of interviews	Number of interview partners	Organizations represented
Government	4	5	IMSS, STPS, CNEGSR, SIPINNA
NGO	2	2	Pacto por la Primera Infancia, ACCLAM
Academia/Research	2	2	IBERO, INSP
International organizations	2	2	UNICEF
Others	1	1	PALMA
Total	11	12	10

ACCLAM, Association of International Board Certified Lactation Consultants in Mexico; CNEGSR, National Center for Gender Equity and Reproductive Health; IBERO, Universidad Iberoamericana Mexico City; IMSS, Mexican Institute of Social Security; INSP, National Public Health Institute; PALMA, Proyecto de Apoyo a la Lactancia Materna; SIPINNA, National System for the Protection of Children and Adolescents; STPS, Secretariat of Labor and Social Welfare; UNICEF, United Nations Children's Fund.

unidirectional (i.e., from actor A to actor B but not from actor B to actor A) or bidirectional (i.e., from actor A to actor B and vice versa). Connections were color-coded based on the four types of connection presented to the interview partners (Table 1). (3) *Power mapping* – the goal of this last step was to identify and visualize the relative power/level of influence that actors on the map had over one another in relation to the successful design and implementation of workplace breastfeeding interventions in Mexico. To complete this last activity, interview partners were asked to assign relative power to each actor on a scale from 0 (this actor does not at all influence the success of workplace breastfeeding interventions in Mexico) to 5 (this actor influences the success of workplace breastfeeding interventions in Mexico).

2.2.1. Identification of interview partners

A preliminary list of potential interview partners was identified by two of the co-authors (SH-C and MV-C) based on the co-authors' comprehensive knowledge of and engagement with breastfeeding policies in Mexico. The list included individuals representing government agencies, NGOs, academic organizations/research institutions, international organizations as well as business associations, all of which were expected to have knowledge on workplace breastfeeding interventions in Mexico. Informal invitations, including a short description of the study, were sent via email by the co-authors (VL-M or SH-C) to clarify general interest for participation. Invitees that expressed interest in interview participation received a formal invitation by email. After acceptance, the interview partners received a written privacy statement with the request to sign and send back to the research team prior to the interview. In total, 14 formal invitations to representatives of government agencies ($n = 4$), NGOs ($n = 2$), academic organizations/research institutions ($n = 2$), international organizations ($n = 2$) and others ($n = 4$) were sent. Of the 14 key informants invited, 12 agreed to be interviewed.

2.2.2. Interviews

All but one of the interviews were conducted with one interviewee. One interview was conducted with two interview partners following the request of the participating organization given the complementary expertise of the two interview partners. This resulted in a total of 11 interviews with 12 interview partners. Of the 11 interviews, four interviews were conducted with representatives from governmental organizations, two interviews each were held with representatives from NGOs, academia/research institutions, and international organizations, respectively, and one interview was conducted with a representative of an organization classified as "other." Table 2 gives an overview of the organizations represented during the interviews. The interviews were conducted in English ($n = 6$) or in Spanish ($n = 5$) depending on the preference of the interview partner. All but one of the interviews were conducted online using the Zoom platform. The one in-person interview was conducted in the office of the interviewee.

Interviews were led by a co-author (KL or VL-M) using a semi-structured interview guide developed for this study (Supplementary material). The other co-author (VL-M or KL) acted as note taker during the interviews. All interview materials were developed in English and then translated into Spanish. For the online interview, a Microsoft PowerPoint template (Supplementary Figure 1) was developed including color-coded text boxes for the actor mapping, color-coded arrows for the linking actor networks and pictograms of "power towers" for the power mapping activity. The Microsoft PowerPoint template was shared with the interview partner using the "share screen" function on the Zoom platform and remote control was given to the interviewees to populate the maps. For the in-person interview, a white board, color-coded self-adhesive paper notes for the actor mapping, white-board markers for the linking actor networks activity and "power tower" notes for the power mapping activity were used. Upon request of the interviewees, the guiding interview questions were shared with the interviewees prior to the interview.

During each interview, three maps – one per interview step (described earlier), were created and a picture of each of the maps was taken. All interviewees agreed for the interviews to be video and/or audio recorded. The interviews were conducted between October 13, 2022, and December 2, 2022, and lasted between 80 and 110 min, each.

2.3. Data management and analysis

Data on actor names, actor group allocation, assigned relative power as well as links between actors were entered in a separate Microsoft Excel sheet for each interview. Actor data (name and group allocation) were compared across all 11 interviews to ensure consistency. Any inconsistency in actor data was recoded based on the majority of responses across the interviews. During the recoding, a new actor group labeled "UN Organization" was added. Recoding was discussed among the co-authors until a consensus was reached.

Actor data from all 11 interviews were merged and the number of actor citations were reported from the combined data. A weighted average relative power for each actor based on the formula presented below was calculated (Eq. 1). For every interview j that did not mention actor i, we assigned a relative power of 0 to actor i for interview j. To reduce the background noise potentially created by actors cited only once, we excluded all single-cited actors from the analysis if the single-cited actor was assigned a relative power equal or less than 1 and was not part of any links among actors ($n = 9$, see Supplementary Table 1). This decision was made since it is unlikely that actors that were only cited by one interviewee, were assigned a low relative power and that were not part of any interaction will play an important role in the successful design and implementation of workplace breastfeeding interventions in Mexico.

Equation 1. Formula to calculate weighted average relative power for actor i across all j interviews.

$$\text{Weighted average relative power for actor } i = \frac{\sum_{j=1}^{11} \text{Relative power}_{ij}}{11}, \text{ where } j \text{ the interview}$$

Data from the linking actors network step from all 11 interviews were merged into one Microsoft Excel sheet for every type of connection (i.e., advice, command, funding, and information). Every link was weighted according to how many times the directed relation between the two actors was mentioned across the interviews (i.e., a relationship from one actors to another received a weight of 1 if it was mentioned in only one interview and a weight of 11 if it was mentioned in all 11 interviews). All merged data sheets were imported into the Gephi (version 0.10.0) network analysis software (20).

A network of actors was created for every type of connection (i.e., advice, command, funding, and information). Every network consists of actors represented by a node and links represented by arrows connecting two actors together. Nodes were color-coded based on their actor group (pink for governmental organizations, yellow for NGOs, green for academic/research institutions, blue for UN organizations and orange for "others") and sized proportionally to their weighted average relative power. Larger nodes represent actors with higher weighted average relative power. The maps of actor networks are a representation of the summative views and experiences of the interview partners as actors and links are reported as stated by the interviewees. Each map was created by using the Yifan Hu algorithm (21). Following the Yifan Hu algorithm, minor cosmetic adjustments were undertaken to increase the readability of the maps: Overlapping nodes (actors) were moved such that they do not overlap in the final map, using the Gephi's Noverlap plugin (22).

Network density and average degree as well as measures of centrality are used to describe the networks. While network density and average degree are network-level descriptive statistics and help to understand the network as a whole, measures of centrality are node-level descriptive statistics and help to understand the role of single actors within the network and compare actors within the same network. An overview of used network statistics, their definition and importance can be found in Table 3. In the following, we will report the actor distribution across actor groups (i.e., government agency, NGO, UN organization, university/research institution, and others), the number of times each actor was cited across all interviews as well as an evaluation across the four resulting networks.

TABLE 3 Definition and importance of statistics used to describe social networks (23, 24).

Statistics	Definition	Used for
Network-level		
Average degree	Average number of connections per node	To describe connectivity of network
Density	Percentage of all possible links that exist in the network	To describe connectivity of network
Node-level		
Degree/degree centrality	Number of connections a node has	To find very connected nodes
In-degree centrality	Number of incoming connections a node has	To find nodes that are largely receiver of a connection
Out-degree centrality	Number of outgoing connections a node has	To find nodes that are largely starting a connection
Betweenness centrality	Extend to which a node connects other nodes that are not otherwise connected	To find nodes that influence the flow around the system
Closeness centrality	Measure for how close a node is to all other nodes	To find nodes that are well placed to influence other nodes
Weighted average relative power	Average power an actor has to influence the success of workplace breastfeeding interventions in Mexico	To find influential actors

3. Results

3.1. Characteristics of actors

After excluding single-cited actors with an assigned relative power of 0 or 1 and without any links in any of the networks, a total of 83 actors were included in the analysis. The majority of actors ($n = 37$) were from governmental organizations, followed by organizations labeled as "Others" ($n = 17$), NGOs ($n = 14$), academic or research institutions ($n = 9$) and UN organizations ($n = 6$; Figure 1). Examples of actors that were assigned to the actor group "Others" included "Employers," "Business groups," "Women," "Private companies," "Families and colleagues," "Social media," "Healthcare professionals," and "Media." The actor group of "UN Organization" was introduced after the researchers examined the interview data. Supplementary Table 2 lists all actors, their assigned actor group as well as the number of citations and the weighted average relative power.

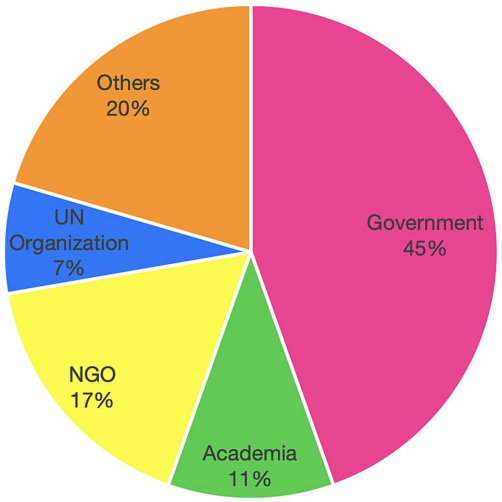

FIGURE 1
Distribution of actors participating in the design and implementation of workplace breastfeeding interventions in Mexico; based on actor groups.

Of the 83 actors, 42 actors were only mentioned in one interview (Figure 2). The Mexican Institute of Social Security (IMSS: Instituto Mexicano del Seguro Social), the Secretariat of Labor and Social Welfare (STPS: Secretaría del Trabajo y Previsión Social) and UNICEF were mentioned in all 11 interviews. The Universidad Iberoamericana Mexico City (IBERO) was mentioned in 10 of the 11 interviews, the Secretariat of Health (SALUD) was mentioned in 9 interviews, and the Association of International Board Certified Lactation Consultants in Mexico (ACCLAM: Asociación de Consultores Certificados en Lactancia Materna), the National Public Health Institute (INSP: Instituto Nacional de Salud Pública) and La Leche League were mentioned in 8 interviews.

The weighted average relative power of actors ranged from 0.00 to 4.00 (Figure 3). IMSS (4.00), STPS (3.82), and UNICEF (3.73) were the actors with the highest weighted average relative power. Five actors (Employers, INSP, IBERO, Federal legislators and SALUD) had weighted average relative powers between 2.01 and 3.00 while 9 actors (Chamber of Deputies, State Secretariats for Health, Institute for Social Security and Services for State Workers, La Leche League, breastmilk substitute industry, ACCLAM, Women, Pacto por la Primera Infancia, and Business groups) had weighted average relative powers between 1.01 and 2.00. The majority of the actors ($n = 66$) had weighted average relative powers between 0.00 and 1.00 with 49 actors with a weighted average relative power between 0.09 and 0.39 and 2 actors (Media and Labor unions) with a weighted average relative power of 0.00 indicating that these actors were perceived by the interviewees as not influencing the success of workplace breastfeeding interventions in Mexico.

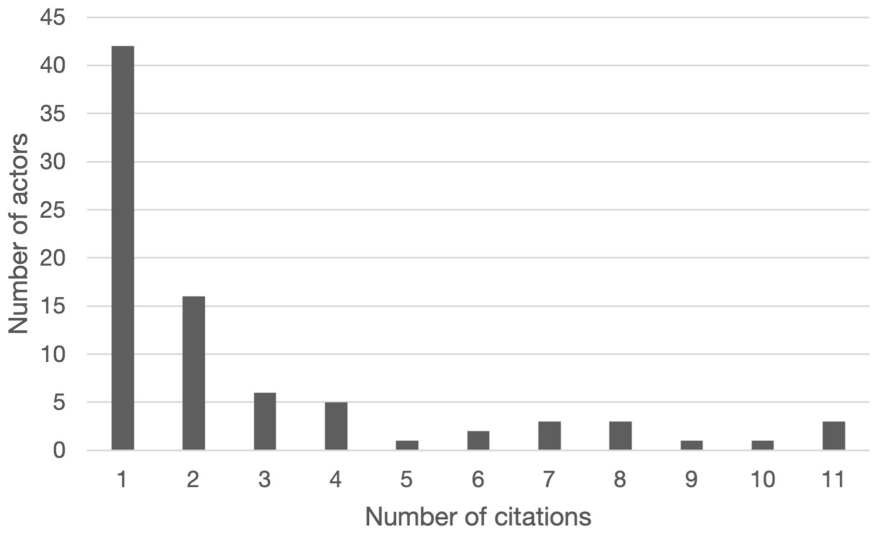

FIGURE 2
Frequency at which actors were mentioned during the 11 NetMap interviews to identify actors participating in the design and implementation of workplace breastfeeding interventions in Mexico.

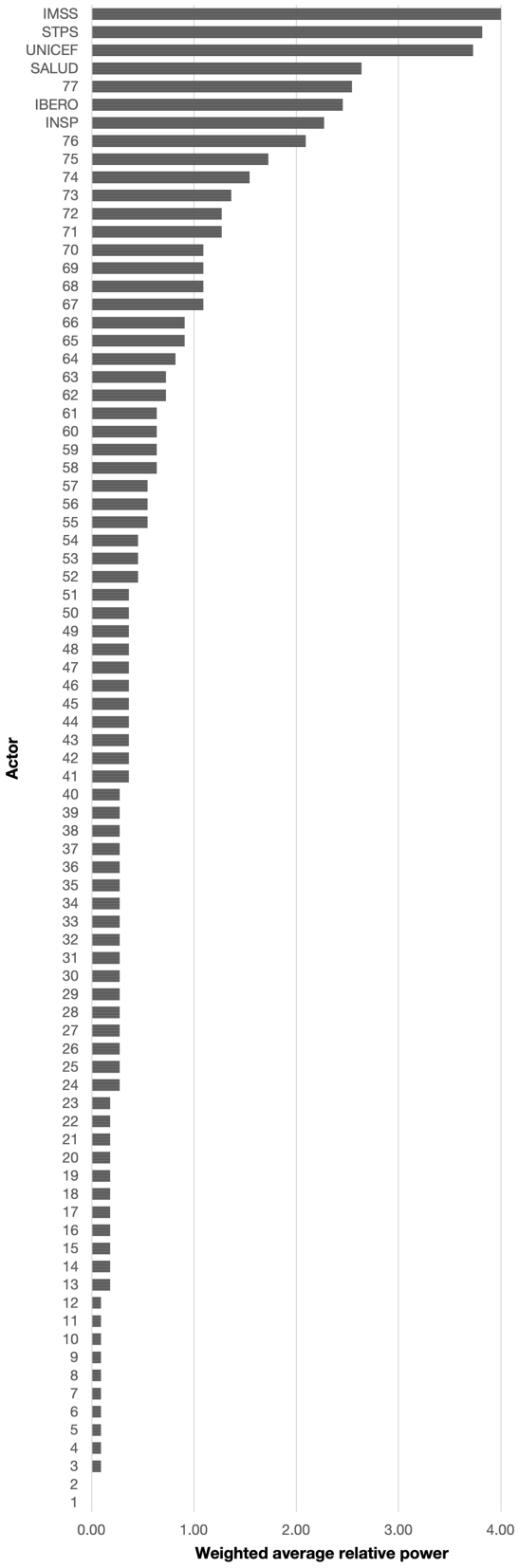

FIGURE 3
Weighted average relative power for all actors participating in the design and implementation of workplace breastfeeding interventions in Mexico; ranked from lowest to highest. 1: Media; 2: Labor unions; 3: National Council to Prevent Discrimination (CONAPRED); 4: Governor of the State of Sinaloa; 5: Infancia plena; 6: Secretariat of Labor and Employment Promotion; 7: State Secretariats of the Treasury and Public Credit; 8: Monterrey Institute of Technology and Higher Education; 9: University of Guadalajara; 10: UN Global Compact; 11: Autonomous University of the State of Hidalgo; 12: Volunatriodo de la Secretaría de Salud (Volunteer of the Ministry of Health); 13: Fundación DIANUI; 14: National Institute for Perinatology; 15: National Polytechnic Institute; 16: Mariana Villalobos; 17: Multi-stakeholder platforms (e.g., Centro Mexicano para la Filantropía); 18: Secretariat of Public Education (SEP); 19: State legislators; 20: Metropolitan Autonomous University; 21: State governments; 22: World Health Organization (WHO); 23: Private health sector; 24: Asociación Pro Lactancia Materna (APROLAM); 25: Chamber of Senators of the Honorable Congress of the Union; 26: National Center for Child and Adolescent Health (CeNSIA); 27: Child daycare centers; 28: Center for Economic and Budgetary Research (CIEP); 29: Parliamentary Front against Hunger (FPH) of the Chamber of Deputies of the General Congress of the United Mexican States; 30: IMSS-Bienestar; 31: Local offices of the Mexican Institute of Social Security; 32: Local offices of the National Institute for Women; 33: Punto de lactancia; 34: Save the Children; 35: Secretariat of Communication and Transportation; 36: Secretariat of Municipal Public Services; 37: Women's NGOs (e.g., GIRE); 38: Un Kilo de Ayuda; 39: UN Women; 40: International Labor Organization (ILO); 41: Alianza por la Salud Alimentaria; 42: Committee on Children and Adolescent of the Chamber of Deputies; 43: Committee on Health of the Chamber of Deputies; 44: Committee on Social Security of the Chamber of Deputies; 45: Secretariat of Public Services; 46: Secretariat of National Defense; 47: Naval Secretariat; 48: Judiciary; 49: National System for the Protection of Children and Adolescents (SIPINNA); 50: National Autonomous University of Mexico (UNAM); 51: Doctors and Researchers in the Fight against Breast Cancer (milc); 52: Support groups of mothers; 53: Supreme Court of Mexico; 54: Social media; 55: Healthcare professionals; 56: Federal Commission for the Protection against Health Risks (COFEPRIS); 57: Proyecto de Apoyo a la Lactancia Materna (PALMA); 58: Corporate foundations; 59: PEMEX (Mexican state-owned petroleum company); 60: National Center for Gender Equity and Reproductive Health (CNEGSR); 61: National Institute for Women; 62: Secretariat of the Treasury and Public Credit; 63: Private companies; 64: State Secretariats of Labor and Social Welfare; 65: Families and colleagues; 66: Pan American Health Organization (PAHO); 67: Chamber of Deputies; 68: State Secretariats of Health; 69: Institute for Social Security and Services for State Workers (ISSSTE); 70: La Leche League; 71: Breastmilk substitute industry; 72: Association of International Board Certified Lactation Consultants in Mexico (ACCLAM); 73: Women; 74: Pacto por la Primera Infancia; 75: Business groups (e.g., chamber of commerce, COPARMEX); 76: Employers; 77: Federal legislators; IBERO: Universidad Iberoamericana Mexico City; IMSS: Mexican Institute of Social Security; INSP: National Public Health Institute; SALUD: Secretariat of Health; STPS: Secretariat of Labor and Social Welfare; UNICEF: United Nations Children's Fund.

3.2. Networks of actors participating in workplace breastfeeding interventions in Mexico

All four networks are described below. Each network is depicted as a network map in which the actors that are participating in a respective connection are depicted as a node while the directed connections are shown as arrows going from the actor that initiates the relationship to the actor that is the receiving actor of this relationship. Table 4 gives an overview of the statistics of each of the four networks. Networks including their maps and statistics were developed as a result of the four types of connections between actors: Advice, Command, Funding and Information. Among the 83 actors, 4 actors (Labor unions,

TABLE 4 Network statistics of actors participating in the design and implementation of workplace breastfeeding interventions in Mexico for the Advice, Command, Funding, and Information networks.

	Advice network	Command network	Funding network	Information network
Actors participating in links	62	34	51	42
Number of links (incl. multiple citations)	218	72	105	185
Number of unique links	168	51	84	143
Network density	0.025	0.007	0.012	0.021
Average degree	2.024	0.614	1.012	1.723
Average weighted degree	2.627	0.867	1.265	2.229
Betweenness centrality – Top 1 actor	UNICEF (492.95)	STPS (37.17)	UNICEF (144.00)	SALUD (446.36)
Betweenness centrality – Top 2 actor	SALUD (429.74)	IMSS (27.33)	SALUD (77.50)	UNICEF (286.10)
Betweenness centrality – Top 3 actor	IMSS (404.45)	SALUD (24.17)	Private companies (23.00)	STPS (198.05)
Weighted Degree – Top 1 actor (Centrality/weighted centrality)	UNICEF (28/51)	SALUD (16/24)	UNICEF (16/27)	SALUD (32/44)
Weighted Degree – Top 2 actor (Centrality/weighted centrality)	SALUD (30/44)	STPS (12/21)	Chamber of deputies (14/16)	STPS (24/35)
Weighted Degree – Top 3 actor (Centrality/weighted centrality)	IMSS (27/41)	IMSS (10/17)	SALUD (10/15)	IMSS (22/30), UNICEF (20/30)
Weighted In-Degree – Top 1 actor (Centrality/weighted centrality)	IMSS (18/31)	Employers (5/13)	INSP (5/10)	SALUD (20/30)
Weighted In-Degree – Top 2 actor (Centrality/weighted centrality)	Federal legislators (16/21)	IMSS (6/10)	IMSS (7/9)	STPS (15/23)
Weighted In-Degree – Top 3 actor (Centrality/weighted centrality)	STPS (14/20)	ISSSTE (4/6)	UNICEF (6/7)	Federal legislators (15/18)
Weighted Out-Degree – Top 1 actor (Centrality/weighted centrality)	UNICEF (22/44)	SALUD (14/22)	UNICEF (10/20)	UNICEF (14/20)
Weighted Out-Degree – Top 2 actor (Centrality/weighted centrality)	SALUD (21/26)	STPS (7/16)	Chamber of deputies (14/16)	IMSS (9/15)
Weighted Out-Degree – Top 3 actor (Centrality/weighted centrality)	ACCLAM (14/19)	Women's NGO (e.g., GIRE) (7/7), IMSS (4/7)	BMS industry (9/11)	SALUD (12/14)

ACCLAM, Association of International Board Certified Lactation Consultants in Mexico; BMS industry, breastmilk substitute industry; IMSS, Mexican Institute of Social Security; INSP, National Public Health Institute; ISSSTE, Institute for Social Security and Services for State Workers; SALUD, Secretariat of Health; STPS, Secretariat of Labor and Social Welfare; UNICEF, United Nations Children's Fund.

Chamber of Senators, Judiciary and the Supreme Court of Mexico) had no links with other actors. A total of 22 actors were part of all four networks. The number of actors participating in the network ranged from 34 in the Command network to 62 in the Advice network. The Information network had a total of 42 participating actors while 51 actors participated in the Funding network.

The interview partners mentioned a total of 580 connections. Excluding all multiple mentioning of connections, a total of 446 links across all type of connections could be counted (referred to as "unique links"). The Advice network had the highest number of unique links ($n = 168$) followed by the Information network ($n = 143$), the Funding network ($n = 84$) and the Command network ($n = 51$).

3.2.1. Advice network

Actors that exchange advice about workplace breastfeeding interventions are described in the Advice network (Figure 4A). The Advice network was the biggest network with 62 actors and 168 unique links. Four out of 10 actors with links in this network were governmental organizations (40.32%) and 20.79% were from "other" organizations. The network density was 0.025, thus 2.5% of all possible links between all actors in the network had been achieved. The average distance between any two actors in the network was 2.70. UNICEF was the actor with the greatest betweenness centrality (492.95) suggesting an important role in connecting other actors in the network to each other. UNICEF was also the actor with the highest number of links (weighted degree = 51) and who provided most of the advice to the other actors (weighted out-degree centrality = 44). Of the 22 unique links from UNICEF to other actors, 14 links were going to governmental actors. IMSS was the actor that received most of the advice (weighted in-degree centrality = 31). Of the total 18 incoming unique links to IMSS, six links came from a UN Organization and five links came from a governmental actor.

3.2.2. Command network

The Command network (Figure 4B) describes actors that provide or receive command regarding workplace breastfeeding interventions.

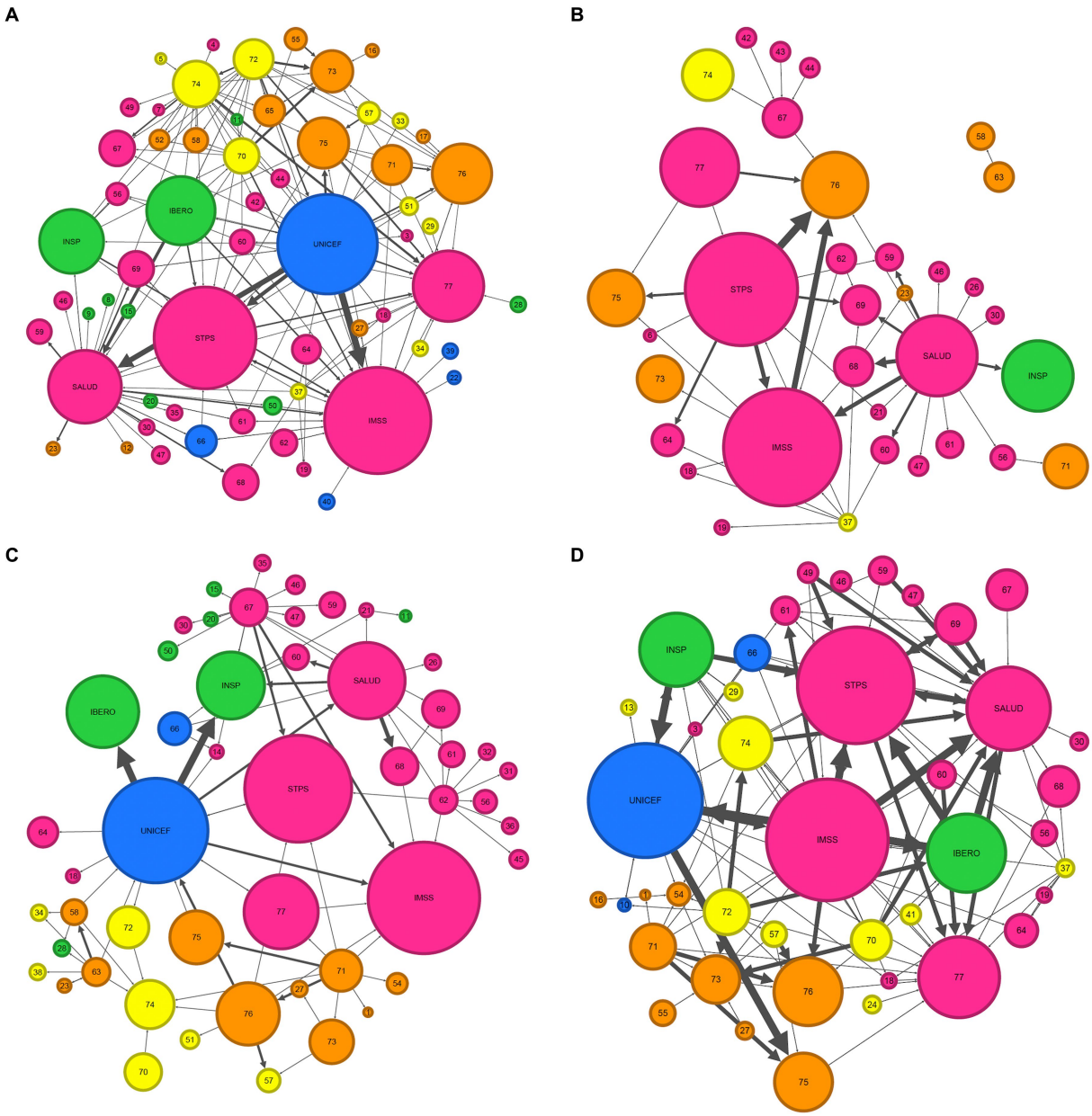

FIGURE 4
Maps of the Advice **(A)**, Command **(B)**, Funding **(C)**, and Information **(D)** networks of actors participating in the design and implementation of workplace breastfeeding interventions in Mexico. Actor group colors: pink = government, yellow = NGO, green = academia/research institution, blue = UN organization, orange = others. Arrows: Arrows indicate the direction of relationship, e.g., the direction of advice [which actor is providing advice to which actor in the Advice network **(A)**]. The thickness of the arrow represents the number of citations this relationship has been mentioned during the NetMap interviews, i.e., the weight of the link, and thus, the robustness of the relationship with thinner arrows only having a single citation and thicker arrows representing connections that have been cited multiple times across the NetMap interviews. Actors: 1: Media; 3: National Council to Prevent Discrimination (CONAPRED); 4: Governor of the State of Sinaloa; 5: Infancia plena; 6: Secretariat of Labor and Employment Promotion; 7: State Secretariats of the Treasury and Public Credit; 8: Monterrey Institute of Technology and Higher Education; 9: University of Guadalajara; 10: UN Global Compact; 11: Autonomous University of the State of Hidalgo; 12: Volunatriodo de la Secretaría de Salud (Volunteer of the Ministry of Health); 13: Fundación DIANUI; 14: National Institute for Perinatology; 15: National Polytechnic Institute; 16: Mariana Villalobos; 17: Multi-stakeholder platforms (e.g., Centro Mexicano para la Filantropía); 18: Secretariat of Public Education (SEP); 19: State legislators; 20: Metropolitan Autonomous University; 21: State governments; 22: World Health Organization (WHO); 23: Private health sector; 24: Asociación Pro Lactancia Materna (APROLAM); 26: National Center for Child and Adolescent Health (CeNSIA); 27: Child daycare centers: 28: Center for Economic and Budgetary Research (CIEP); 29: Parliamentary Front against Hunger (FPH) of the Chamber of Deputies of the General Congress of the United Mexican States; 30: IMSS-Bienestar; 31: Local offices of the Mexican Institute of Social Security; 32: Local offices of the National Institute for Women; 33: Punto de lactancia; 34: Save the Children; 35: Secretariat of Communication and Transportation; 36: Secretariat of Municipal Public Services; 37: Women's NGOs (e.g., GIRE); 38: Un Kilo de Ayuda; 39: UN Women; 40: International Labor Organization (ILO); 41:Alianza por la Salud Alimentaria; 42: Committee on Children and Adolescent of the Chamber of Deputies; 43: Committee on Health of the Chamber of Deputies; 44: Committee on Social Security of the Chamber of

(Continued)

FIGURE 4 (Continued)
Deputies; 45: Secretariat of Public Services; 46: Secretariat of National Defense; 47: Naval Secretariat; 49: National System for the Protection of Children and Adolescents (SIPINNA); 50: National Autonomous University of Mexico (UNAM); 51: Doctors and Researchers in the Fight against Breast Cancer (milc); 52: Support groups of mothers; 54: Social media; 55: Healthcare professionals; 56: Federal Commission for the Protection against Health Risks (COFEPRIS); 57: Proyecto de Apoyo a la Lactancia Materna (PALMA); 58: Corporate foundations; 59: PEMEX (Mexican state-owned petroleum company); 60: National Center for Gender Equity and Reproductive Health (CNEGSR); 61: National Institute for Women; 62: Secretariat of the Treasury and Public Credit; 63: Private companies; 64: State Secretariats of Labor and Social Welfare; 65: Families and colleagues; 66: Pan American Health Organization (PAHO); 67: Chamber of Deputies; 68: State Secretariats of Health; 69: Institute for Social Security and Services for State Workers (ISSSTE); 70: La Leche League; 71: Breastmilk substitute industry; 72: Association of International Board Certified Lactation Consultants in Mexico (ACCLAM); 73: Women; 74: Pacto por la Primera Infancia; 75: Business groups (e.g., chamber of commerce, COPARMEX); 76: Employers; 77: Federal legislators; IBERO: Universidad Iberoamericana Mexico City; IMSS: Mexican Institute of Social Security; INSP: National Public Health Institute; SALUD: Secretariat of Health; STPS: Secretariat of Labor and Social Welfare; UNICEF: United Nations Children's Fund.

It was the smallest network with 34 participating actors and 51 unique links. Governmental organizations made 70.59% of participating actors followed by actors grouped as "others" (20.59%). There were no actors from UN Organizations participating in the Command network. The network density of the Command network was 0.007 and the average distance between any two actors was 1.75. With a betweenness centrality of 37.17, STPS was the most central actor in the network. SALUD was the actor with the largest weighted degree (weighted degree = 24) and the largest number of out-going links (weighted out-degree centrality = 22). Of the 14 outgoing unique links from SALUD to other actors, 11 links were going to other governmental actors. Employers were the actor with the highest weighted in-degree centrality (weighted in-degree centrality = 13). All incoming links came from governmental actors. There was one isolated group of two actors that was not connected to the other actors in the network: Private companies provided command to Corporate foundations. Neither of these two actors was connected to any of the actors that were linked in the Command network.

3.2.3. Funding network

Actors that provide or receive funding for the design or implementation of workplace breastfeeding interventions are included in the Funding network (Figure 4C). It consisted of 51 participating actors and had 84 unique links. Of the 51 participating actors, 49.02% were categorized as governmental organizations and 19.61% as "others." The actor groups "Academic and research institutions" and "NGO" represented 13.73% of participating actors. The network density of the Funding network was 0.012 and the average distance between any two actors was 2.16. The actor with the highest betweenness centrality was UNICEF (betweenness centrality = 144.00). UNICEF was also the actor with the highest weighted degree (weighted degree centrality = 27.00) and weighted out-degree centrality (weighted out-degree centrality = 20.00). Of the 10 outgoing unique links from UNICEF, 7 links were going to governmental actors. The actor with the largest weighted in-degree centrality was INSP (weighted in-degree centrality = 11.00). Of the 5 incoming unique links to INSP, 3 were coming from governmental actors and 2 were coming from UN Organizations.

3.2.4. Information network

The Information network (Figure 4D) includes all the actors that are providing or receiving information about workplace breastfeeding interventions. It had 42 participating actors and 143 unique links. Of all participating actors, 45.24% were governmental actors, followed by NGO and "other" actors with each 21.43%. The network density of the Information network was 0.021 and the average distance between any two actors was 2.84. SALUD had the greatest betweenness centrality (446.36) indicating that SALUD is very central in the network. The highest out-degree centrality had UNICEF (weighted out-degree centrality = 20.00). Of the 13 unique links going from UNICEF to other actors, seven links were going to governmental actors. SALUD was the actor with the highest weighted in-degree centrality (weighted in-degree centrality = 30.00). Of the 20 incoming unique links to SALUD, 13 were coming from governmental organizations.

The interview data allowed us to describe the field of actors participating in the design and implementation of workplace breastfeeding interventions in Mexico in four networks: the Advice network, Command network, Funding network and Information network. Each of the networks describe how the different actors in the field are connected to each other based on the type of relationship they share. In all four networks, the majority of actors belonged to the actor group of "governmental organization" indicating the important role of the government in designing and implementing workplace breastfeeding interventions in Mexico. Based on the network statistics and given their position in the different networks, IMSS, STPS, UNICEF, and SALUD were identified as key actors in the design and implementation of workplace breastfeeding interventions in Mexico with UNICEF being the only non-governmental actor in this group of key actors.

4. Discussion

This is the first study to identify the key actors for designing and implementing successful workplace breastfeeding interventions in the Mexican context. Out of the 83 actors from governmental, academic/research, NGO, UN or "other" organizations, we identified patterns in the top actors and the relationships between them. The actors IMSS (the Mexican Institute of Social Security), STPS (the Mexican Secretariat of Labor and Social Welfare), UNICEF, and SALUD (the Mexican Secretariat of Health) consistently emerged as the top actors with respect to different centrality measurements in the four analyzed networks of Advice, Command, Funding, and Information. This indicates that these four players hold an important role in the design and the implementation of successful workplace breastfeeding interventions in Mexico. More generally, our analysis identified that governmental actors were perceived by the interview partners to play an important role in the design and implementation of workplace breastfeeding interventions in Mexico. This perceived importance of governmental actors was further supported by network centrality measures and weighted average relative power. Besides always ranking among the top actors in means of centrality measures and weighted

average relative power in each network, connections to and from governmental actors were also responsible for the high degree centrality of top actors in the respective networks. This suggests that governmental actors such as the IMSS, STPS and SALUD together with UNICEF need to be included in initiatives to change workplace breastfeeding interventions and policies in Mexico.

We want to highlight several points. The current analysis adds knowledge to a growing body of literature discussing social networks in the field of breastfeeding and more generally in the field of infant and young child feeding such as a previous NetMap analyses of breastfeeding policy and programming in Mexico (25), infant and young child feeding in India (26), and breastfeeding policies and programs in Ghana (27). Compared to a previous NetMap in Mexico (25), the current analysis identified a much larger number of actors in the field. This is remarkable as the analysis by Buccini and colleagues is a description of actors participating in the field of general breastfeeding policy and programming in Mexico rather than focusing on workplace breastfeeding interventions as this analysis has done. It is reasonable to assume that the number of participating actors would decrease when going from the general to a more specific perspective. As a consequence, this indicates that when discussing the design and implementation of workplace breastfeeding interventions in Mexico, more actors need to be included in the discussion which leads to the need for good coordination in order to be effective. Actors well positioned to take over the lead of such conversations are actors with a high centrality and a high relative power. Of the 83 identified actors, only three actors had a weighted average relative power above 3.50 while the remaining actors all showed a weighted average relative power below 3.00 indicating that IMSS, STPS and UNICEF are the most influential actors in the field, and thus, need to be included when discussing workplace breastfeeding interventions and policies in Mexico. Comparisons to other NetMap analyses in the fields of breastfeeding and infant and young child feeding vary. While the previous NetMap analysis in Mexico by Buccini et al. (25) and the NetMap analysis for infant and young child feeding in India (26) identified more actors with higher relative power, the NetMap analysis for breastfeeding policies and programs in Ghana (27) also identified only a small number of actors with a high relative power. Comparisons across different analyses is always difficult as the result of each analysis highly depends on contextual factors present at the time of the analysis. The fact that the current analysis presented only three actors with a middle range weighted average relative power (possible range went from 0.00 indicating that the actor does not at all influence the success of workplace breastfeeding interventions in Mexico to 5.00 indicating that the respective actor highly influences the success of such interventions) indicates that those three actors need to be at the table when workplace breastfeeding interventions are discussed but it also indicates that those actors have the opportunity to even strengthen their influence on the success of workplace breastfeeding interventions in Mexico by strengthening their focus, and thus, strengthening the entire field.

Governmental actors along with UNICEF were identified as most influential actors in the design and implementation of workplace breastfeeding interventions in Mexico throughout the different dimensions of Advice, Command, Funding, and Information. UNICEF was identified to be best positioned to coordinate advice and funding between the different actors while STPS was identified to be best positioned to coordinate command and SALUD had a central position to coordinate information among the participating actors. Among all four networks, there were only two actors not belonging to governmental agencies that had top-3 betweenness centrality measures in the respective networks: UNICEF and Private companies. The importance of governmental actors can also be seen, when looking at the breakdown of links of actors with highest degree measurements. Connections from or to governmental organizations were the main driver for the high degree centrality measures of top actors. Other studies also identified governmental actors as important for policies and programing of breastfeeding and more in general infant and young child feeding (25–28). While SALUD emerged to be the most central actor among all four networks in the analysis by Buccini et al. (25), SALUD was only best positioned to coordinate the flow of information between the actors in the current analysis. It is important to mention that the analysis by Buccini and colleagues focused on general breastfeeding policies and programing while the presented analysis focused especially on workplace breastfeeding interventions. It is therefore reasonable that other players such as STPS are attributed an important position in the field. But the lost importance of SALUD is likely also a result of political changes in Mexico that happened between the two analyses. Between 2006 and 2018, the Mexican government invested in a national breastfeeding strategy to promote, protect and support breastfeeding, thus, providing funding for respective initiatives. The current administration that is in office since 2018 did not continue the political and financial commitment from its previous administrations (29), thus forcing governmental actors to open up the space for other actors, in particular UNICEF. While governmental actors might have lost some of their previous importance in promoting, protecting, and supporting breastfeeding through respective interventions, this study shows that they are still important and need to be included in any discussions about the topic. As such, it is important to remind that governmental organizations need to work on the dissemination of and announcements about working mother's rights to breastfeed after their return to work (30). Given the wide variety of backgrounds and expertise of our interview partners, we concluded that the sum of interviews will level out any potentially biased results and that the prominent appearance of governmental actors is a true result rather than an artifact, and thus, that governmental actors need to have a seat at the table when discussing and coordinating the design and implementation of workplace breastfeeding interventions in Mexico.

While the key role of governmental actors was to be expected to some extent, the authors were surprised by the relatively small role the actors "Employers" and "Women" played. As the implementer of any workplace breastfeeding policy, employers are a key actor, and their buy-in is critical to achieve the desired outcome of an environment that allows women to feel safe and comfortable making breastfeeding choices. Women are the ultimate end user of the policy, and their relatively small role in our results indicates a top-down approach that does not include the end user in formulating workplace breastfeeding interventions in Mexico. While the actor "Employers" was mentioned in 7 out of the 11 interviews with a weighted average relative power of 2.09, the actor "Women" was only mentioned in 3 interviews and received a weighted average relative power of 1.36. The relative low importance of the actors "Employers" and "Women" can also be seen when comparing their weighted average relative powers to the weighted average relative power of IMSS (4.00): The actor "Employers" has only about half of IMSS' influence on the success of workplace

breastfeeding interventions while the actor "Women" only has about a third of IMSS' influence. It is possible that the way the questions during the interviews were framed gave rise to this low representation of "end users" of workplace breastfeeding interventions. The emphasis of "successful design and implementation" of workplace breastfeeding interventions could have been one reason interview partners did not immediately think of "Employers" and "Women" as important actors in the field. To ensure an uptake of workplace breastfeeding interventions and regardless of the result of the present analysis, it is critical that discussions around the design and implementation of workplace breastfeeding interventions center on women and their employers.

Low network densities are consistently reported in social network analyses in the field of breastfeeding and infant and young child feeding (25, 27, 31). Nevertheless, and when compared to the only NetMap analysis available for breastfeeding interventions in Mexico, network densities resulting from the present analysis seem to be particularly low. Compared to the previous NetMap analysis of breastfeeding policy and programming in Mexico by Buccini et al. (25), there was a notable drop in network density. Reasons for this drop can be manifold and a direct comparison between the two studies is to be taken with care. First of all, our analysis focused on the system that gives rise to workplace breastfeeding interventions while the analysis by Buccini et al. analyzed the more general breastfeeding governance system in Mexico not solely focusing on workplace breastfeeding interventions. Secondly, there are 5 years between the two analyses. The analysis by Buccini and colleagues' is based on interviews conducted between November and December 2017 while the interviews for the present analysis were conducted between October and December 2022. In December 2018, and thus in between the two analyses, the current administration came into office. As previously mentioned, investments into national breastfeeding policies and programs that were initiated and supported by the administrations between 2006 and 2018 were not anymore supported by the new administration (29). It is further to mention, that the low density measurements are likely to be a result of chosen methodologies. We used the full list of all 83 actors for all four different networks instead of only using the list of participating actors per network, i.e., a list of actors that had connections to other actors in the respective network. This increased the denominator to calculate the percentage of all possible links within a network thus decreasing the resulting density measurements as well as the average degree measures. It is to mention that a network density (i.e., the percentage of all possible links that exist in a network) towards 1 is also not desirable as it seems very inefficient if everyone is connected to everyone (27). Furthermore, networks with lower connectivity (indicated by a low network density) also provide an opportunity for actors within the network to take the lead in connecting other actors and leading the development of the field. The identified key actors IMSS, STPS, UNICEF, and SALUD are in the prime position to take over the lead in strategically designing and implementing workplace breastfeeding interventions in Mexico by including the actors "Employers" and "Women."

The previously mentioned decision of the current administration in Mexico to discontinue its commitments in a national breastfeeding strategy (29) can be understood as the contextual factor that results in the presented network characteristics. The high number of actors together with a high percentage of single-time actor citations and high proportion of unique links in relation to the total number of links are an indication for low agreement in the field about participating actors. The sudden removal of a national breastfeeding strategy is likely to have led to a disorganization of the field which can lead to a low agreement about actors in play. The previously discussed low network densities identified in this analysis and the seemingly low agreement among interviewees on participating actors lead to the conclusion that the design and implementation of workplace breastfeeding interventions is unstructured. Thus, in order to increase the efficiency and the success of workplace breastfeeding interventions, it is strongly recommended to re-introduce a national breastfeeding strategy for Mexico that includes policies for workplace breastfeeding interventions.

The analysis showed that perceived actor influence as rated by the interview partners was congruent with network statistics indicating relevance of actors. Each interview partner assigned a relative power (on a scale from 0 to 5, with 0 indicating that the actor does not at all influence the success of workplace breastfeeding interventions in Mexico and 5 indicating that the actor influences the success of workplace breastfeeding interventions in Mexico). The actors with the highest weighted average relative power IMSS (4.00), STPS (3.82), UNICEF (3.73), and SALUD (2.64) are the actors that were identified as the most influential actors based on different network statistics such as betweenness centrality and degree centrality. Furthermore, most of the top-10 actors based on weighted average relative power are also among the top-3 actors when looking at network centrality measures. Thus, despite that relative power is a measurement of influence perceived by the single interviewees, its aggregated form of weighted average relative power is a good first estimation of actor's influence in the field in situations where there are no resources to conduct a full social network analysis.

To our knowledge, this analysis is the first published study using the NetMap methodology with a combination of online and in-person interviews. Based on personal preferences and time availability of our interview partners, the interview partners could choose between in-person and online interviews. We could not find any difference between the two interview types. There was no difference in the number of mentioned actors (23 actors were mentioned in the in-person interview vs. a median of 22 actors in the online interviews, data not shown) as well as in the number of mentioned relationships. Thus, our study could show that using online interviews instead of in-person interviews during the NetMap process is a valid alternative.

Besides being the first social network analysis of workplace breastfeeding interventions in Mexico with a clear identification of key actors following a robust study methodology and methodological contributions, our study is not without limitations. While our interview partners had a diverse background, all our interviewees worked on the national level rather than on the local level. Thus, our analysis does not allow us to identify local key actors for designing and implementing workplace breastfeeding interventions in Mexico. Furthermore, we conducted the interviews mostly with one representative of the respective organization. Only UNICEF and the National Center for Equity and Reproductive Health were represented by two interviewees. In both cases, the expertise of the interview partners was different between the two interviewees such that the additional representative added a second perspective while being associated with the same organization. In addition, we interviewed a

relatively small number of participants, although they did identify a large number of actors. Given that the top ranked actors (UNICEF, IMSS, STPS, and SALUD) were mentioned in most of the interviews and mostly have been assigned similar relative powers across the interviews, we concluded that we reached saturation of information after the 11 conducted interviews. However, we acknowledge that there is the possibility that interviewing more than one representative from each organization and interviewing more interview partners in general could have led to more insights. But by consistently identifying the same actors as key actors, we feel comfortable that we did not miss an important actor that is participating in the design and implementation of workplace breastfeeding interventions in Mexico. Furthermore, even though we feel confident in our results we cannot rule out the introduction of two potential biases in our study. First, participants' selection bias may have been present as the preliminary list of possible interview partners was identified through the co-authors' networks in topics related to breastfeeding. Second, a bias may have been introduced because the relative power attributed to each actor was based on the subjective assessment of each interviewee. Those possible biases could only have been eliminated by expanding the number of interview participants which would have exceeded the scope of the study. We would also like to mention that the interview questions allowed us to identify actors that are currently participating in the successful design and implementation of workplace breastfeeding interventions but did not allow us to identify potential actors that currently do not participate but have the potential to do so. While the questions did not prompt the interview partners to actively think about actors that should, and have the potential, to play a role, the method of network analysis allows to at least identify potential for identified actors to adapt their roles. For example, the betweenness centrality of the identified key actors UNICEF, IMSS, STPS, and SALUD shows their potential to strengthen the field by fostering further connections between additional players. The actors "Employers" and "Women" have the potential to be such additional players. While the analysis did not reveal the actors "Employers" and "Women" as actors of high influence, we made the case that those two actors have the potential to, and thus, should be included to strengthen the success of workplace breastfeeding interventions. One possibility why we were not able to identify "Employers" and "Women" as actors of high influence is the fact that we did not have representatives of those two actor groups as interview partners. Another possibility is that until now they have not really played an influential role in setting or implementing workplace breastfeeding policies and programs. We recommend that research discussing the design of workplace breastfeeding interventions should include working mothers as well as employers for example by following the human-centered design approach (32, 33). Lastly, we would like to acknowledge that our study is a pure analysis of the system of actors. To be able to structure priorities in the field, it is not enough to only know the actors. Rather the entire system needs to be evaluated (34). So far and to the knowledge of the authors, there is currently no system analysis about the design and implementation of workplace breastfeeding interventions in Mexico available. Thus, and in order to strengthen the national efforts to support parents in reaching their breastfeeding goals, we would recommend conducting such a system analysis for workplace breastfeeding interventions in the Mexican context.

In order to best support working parents in reaching their breastfeeding goals at the national level, there needs to be clarity about who needs to be involved and about the choice of policy instruments. This analysis provides an overview of actors that participate in some capacity in the design and the implementation of workplace breastfeeding interventions in Mexico. It therefore can serve as a starting platform to discuss the best instruments or mix of instruments with the most important actors. Workplace breastfeeding policies in Mexico are currently mainly supported by regulatory instruments (the Mexican constitution as well as the Mexican labor law defines the women's right for two 30-min extra breaks a day to nurse their infants during the first 6 months of life (35)). Discussions with involved actors need to involve discussions about other possible instruments such as economic and financial instruments as incentives for employers to implement workplace breastfeeding interventions, e.g., tax exemptions or subsidies for employers implementing workplace breastfeeding interventions. By discussing the best mix of interventions, the actors should also always remember that the interventions need to be flexible enough to be adapted to the context in which the intervention will be implemented (17). Families will be most supported if knowledge about workplace breastfeeding interventions as well as knowledge about policy instruments is applied in combination.

In conclusion, using the NetMap methodology, we identified IMSS (the Mexican Institute of Social Security), STPS (the Mexican Secretary of Labor and Social Welfare), UNICEF, and SALUD (the Mexican Secretary of Health) as key actors in designing and implementing workplace breastfeeding interventions in Mexico when looking at Advice, Command, Funding and Information relationships between actors. Our analysis also showed that besides these four key actors, in general governmental organizations played an important role. Furthermore, we laid out why the actors "Employers" and "Women" should also be included in future discussions around workplace breastfeeding interventions. The high number of actors together with a high number of unique relationships between actors were an indication for a fairly fragmented field. This bears the opportunity for interested actors to take over the lead to structure and develop the design and implementation of workplace breastfeeding interventions in Mexico. Therefore, findings from this analysis should be used as a starting point for directed discussions with actors that are positioned best to address policy recommendations in order to reach the best possible results for working mothers and their families.

Data availability statement

The datasets presented in this article are not readily available because the confidentiality of the participating interview partners needs to be protected. Requests to access the datasets should be directed to KL, kathrin.litwan@yale.edu.

Ethics statement

The studies involving humans were approved by the Ethics Committee from Universidad Iberoamericana Mexico City and received IRB exemption from the Institutional Review Board of Yale

University. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

KL designed the study with the help and input from SH-C, MV-C, and RP-E. KL led the development of the data collection tool and designed and conducted the data analysis with contributions from SH-C, MV-C, and RP-E. KL led the English NetMap interviews. VL-M led the Spanish NetMap interviews. VL-M led the interview administration under the supervision of KL and SH-C. KL led the development of the manuscript with input from VL-M, TC, SH-C, MV-C, and RP-E. 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 relationship 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/fpubh.2023.1192600/full#supplementary-material

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Case Report: I feel like a mother to other babies: experiences and perspectives on bereavement and breastmilk donation from Vietnam

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There is a growing recognition globally that care regarding lactation following a perinatal death needs to potentially offer the opportunity for maternal donation. This article discusses this experience and perspectives from a human milk bank (HMB) in Vietnam. This is a descriptive exploratory case study that has a long tradition in both the social and health sciences. Triangulated data collection involved a review of video data, interview data with the donor, and data review for the Da Nang HMB, a Center for Excellence in Breastfeeding. We found that although it is common for mothers in Vietnam to donate breastmilk to HMBs, it is less common for this to occur following perinatal loss. We offer a descriptive case study of the maternal loss of twins and a subsequent choice to donate for approximately 1 month to the Da Nang HMB, the first HMB in Vietnam. We discuss four reasons derived from this case regarding donation following perinatal loss. (1) A strong motivation to donate breastmilk when aware of the service, (2) donating breastmilk helped her deal with grief, (3) family members supported her through this tough time and supported her decision, and (4) health staff supported her decision. While human milk sharing (e.g., wet nursing) has been practiced in Vietnam, breastmilk donation from bereaved mothers has neither been discussed nor well-researched. Because maternal grief is complex and individual, deciding to donate breastmilk is a personal decision that needs to be supported, without creating guilt for those who do not wish to donate.

KEYWORDS

anthropology, bereavement, breastfeeding, child health and nutrition, human milk, human milk banking, neonatal mortality, vulnerable babies

1. Introduction

UNICEF reports that children "face the highest risk of dying in their first month of life at an average global rate of 17.6 deaths per 1,000 live births in 2021, down by 54 percent from 37 deaths per 1,000 in 1990" (1). UNICEF also reports that these same rates have also dropped significantly in Vietnam, and report that the Vietnamese neonatal mortality rate in 2020 is now under 10 per 1,000 live births, as was indicated in a survey conducted by the General Statistics Office in Vietnam and supported by UNICEF (2). Despite these reductions, neonatal deaths still occur, the suffering of the families that experience the loss is present. Research indicates that parental, particularly maternal, grief is present and highly complicated, especially when considering physiological experiences (e.g., hormonal

changes, breast engorgement, uterine contractions, and weight loss) associated with lactation, as well as emotional and psychological changes (e.g., grief, sadness, anger, and guilt) (3, 4). There is a growing global recognition that healthcare providers need to support maternal grieving paying attention to physiological changes which will be experienced following the death of infant (s), validating decisions regarding continuing lactation while recognizing that for some people donation can potentially help to alleviate the emotional pain associated with perinatal loss (5). In this article, we concentrate on a specific case from Vietnam. Throughout the discussion of this specific case following the death of twins, and maternal donation to the first human milk bank (HMB) in Vietnam, we present this case to help shed light on an under-researched part of the world regarding the topic of bereavement, lactation, and potential maternal milk donation.

1.1. Human milk donation is common across the world

The twenty-first century has seen an exponential expansion of HMBs around the world, with almost 800 services being recognized in approximately 70 countries around the world, including increasing numbers in low- and middle-income countries (6). The World Health Organization (WHO) has recommended the optimal use of donor human milk from an HMB in preference to commercial milk formula and especially for small or sick infants, including low- and middle-income countries (7, 8). The demand for donor milk is high for vulnerable infants (9, 10), even for full-term newborns (9). Previous studies have shown that prelacteal feeding, especially with commercial milk formula, is negatively associated with exclusive and continued breastfeeding (11–13). There is a need for the development of HMB guidance, further expansion of the HMB network, and the inclusion of HMB as a part of breastfeeding policy (6, 14).

1.2. Human milk donation among bereaved mothers is not well-studied

Recently we are seeing increased research on HMB donations following a perinatal loss (15–17), although there is a long tradition of looking at this topic in North America (18) and Ireland (19). In 2019 the non-profit PATH released "A Resource Toolkit for Establishing and Integrating Human Milk Bank Programs," with its final chapter dedicated to how to engage bereaved mothers in HMB services. The authors suggested that this complex and sensitive issue should be discussed with global health considerations (20).

Until recently, this topic has not been well-researched, and in some cultures, it has not even been discussed (21). Research shows, however, that donating breastmilk can have advantages for families, including helping with grief by acquiring a donor identity (15), and providing structure around bereavement (16). Yet, donating under these circumstances is not a decision that every person may choose or be able to make, or be offered. For women who wish to become a donor but are physically unable to, further support must be provided to avoid an additional sense of loss as well as mental and emotional health complications. At the same time, we must also make sure that under these very emotional circumstances that a potential donor is in no way feeling coerced to donate. This form of donation must be to help the mother through her journey of grief, and therefore may not be a part of the journey for everyone.

1.2.1. Purpose of study

This study was designed to offer a descriptive exploratory account of breastmilk donation after perinatal death in the first HMB in Vietnam. We described a rare case of a donor who after the death of her twins started donating her milk to the first HMB in Vietnam, an important internationally recognized center of breastfeeding excellence, and explain the reasons/motivations for her donation.

1.2.2. Type of research

This is a qualitative exploratory descriptive case study (22, 23), which involves exploring a specific phenomenon (in this case bereavement and donation) using more than one method (documentary analysis, interview, and mini ethnography), to describe in detail a specific case of bereavement and breastmilk donation in Vietnam. We then used pragmatic ethnographic analysis, to unpeel the layers of meanings (24) of the bereaved mother, the HMB, and Vietnamese culture in general (25, 26).

1.2.3. Research questions

Why did a mother start or continue donating her milk after her children passed away? What are the barriers for her to do so? What are the facilitating factors? What can a health worker do to support the woman's and her family's decision?

2. Methods

2.1. Conceptual model and theory

Our study is informed using a form of mini ethnographic data collection which has been linked by Fusch et al. (27) and has been used for teaching particularly in South Asia (28) and linking these educational aspects to case study designs (26, 29). Ethnography has its roots in both anthropological and sociological theories, and some have argued it "has long been synonymous with case studies, typically conceived of as grounded in the local and situated in specific, well-defined, and self-contained social contexts" (29, 30). Using a mini form of ethnography follows the tradition in health services of having shorter, more focused ethnographic data collection, while still framing analysis around an ethnographic epistemology. Ethnographers and qualitative researchers in general explore how individuals make sense of their social worlds and wish to further understand those worlds or cultures, we can employ pragmatic ethnographic frames, giving a detailed description

and interpretation of the culture or social world. In this case, we are describing the maternal social world of bereavement and donation in Vietnam.

2.2. Setting and study context

The case study was conducted at Da Nang Hospital for Women and Children (the hospital), a facility with a reputation for excellence. The hospital is designated as one of the three World Health Organization's Centers of Excellence for newborn care in Vietnam (31). It is also a referral hospital responsible for technical support and supportive supervision of district hospitals in Da Nang and other provinces in central Vietnam. Each year, 15,000 births take place in the hospital, and the facility supports 420,000 outpatients as well as over 30,000 women and 65,000 children inpatients. Because of its solid foundation for maternal and newborn care, including maternity promotion, protection, and support, the hospital was selected as the site for the first HMB in Vietnam (14).

The HMB in Da Nang, established in February 2017 is the first HMB in Vietnam and the model for establishing and improving HMBs in Vietnam and other East and Southeast Asian countries (14). Onsite events, social media, televisions, posters, and leaflets help to raise awareness of the HMB. Some donors contact health workers, mostly from the HMB or neonatal units to express their interest in becoming breastmilk donors. Health staff also directly contact potential donors, mostly from neonatal units, to recruit them (10). Upon consent to be a human milk donor, all donors are tested for HIV, hepatitis B and C, and syphilis. They also complete screening questions regarding recent treatments (e.g., blood transfusion or blood products, tuberculosis or cancer, medications contraindicated for breastfeeding), vaccination within 4 weeks, and risky behaviors (e.g., smoking, alcohol drinking, drug use, unsafe sex). The staff equips donors with essential skills in hygienic practices, expression, and storage of donor breastmilk. Donors have the choice to use their breast pump or use or borrow hospital breast pumps if they do not prefer hand expression. For donors from the surrounding community, HMB staff visit their residences weekly to check in with families, ensure maternal milk safety, provide clean containers, and collect the donated milk (10). In addition, throughout the entire process, healthcare workers provide breastfeeding support, including psychological support, lactation assistance, and milk donation guidance. This support encompasses, but is not limited to, answering questions, addressing concerns, helping women overcome breastfeeding and donation challenges, and assisting in making decisions about ending donations.

As of April 2022, monitoring data showed an enormous impact —516 breastmilk donors together donated 9,777 liters of milk that was pasteurized to support and nourish 24,079 newborns.

2.3. Sampling

This case study, like many qualitative studies, involves purposive sampling and non-random sampling, which can support an in-depth study and give information-rich cases (32). Since the opening of the HMB in Da Nang there have been 516 donors, and three donated breastmilk after their children's death at the hospital. Two donors started donating when their newborns were under treatment at the neonatal unit. They continue donating for a few days after the deaths of their newborns. They returned to their home provinces, and thus stopped donating breastmilk. The remaining donor was the only one who started donating milk after her newborns passed away and continued doing so for about a month. Even though the case was from 2018, the hospital staff stayed in contact with her. Furthermore, she was featured in a national TV program. We contacted her to seek her permission to use her story for this write-up and she agreed. We also contacted and obtained permission to use the content from the TV program for our write-up, and had it translated into English with subtitles. This was then made available for the first time during a webinar hosted by the UK Association of Milk Banking (UKAMB) and Dublin City University (DCU) in November 2021 and is available on YouTube (33). The third coauthor supported the donor through the entire process and stayed connected with her after the last donation. This author was the focal contact with this woman for this article. Ethical approval was obtained for this case report from the Scientific and Ethics Board of the hospital and written informed consent and permission from the mother were obtained.

2.4. Data collection

We used three data collection sources for our mini-ethnographic case study, which supports a sense of triangulated confidence in our results.

First, we gathered data from the HMB regarding the social and cultural context around donation in Vietnam (9).

Second, we gathered information about this case. The mother donated in 2018 and participated in a documentary about the HMB. The original Vietnam Television Channel 1 (VTV1) documentary also aired in late 2018 (34). Staff had stayed in touch with the donor, and so we contacted her again when we were preparing this research to get her consent to be part of the study in late 2021 for the presentation and early 2022 for this case study. The VTV1 original documentary was clipped to include only the materials related to this donor and English subtitles were produced and presented at a webinar hosted by Dublin City University (DCU) School of Nursing, Psychotherapy, Community Health (SNPCH) and the UK Association of Milk Banking (UKAMB) (33).

We also engaged with the donor after that through contact with the health worker, who is the third co-author of this article. To prepare for this case study, in early 2022, the health worker contacted her again to seek her approval for her story to be used in this research article and sought additional information to help frame our discussion. This documentary and interview data were triangulated with a review of data from the HMB, to build a social and cultural mini-ethnographic context around donation in Vietnam.

2.5. Data analysis and presentation

Ethnographic data analysis is an iterative, spiral, and self-reflective process (35). All of the authors have been involved in the HMB since before it existed, as the collaboration occurred during a separate ethnographic study on donor human milk services which one of the authors conducted in the UK (21). In addition, one author conducted the donor interviews, and all authors reviewed the documentary as mini-ethnographic data, especially after English subtitles were available (35). Although a descriptive case study (22), our data was analyzed from an abductive ethnographic frame (36), which offers some explanatory discussions related to the details discussed in this case.

3. Results

3.1. A case study: donating breastmilk after bereavement

Ms. Hoa (pseudonym, even though she consented to using her real name) was 20 years old and working in a factory in Da Nang, Vietnam. In early 2018, she married her husband, and soon announced her first pregnancy. At her first trimester check-up, Ms. Hoa and her husband were thrilled to learn that she was expecting twins. However, 5 months into her pregnancy, the unimaginable happened—after feeling signs of labor in the night, she experienced a spontaneous membrane rupture. She was immediately rushed to the hospital, but her babies could not be saved. The unexpected loss left Ms. Hoa, as well as her family, grieving and devastated.

In the following days, while she was still in the hospital, Ms. Hoa experienced breast engorgement. Her doctor offered medications to stop her milk production, but Ms. Hoa had seen information about the HMB and contacted hospital staff to learn more about the process. Ms. Hoa was moved by the idea of becoming a breastmilk donor, thinking about the support and relief she could give to other mothers, as well as the vital, life-saving nutrition she could provide to vulnerable newborns. Over the next month, she donated a total of 5.3 L of breastmilk, which was used to support preterm and sick newborns. All the breastmilk Ms. Hoa donated met the strict safety standards set by the HMB.

The above story was told by Ms. Hoa and the HMB staff (the third author) and was featured on a National Television program (34).

In Vietnam, it is uncommon for bereaved mothers to donate breastmilk, and the option is rarely offered. This makes the donation from Ms. Hoa even more unique. Only three generous donors at the Da Nang HMB, out of 516 total donors over the past 5 years, have been bereaved mothers—including Ms. Hoa.

According to the Vietnam Ministry of Health, in the main catchment area of the hospital (Da Nang City, Quang Nam and Quang Ngai provinces), the number of neonatal deaths is approximately 84 out of 54,000 annually, translating to 420 deaths over 5 years at a neonatal mortality rate of 1.54 per 1,000 live births. Nationwide, 17,000 neonatal deaths (at a neonatal mortality rate of 11 per 1,000 live births) were recorded in 2018 (37). Thus, the potential number of donors like Ms. Hoa could be much higher.

3.2. Why Ms. Hoa donated after her loss

Ms. Hoa had a strong motivation to donate breastmilk. She said:

> *"I did not take medications to stop my milk production—I wanted to bring my breastmilk to other babies, especially premature babies like the ones I lost, or babies who are sick or whose mothers can't produce breastmilk. I wanted to bring breastmilk to the babies who don't have access to it. As long as I can produce milk, I know I'm doing something to support other babies—I will keep on giving every drop until it naturally stops. I hope that each drop of love will help babies grow up strong."*

For Ms. Hoa, donating breastmilk also helped her deal with her grief. She said:

> *"Expressing breastmilk gave me the strength to continue with my life. My babies will not come back to me. But through donating the milk I made for the babies I lost, I feel like a mother to other babies."*

Ms. Hoa had family members who supported her through this challenging time. The HMB staff observed:

> *"Ms. Hoa and her family lived in a small house in a poor, suburban area of Da Nang City. They had only one old fridge to store food. When I explained the requirement of a clean fridge to store breastmilk, her mother-in-law, without hesitation, took out the food in their only fridge, cleaned it, and gave Ms. Hoa the space to store donor breastmilk."*

HMB staff supported Ms. Hoa both physically and mentally and formed a close relationship that allowed her to donate milk during her hospital stay and after discharge. They helped her to channel her grief, as she missed her babies during milk expression. Later Ms. Hoa reflected: "the staff helped me understand that to give is to receive. Indeed, I received happiness in return. I got to provide for other babies, and then, I got pregnant again and have now two beautiful and healthy children."

4. Discussion

4.1. The culture of human milk sharing in Vietnam

Human milk sharing (e.g., wet nursing), either paid or unpaid, has been practiced since ancient times in many countries in the world (38, 39). Wet nurse—"a woman who breastfeeds another's child"—was considered a popular, well-paid, and highly organized profession (38). Some countries, including France, required wet nurses to register at a municipal employment bureau and had laws to regulate their employment (38).

In Vietnam, nursing mothers were commonly hired by well-off families (e.g., in North Vietnam before 1954 and South Vietnam before 1975) in exchange for money, food, and lodging (40). However, public opinion considered wet nursing an evil of feudalism and colonialism, and exploitation of women, and the practice eventually became less commonplace (40). Similar exploitive features of wet nursing also led to its pejorative features in other countries around the world, contributing to the reason that many HMBs around the world do not have commercial payments associated with milk donations. Wet nursing, a woman breastfeeding another's child without payment, often occurs around the world and has been labeled by some as "cross nursing" to avoid the pejorative links to the term wet nursing (41).

HMBs are to provide human milk to small and sick infants while addressing the concern of transmitting diseases associated with wet nursing such as HIV, hepatitis B and C, and syphilis (14, 42). However, the donation of breastmilk to an HMB is a new concept in Vietnam. Based on our estimation, the current four active HMB in Vietnam only cover a catchment area of about seven percent of the total number of newborns throughout the country. Also, not all mothers have the capacity or are willing to donate their milk. In the last 5 years, only 516 mothers out of the 75,000 births became donors at the Da Nang HMB.

Mothers who have lost their babies typically do not start or continue donating their breastmilk. Of the three bereaved donors in Da Nang, Ms. Hoa is the only one who continued donating for an extended period. The other two donors donated only briefly after their children passed away. Potential reasons could include that donating reminds mothers of their loss. Mothers and other family members may also think that continuing to express breastmilk may prolong their grief.

It is worth noting that abortion, miscarriage, and surrogacy mothers also experience similar feelings of loss especially when the women have the capacity of lactating, although these experiences may be different in important ways as well (43, 44).

For Ms. Hoa and her family, the breastmilk donation helped to alleviate her sadness significantly. This "maternal generosity" and the communal nature of maternity underlies donor human milk services (21). The support from Ms. Hoa's mother-in-law and family members is also important given their influence in decision-making about maternal, infant, and young child nutrition (45, 46). However, due to the complicated and individual nature of grief, bereaved families should never be made to feel like they must or should donate.

In the 5.5 years of operation, HMB staff often do not actively approach mothers who have lost their babies for donation. First, the staff are afraid of touching the mothers' and families' sadness. However, donations can alleviate sadness as in this case study. We need to be incredibly careful if presenting this option that donors in no way feel a sense of coercion, but instead are aware that some people have found this helpful and that they were only being told in case they also found it helpful. Second, the volume of donor breastmilk received from other mothers is in surplus for the Da Nang HMB to be used by the hospital and hospitals in need in Da Nang and neighboring provinces (9).

4.2. What more do we need to learn?

Knowledge is limited to mothers' opinions on milk donation after losing their babies. For example, what would a mother do to her frozen stored milk? Why does a grieving mother start or continue donating her milk? Does the donation improve or aggravate their feelings of loss? What is the perception of recipients' families on donor milk from bereaved mothers? Are there cultural variations in bereavement and donation?

5. Conclusion

In this case study, we found that the bereaved mother donated milk for a month with the support of her family and health workers. The woman was determined to donate milk, and this act helped her alleviate her feelings of loss after losing two children.

Because maternal grief is complex (4) and individual, deciding to donate breastmilk is a personal decision that needs to be supported, without creating guilt for those who do not wish to donate. Health workers, including those working in the HMB, should make sure that mothers and other family members know that a donation is an option if they wish to do so. HMB networks around the world need to exchange information, experience, lessons learned, research findings, and appropriate policies to bolster learning on this topic. The HMB networks can also develop culturally appreciative guidelines to help shape the practice globally. With the world of online information and support from HMB staff, bereaved families may learn about practices and policies in their own and other countries to have informed, appropriate decisions.

Key messages

- Few bereaved mothers donate breastmilk, and the phenomenon is not well understood.
- A case of a mother who became a breastmilk donor after her twins' death showed that she had a strong motivation to

donate, felt eased with the donation, and had support from family members and health workers.
- Because maternal grief is complex and individual, deciding to donate breastmilk is a personal decision that needs to be supported.
- Health workers and human milk bank networks play an important role in sharing information, guiding, and supporting bereaved mothers with informed appropriate decisions.

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 author.

Ethics statement

The studies involving humans were approved by Scientific and Ethics Board of Da Nang Hospital for Women and Children and Da Nang Health Department (protocol code 1922, dated on 6 March 2020). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Conceptualization, HT, TN, RM, and TC; investigation, HT, ON; writing – original draft preparation, TN, ON, and TC; writing – review and editing, HT, TN, RM, and TC. 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

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A descriptive analysis of human milk dispensed by the Leipzig Donor Human Milk Bank for neonates between 2012 and 2019

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Background: Human milk banking has become an important aspect of Nutritional medicine. It is not just about the provision of mother's own milk (MOM) or donor human milk (DHM) in the hospital, but also a strategy to encourage breastfeeding in the clinical setting and beyond.

Objective: To describe the feeding patterns of hospitalised infants including human milk dispensed by the Leipzig Donor Human Milk Bank (LMB).

Design: A descriptive analysis of daily data on milk feeds dispensed by LMB for hospitalised infants distinguishing between MOM or DHM, either fresh or frozen, and raw/pasteurised milk from 2012–2019.

Results: We included 2,562 infants with median hospitalisation of 23 days, for whom human milk was dispensed on median 76% of those days and other nutrition on the remaining days. Raw MOM and raw DHM comprised 52% and 8% of the dispensed milk, respectively. Dispensing exclusive DHM instead of MOM for at least one full day was required for 55% of the infants, mostly at the beginning but also later during hospitalisation. Exclusive raw DHM was dispensed on at least 1 day for 37% of the infants, in different birthweight strata <1,000 g: 10%, 1,000-1500 g: 11%, 1,500-2500 g: 13% and > 2,500 g: 3%. At discharge, MOM was dispensed for more than 60% of the infants.

Conclusion: During an infant's hospital stay, LMB dispenses various human milk feeds with interspersed DHM resulting in complex intra-individual and time-variant feeding patterns. LMB dispenses raw MOM and especially raw DHM with the intention to retain the properties of human milk unlike a diet containing pasteurised DHM and/or formula. Although raw DHM comprises a small percentage of all dispensed milk, raw DHM is dispensed for a substantial portion of infants. Our results document that dispensing raw DHM, is possible in routine settings.

KEYWORDS

donor human milk (DHM), mother's own milk (MOM), infant feeding patterns, raw DHM, Leipzig Donor Human Milk Bank (LMB)

1. Introduction

Exclusive breastfeeding remains the recommended source of early life nutrition (1). When mother's own milk (MOM) is not available, donor human milk (DHM) from human milk banks is the recommended alternative (2–4). The number of human milk banks is increasing worldwide (5). Yet, knowledge about actual DHM use in neonatal intensive care units (NICU) is limited (4, 6, 7).

During hospitalisation, infants receive MOM, DHM and formula, exclusively or concurrently, depending on parental consent and availability. Several studies comparing DHM vs. formula have found considerable reductions in the incidence of necrotizing enterocolitis (NEC) (8–10). Head-to-head comparison of DHM to MOM is rarely reported for infant health outcomes such as NEC, growth, mortality and others; most studies (11–14), combine MOM and DHM into a single measure of human milk feeding. Information about the actual percentage contributions of MOM and DHM across the overall hospital stay would be important because MOM is be prioritised when DHM is available, and when pursuing strategies to improve human milk supply (15).

Moreover, little is known about the intra-individual feeding pattern during hospitalisation. However, we are only aware of one recent study (16) that applied unsupervised machine learning to cluster different groups of hospitalised infants by nutritional patterns and other characteristics. Yet, understanding nutritional course and clustering is of great value for insight into the diversity within common clinical profiles or certain treatment practices (4, 16). Notably, the availability of DHM reduces the time with initial parenteral nutrition (10) but the subsequent course of nutrition is underexplored.

Furthermore, DHM is collected and distributed according to standard operating procedures and is generally pasteurised (5, 17, 18). However, some human milk banks in Germany and also in Norway provide both raw and pasteurised DHM used in accordance with local clinical judgement and health of both recipient and donor (19). The Leipzig Donor Human Milk Bank (LMB) is one of the oldest and largest in Germany providing raw and pasteurised, as well as, fresh and frozen DHM and MOM. Therefore, we sought to describe (i) the variety of intra-individual patterns of dispensed milk and (ii) the output of LMB between 2012 and 2019.

2. Methods

2.1. Data sources and study variables

The Leipzig Donor Human Milk bank (LMB) of Leipzig University Medical Center seamlessly distributes human milk, both MOM and DHM for infants in need especially preterm infants admitted within the Neonatal Intensive Care Unit (NICU) and outside the NICU on other children's wards in the clinic. Paper-based logs of human milk dispensed daily from LMB date back to at least 2008. Up to now, two medical students under close supervision digitized data from 01/2012 and up to 12/2019 manually into a Microsoft Access database. Although double data entry was not done, visual data checking was done to identify unlikely values, which comprised observations excluded from the current analysis (Figure 1). For this, data extracts with the respective patient identifiers and variables in question were generated and output from SAS statistical software following calculation of the difference between specific dates. For instance, the difference between the date on which milk was pasteurised and the date on which milk was dispensed was calculated. Observations with an unlikely value were assigned a code and these extracts were used to verify the values on the paper documentation. Those with typing errors were corrected, but if values were implausible, these were excluded. The Ethics board of Leipzig University granted Ethical Approval.

Records included the date when human milk was dispensed, patient identifiers, the specific types of human milk dispensed on each day (i.e., raw and pasteurised, as well as, fresh and frozen MOM or DHM) and the date of pasteurization if applicable. Of note, fresh milk was kept at 4°C for a maximum of 3 days, after which it was frozen and stored at −20°C for a maximum of 6 months. DHM was pasteurised if the donor was positive for cytomegalovirus infection (CMV) and/or their skin swab for coagulase-negative Staphylococci colony-forming unit (CFU) was $>10^4$/ml. DHM was discarded if any Enterobacteriaceae were detected. MOM was pasteurised for infants <28.0 gestational weeks, for CMV-positive mothers up to 32.0 gestational weeks, and/or if MOM contained $>10^5$/ml total bacterial count. Pasteurisation was also dependent on how long the milk would had sat before or until it arrived at LMB after being pumped/expressed by the donor or the mother. Raw milk (MOM or DHM) refers to non-heat treated milk, kept chilled or frozen in the refrigerator or freezer, respectively. Availability of frozen milk was also dependent on milk intake, more milk at long intervals from dispensing, which translates to more milk in the freezer. There was neither information on actual milk volume, biological composition of MOM or DHM, nor on fortification. Further, we had no documentation of actual milk fed to the infant but used daily dispensed human milk as a proxy for intra-individual feeding patterns, which may be prone to some error, e.g., if clinically required nutrition changed during the day.

Data for the current analysis were coded to represent eight different types of human milk: fresh raw MOM (rMOM), frozen rMOM, fresh pasteurised MOM (pMOM), frozen pMOM, fresh raw DHM (rDHM), frozen rDHM, fresh pasteurised DHM (pDHM), and frozen pDHM. These data were merged with information on administrative data, admission and discharge dates, age at admission, and gestational age obtained from hospital records by our hospital's Data Integration Center. Days during hospitalisation on which the infant had no milk dispensed from LMB were coded as "other nutrition" comprising other forms of parenteral and/or enteral nutrition (e.g., specialized/created diets, infant formula, days when an infant was breastfed). Initially, data from both sources were available from 2,954 infants (Figure 1). We finally included 2,684 administrative cases belonging to 2,562 infants; $n = 90$, $n = 13$ and $n = 2$ infants were admitted twice, three and four times, respectively, and we merged their administrative cases.

2.2. Statistical analysis

During the infant's hospitalisation, each day comprised exclusively one, a mix of the eight human milk types mentioned above, or – if no feeds were dispensed – other nutrition. A modified lasagna plot was used to depict the intra-individual

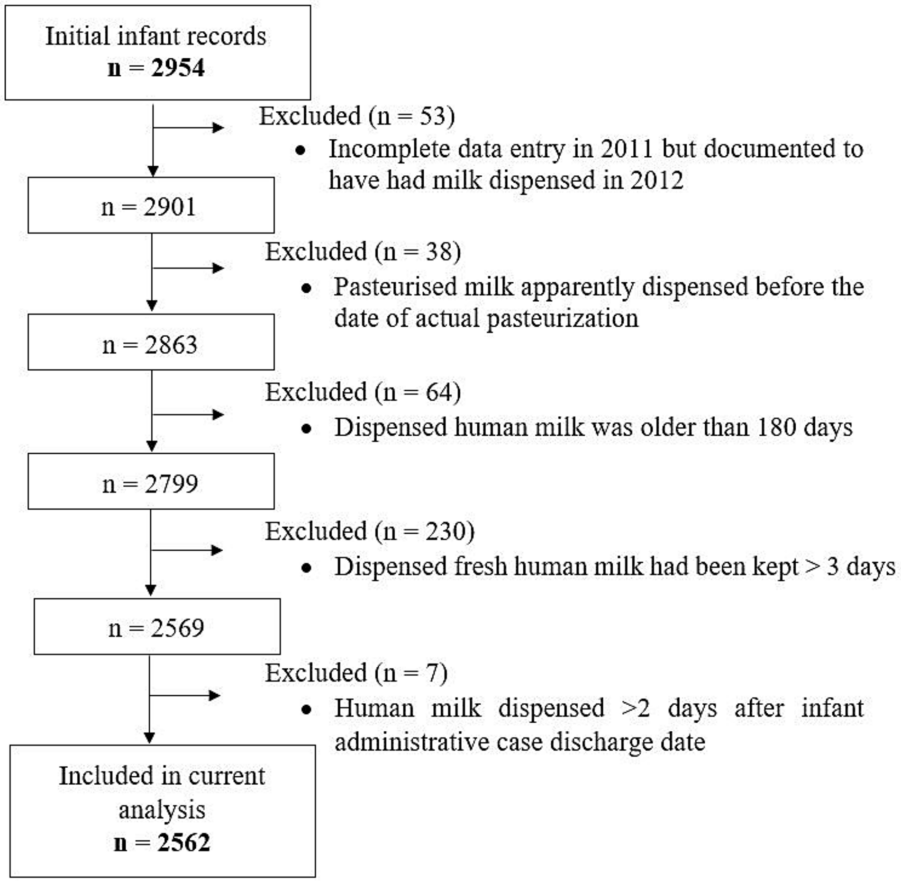

FIGURE 1
Flowchart of implausible values and observations that were excluded as well as hospitalised infants whose records on dispensed milk types were used for the current analysis.

feeding patterns up to the first 100 days of hospitalisation for a subset of infants born and admitted on the same day in 2019 and hospitalised for at least 7 days. For subsequent analyses, milk types on mixed days were weighted by the inverse of the count of distinct milk types on the given day. For each infant, relative proportions were calculated for each milk type by sum of the weighted days divided by the overall number of days for which human milk was dispensed, i.e., percentage contributions of distinct milk types to the overall milk dispensed during hospitalisation. Summation of distinct milk types was used for aggregation to a higher level, e.g., MOM and DHM without discerning raw/pasteurised or fresh/frozen. A kernel plot was used to visualise the distribution of aggregated MOM and DHM percentage contributions for each infant across all calendar years. The contribution of the milk type covered patient days as a percentage of the summed patient days (i.e., days on which milk was dispensed) per calendar year was visualised in a stacked bar chart. Due to the compositional nature of the percentages of the eight milk types per infant, the data were transformed to *centered log ratios* (20) prior to the Cochran-Armitage test for trend across calendar years. All statistical analyses were done using R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria) and SAS (version 9.4, The SAS Institute, Cary, NC, United States).

3. Results

3.1. Intra-individual patterns of dispensed human milk during the first 100 days of hospitalisation in 2019

The 2019 subset comprised n = 351 infants admitted on the day of birth – *or* – within 24 h after birth and hospitalised for at least 7 days, included $n = 162$ girls and $n = 182$ boys, with an average birthweight and gestational age of 2257.9 g [Q1 = 1,550 g, Q3 = 2,900 g] and 34 weeks [Q1 = 31, Q3 = 37], respectively. A modified lasagna plot (Figure 2), each line representing an individual infant, depicts a variety of intra-individual patterns based on dispensed human milk during hospitalisation, though some common features appeared. Other nutrition was the predominant initial form of nutrition, followed by DHM especially for those with a longer hospital stay, and subsequently followed by MOM, which was then supplied for a longer period. There was also a plethora of instances in which DHM exclusively or a mix of DHM and MOM (i.e., separately dispensed on the same day) was interspersed between days of pure MOM; depicting the successful substitution of DHM for MOM to avoid the use of infant formula. Other nutrition was the only form of nutrition for the entire hospitalisation period for some infants.

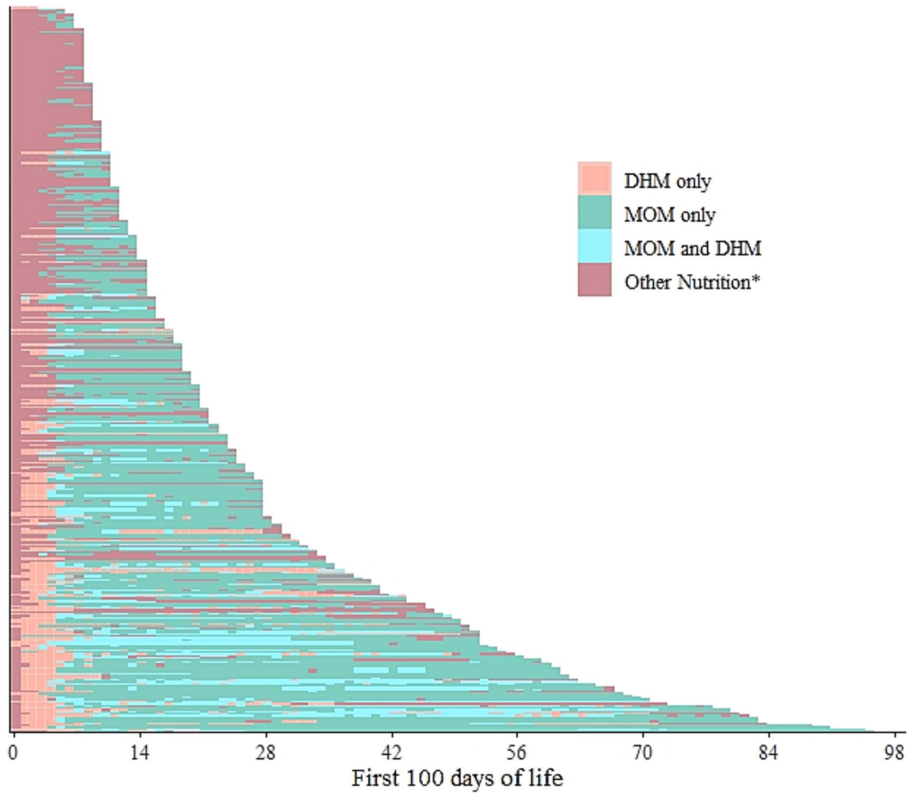

FIGURE 2
Intra-individual feeding patterns, discerning mother's own milk (MOM) and donor human milk (DHM), among the infants admitted on the day of birth in 2019 (n = 351). The first 100 days of life is equivalent to the first 100 days of hospitalisation. Each line represents one infant. *Other nutrition denotes any other form of either parental/enteral feeds that could have included but not limited infant formula, prescribed nutrition, or even fed directly from the breast.

We further categorised the dispensed human milk into raw or pasteurised, disregarding the MOM or DHM category. The resulting modified lasagna plot (Figure 3) showed slightly more complex intra-individual patterns among the same $n = 351$ infants. Initial human milk seemed to be more often raw, i.e., raw DHM (Supplementary Figure S1) and the graph suggested a higher degree of interspersed patterns while dispensing a mix of both raw and pasteurised milk (i.e., separately dispensed on the same day) on the same day may have occurred more often with longer hospitalisation.

3.2. Infants for whom LMB dispensed human milk between 2012 and 2019

In total, LMB dispensed at least one human milk feed for 2,562 hospitalised infants between 2012 and 2019: 2030 singletons, 237 pairs of twins, 18 sets of triplets and 1 set of quadruplets. Almost 90% ($n = 2,273$) of the infants were admitted on the same day they were born; the median age at admission of the remainder was 10 days (Q1 = 2, Q3 = 36, Supplementary Table S1). The median duration of hospitalisation was 23 days [Q1 = 14, Q3 = 41; total of 84,991 days]. On average 70% of the hospitalization, days (62,715 patient-days) were covered with dispensed human milk (i.e., any MOM or any DHM). Other nutrition was the form of nutrition on the remainder of the days (i.e., 30%). For most infants (90%), exclusive MOM was dispensed on at least 1 day, even within the different gestational age strata (Supplementary Table S1), and on average almost 50% of the days of hospitalisation (Table 1); at discharge, MOM was dispensed for 60% of the infants (Supplementary Table S1). Dispensing exclusive raw DHM for at least 1 day, presumably due to lack of raw MOM, was required in more than a third (37%, $n = 955$, Supplementary Table S1) of the infants, in the different birthweight strata (Supplementary Table S2) and on average on 5% of the days of hospitalisation (Table 1). Exclusive fresh raw MOM, i.e., the nutritional gold standard, was dispensed on average on 21% of the days of hospitalisation (Table 1).

For the subsequent analyses, we rescaled the denominator for each infant by omitting days with other nutrition to the total number of days during which any human milk was dispensed. The percentage contributions of MOM and DHM were displayed in a density plot by overlay to emphasize the different distribution shapes (Figure 4). The overlay therefore does not imply that LMB dispensed a mix of MOM and DHM for a given infant. On the right margin of the graph, 100% of the days were covered with dispensed DHM or MOM for $n = 166$ and $n = 1,087$ infants, respectively. For the remainder of the infants ($n = 1,309$), their patient days were covered by a mix of both MOM and DHM (i.e., dispensed separately on the same day). Although there is no clear cut-off, the density of the MOM-curve takes a steeper increase around 75% of the patient days covered with human milk for $n = 1738$ infants. Suggesting there is no clear break in the shape of the

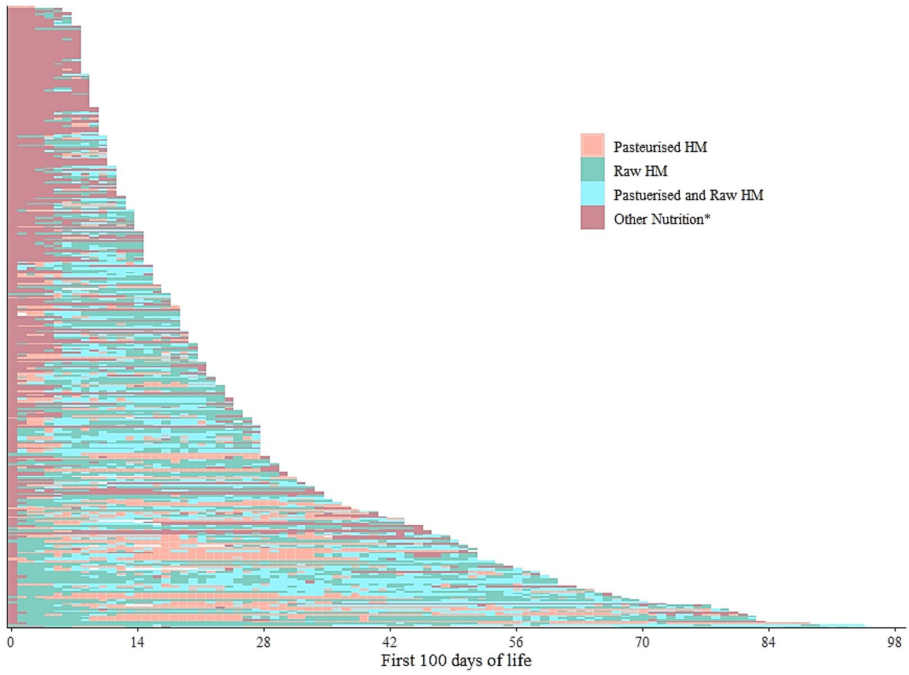

FIGURE 3
Intra-individual feeding patterns, discerning raw and pasteurised human milk, among the infants admitted on the day of birth in 2019 ($n = 351$). The first 100 days of life is equivalent to the first 100 days of hospitalisation. Each line represents one infant. HM- Human milk. *Other nutrition denotes any other form of either parental/enteral feeds that could have included but not limited infant formula, prescribed nutrition, or even fed directly from the breast. Pasteurized and raw HM refer to the respective milks dispensed on the same day but not mixed.

TABLE 1 Average (arithmetic mean and median) percentages of hospitalised days per infant on which exclusive and mixed human milk feeds were dispensed between 2012 and 2019.

Dispensed human milk feed	Mean (sd)	Median [Q1, Q3]
Any human milk was dispensed	70 (27)	76 [50, 94]
Exclusive MOM was dispensed	48 (29)	50 [20, 72]
Exclusive DHM was dispensed	13 (19)	5 [0, 17]
Mixed MOM and DHM were dispensed	9 (18)	0 [0, 9]
Exclusive fresh milk was dispensed	41 (26)	43 [17, 63]
Exclusive fresh unpasteurised milk was dispensed	22 (20)	16 [4, 35]
Exclusive fresh unpasteurised MOM was dispensed	21 (20)	15 [3, 35]
Exclusive unpasteurised DHM was dispensed	5 (10)	0 [0, 6]

MOM, Mother's own milk; DHM, Donor human milk; Q1, First quartile; Q3, Third quartile.

distribution for neither MOM nor DHM. That is, is not possible to pick a meaning cut off for DHM because 75% would not split the sub-population. If we were to, then 50% would be a potentially meaningful cut-off thereof, as it offers a potentially even split.

3.3. Overall output of LMB

Aggregating across all infants, LMB dispensed human milk on 62,715 patient-days (i.e., sum of days on which milk was dispensed) across the eight-year study period (Supplementary Table S1). In each of these years, fresh raw MOM was the most commonly dispensed type (Figure 5; Supplementary Table S3). However, there was borderline statistical significance ($p_{trend} = 0.055$) for an overall trend in dispensing higher or lower percentages of the milk types. There was a marked drop in the percentages of dispensed fresh raw DHM, frozen raw DHM, and fresh pasteurized DHM from 2012 to 2013 accompanied by an increase in the percentage of fresh raw MOM, which may drive the overall and individual trends (Supplementary Table S4). In a more aggregated view, there were statistically significant trends in dispensing less pasteurised and less frozen milk over the years ($p < 0.05$, Supplementary Table S4). Although the proportions of DHM comprised 8% of overall milk types, raw DHM was dispensed exclusively for 37% of the infants, that is, a substantial portion of infants.

4. Discussion

Leipzig Donor Human Milk Bank (LMB) dispensed human milk for 2,562 infants between 2012 and 2019 with a median hospital stay of 23 days, on a median of 76% of those days amounting to 62,715 patient-days covered with human milk. Using daily data on dispensed milk as a proxy for actual infant feeding, we document an explicit variety of intra-individual feeding patterns during the first 100 days of hospitalisation.

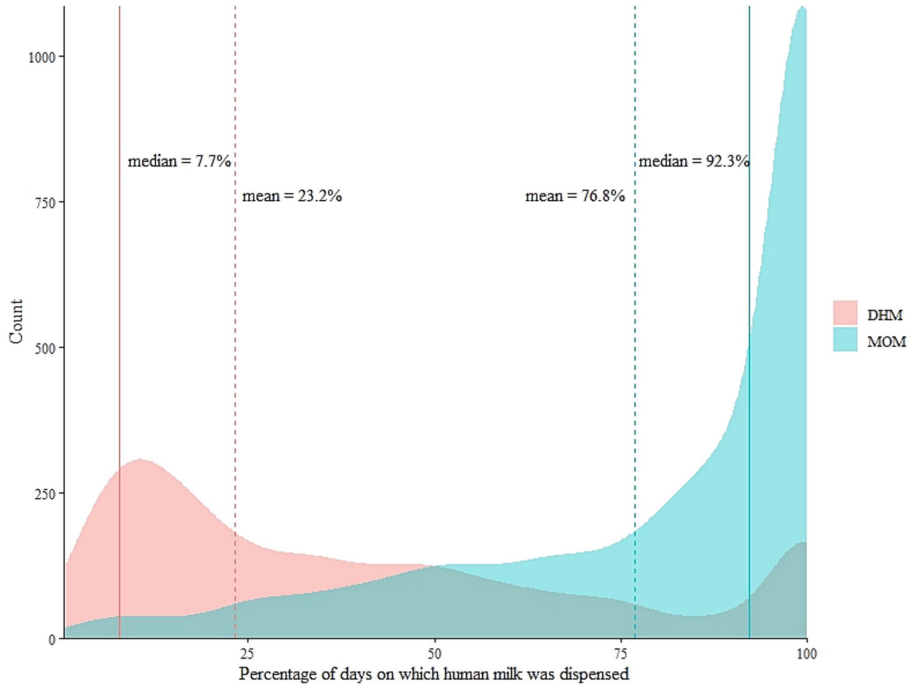

FIGURE 4
The distribution of mother's own milk (MOM) and donor human milk (DHM) as percentages of the total number of days on which any human milk was dispensed for each infant between the years 2012 and 2019. Dotted lines show the arithmetic mean of the percentage contributions of the respective human milk type. *N* = 859 infants fall below the means of MOM, *n* = 1703 fall below the means of DHM.

FIGURE 5
Milk type covered patient days as a percentage of summed patient days per calendar year between 2012 and 2019. Values shown above the plots are the aggregate number of days on which milk was dispensed each year and the number (n) of infants for whom the Leipzig Donor Human Milk Bank (LMB) dispensed milk each year. MOM, mother's own milk; rMOM, raw mother's own milk; pMOM, pasteurised mother's own milk; DHM, donor human milk; rDHM, raw donor human milk; pDHM, pasteurised donor human milk.

Moreover, we were able to discern the percentage contribution of MOM and DHM, that is, the proportion of days on which a specific milk type was dispensed for an infant. These data could potentially improve accuracy in grouping infants into distinct human milk feeds accounting for co-exposure to each milk feed in studies contrasting DHM and MOM exposure. DHM was dispensed to potentially bridge the days until MOM was available (21). The supply of raw milk in general has consistently increased over the years, with raw MOM amounting to almost 60% of all dispensed milk in 2019 when raw DHM had a share of 5%. Although this share of DHM was small it was dispensed for a substantial portion of the infants ($n = 129$, 40%).

We identified plausible intra-individual feeding patterns during the first 100 days of hospitalisation. Notably, the time-variant nature of these potential feeding patterns is likely due to milk availability, but also the infant's clinical state. Although other nutrition was the predominant initial form of nutrition, the ultimate goal is to optimize the use of MOM during early life. In our study, 90% of the infants received exclusive MOM at least once marking a high initiation rate and at discharge, MOM and DHM were dispensed for 60% and 3% of the infants, respectively. However, none of the infants are discharged while dependent on DHM, thus this result should be interpreted with caution, as it may have been subject to documentation error. Compared to nationwide figures (22), all of this suggests that the services of LMB do not impact on breastfeeding rates (23). Similar to other studies (24–27), we show that DHM is dispensed as a bridge until MOM is available, and potentially overcoming initial breastfeeding difficulties. On one hand, other studies reported a decreased use of MOM after the introduction of DHM, despite having received some MOM prior to DHM availability (11, 28). On the other hand, the availability of a human milk bank resulted in a stable use of MOM while the use of infant formula milk in the NICU decreased (29, 30). As such, our results further highlight the importance of a human milk bank in supporting mothers to overcome breastfeeding challenges (24, 31–33).

Describing the complex, time-varying feeding exposures during hospitalisation is an essential first step to ascertain whether the sequence of events supports a causal association of MOM or DHM feeding with the onset of an infant's clinical outcome. We are aware of a recent study (16) clustering infants admitted to neonatal units in England according to clinical and feeding data. While this study shows time curves of the proportions of infants for whom a nutritional component (e.g., MOM, DHM) was dispensed, it does not provide insight into intra-individual sequences but rather identifies clustered groups of infants with varying percentage contributions of milk types to overall feeding. Most other previous studies largely neglect time-variant aspects of human milk feeding and employ crude categories of milk type proportions over the whole hospital stay.

In light of this, it has been pointed out (11), that previously reported beneficial effects and/or associations using such predefined cut-offs, e.g., 75% of feeds during hospitalisation covered with MOM, may well be driven by co-exposure to MOM and DHM. Our results on intra-individual patterns and on distributional aspects of MOM and DHM co-exposure highlight the importance of choosing cut-offs carefully from the underlying research population and desired contrast, rather than from the "learned presentations" or arbitrary cut-offs. As such, the services of the milk bank are not just about supplying hospitalised infants with DHM, but also about creating a strategy that encourages breastfeeding in the clinical setting with a public health perspective. More research is therefore needed to investigate the dose-and time-dependent associations of the potential feeding patterns identified in this current descriptive analysis with infant health outcomes. Moreover, it is only until recently that the use of DHM has been limited only to vulnerable groups of infants based on gestational age cut-off and birth weight (24). This warrants further research to provide more insight in the use of DHM in populations beyond the NICU (12).

Adhering to strict standards and extensive screening (19, 34, 35), LMB dispensed raw DHM for all infants across different gestational ages and birthweight. Without this, these feeds would have had to be covered by pasteurised DHM. Although the nutritional composition of raw DHM is not thoroughly documented (36), the intention therefore is to retain as much of the active biological and microbial contents within the milk (37). However, this practice is not without challenges, as it requires extensive screening, with potentially large volumes of DHM being discarded due to high microbial counts. Still, the use of raw milk in general and particularly raw DHM is still a unique feature of LMB and some other human milk banks in Germany and few other countries.

Although we show a reduced distribution of DHM over the 8 year study period in Leipzig, the reduction was compensated by increased use of MOM, and not by formula feeding. The German Neonatal Network (GNN) (38) shows an increased use of (pasteurised) DHM for enteral feeding in Germany between 2013 and 2019, especially in very low birth weight infants. Similarly, higher rates of DHM use were reported in the United States (3, 7) and United Kingdom (16). Although conclusions from the GNN are based on cumulative data, hospital practices of providing DHM vary by geographical region and institution. DHM provides an indispensable bridge to successful optimal forms of nutrition, but MOM should be prioritised when pursuing strategies to increase supply and provision of human milk (31, 39).

A limitation of our study is the lack of actual volume of milk supplied and fed to the infant, leaving us with taking dispensed milk weighted by the inverse of the count of different milk types per day as a proxy. We lack data on daily caloric intake and on nutritional and biological composition of either MOM or DHM and donor characteristics. Standard fortification was done during the observed time frame and target fortification was only carried out as part of a clinical study during the observation period. However, these data are not included in the records of the LMB. Important strengths of this study are the insights into eight different milk types, actual relative contributions of MOM and DHM can provide, which allow more accurate grouping of infants according to their milk feeds. This allows the comparison of exclusive milk groups thereby reducing the potential confounding effect of combining both MOM and/or DHM in one single group. We also display high-resolution data on plausible feeding patterns during hospitalisation over a defined period, which demonstrates the time-variant nature of feeding that is likely highly relevant for association studies with infant outcomes. This eliminates a major limitation of including MOM and DHM in single matrices, which could lead to inclusion bias and lower generalisability in observational analyses.

In conclusion, forms of nutrition during hospitalisation vary greatly, with interspersed DHM resulting in complex intra-individual time-variant feeding patterns. Thus, cut-offs utilised for classification into predominant MOM or DHM feeding during hospitalisation in previous studies may not always be applicable in otherwise different infant populations. LMB dispenses raw milk, particularly raw DHM

with the intention to preserve the properties of human milk unlike a diet containing pasteurised DHM and/or formula. As such, dispensing raw DHM is possible for a substantial portion of infants.

Data availability statement

The datasets presented in this article are not readily available due to data protection laws, we may not be able to share the raw data. However, the authors are open to sharing aggregate data (for instance, relative concentrations of the different milk types). Requests to access the datasets should be directed to Jon.Genuneit@medizin.uni-leipzig.de.

Ethics statement

The studies involving humans were approved by Ethics board of Leipzig University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LS, CB, EP, CG, and JG: conceptualization. CB, JG, EP, LS, UT, CG, RA, and TW: data acquisition. LS, CB, EP, and JG: analysis. LS, CB, EP, CG, RA, UT, and JG: interpretation. LS and JG: drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JG is the project manager and LS is a scientist on unrestricted research grants from Danone Nutricia Research to Ulm University and to Leipzig University for research into human milk composition within the Ulm SPATZ Health Study and the Ulm Birth Cohort Study.

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.2023.1233109/full#supplementary-material

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Unraveling the effects of maternal breastfeeding duration and exclusive breast milk on children's cognitive abilities in early childhood

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Background: This study investigated the putative associations between mothers' use of exclusive breast milk and the duration of breastfeeding with child cognitive development.

Methods: This study is based on 2,210 Canadian families with children assessed longitudinally from age 4 to 7 years on their memory-span and math skills. These cognitive abilities were measured with standardized tasks. Breastfeeding practices were collected via maternal reports. We applied propensity scores to control the social selection bias for breastfeeding.

Results: Results adjusted for propensity scores and sample weight revealed no significant differences between non-breastfed children with those being non-exclusively breastfed for 5 months or less, and with children being exclusively breastfed for 9.2 months on average, on their early math skills and memory-span. We found that children who were non-exclusively breastfed for 6.8 months on average had a slightly higher levels of memory-span at age 4 than children who were never breastfed, and this small but significant difference lasted up to age 7.

Conclusion: Our findings suggest no significant differences between children being exclusively breastfed and those fed with formula on their early math skills and memory-span. The encouragement of breastfeeding to promote child cognitive school readiness may, in some case (non-exclusive breastfeeding for more than 5 months), show a small but long-lasting advantage in early memory-span.

KEYWORDS

breastfeeding, breast milk, formula, memory-span, math skills, longitudinal design

Introduction

Breastfeeding and human milk are considered the normative standards for infant feeding and nutrition. It has several beneficial effects including nutrition and growth, fostering immune-microbiome interplay, promoting mother–child interaction, and improving neurobehavioral outcomes, especially among premature and very low birth weight children (1, 2). Breastfeeding for 3 months or more has been determined as an adequate period according to guidelines on allergy prevention and nutrition (1). However, the World Health Organization and UNICEF (3) suggests that exclusive breastfeeding for at least 6 months is necessary to observe early cognitive gains in breastfed children.

Multiple possible mechanisms can explain the effect of breastfeeding on children's health and cognitive development. According to the nutrient hypothesis, the docosahexaenoic (DHA) and arachidonic acids found in breast milk are involved in neural maturation, which would enhance the development of cognitive abilities such as problem-solving and memory-span (4–6). This mechanism has been shown in rats, where deficiency of DHA during lactation resulted in poor memory retention during learning tasks, whereas DHA supplementation had the reverse effect (7). Another potential mechanism for the development of cognitive abilities is the mother-infant physiological proximity during breastfeeding. According to this hypothesis, early skin-to-skin contact during breastfeeding would accelerate neuromaturation and thus, the development of cognitive abilities. One additional explanation is that mothers breastfeeding their infant are also more likely to provide a cognitively stimulating environment. Several studies revealed a negligible relationship between breastfeeding and child cognitive functioning after adjusting for maternal and home environment (8–10). This later explanation suggests controlling for the quality of the home environment as breastfeeding may be a proxy for parenting (11, 12).

Yet, despite these possible mechanisms, evidence that breastfeeding promotes child cognitive development is mixed. Some studies support a small but significant direct effect of breastfeeding on child cognitive abilities (2, 13–21). One cluster-randomized trial study found evidence that prolonged and exclusive breastfeeding (intervention group) improved children's IQ scores at age 6.5 years in comparison with controls (18). One observational US study also revealed that breastfeeding remained significantly associated with the child cognitive functioning measured with the Bayley Scales of Infant Development (BSID-II) at age 2 years, even after matching children being breastfed to those not being breastfed (14). In contrast, other studies found little to no relationship between breastfeeding practices and child cognitive development (8, 22–24). In a US national cohort study of 5,475 children of normal birth weight, ever being breastfed was associated with almost a 5-point higher child IQ, but this effect disappeared after adjustment for confounders (23). Another study in the US found no significant effects of any breastfeeding, breastfeeding duration, or exclusive breastfeeding on child executive function during mid-childhood (22). Another study revealed no significant association between breastfeeding for 3 months or longer and child math and reading skills at age 4 (8).

One possible explanation for these mixed findings is that previous studies have not adequately disentangle the duration of breastfeeding and whether children were exclusively and/or predominantly breastfed (20). Previous studies examining how breastfeeding practices are associated with children's cognitive development have also been limited by their study design, with most studies not controlling the selection bias for breastfeeding (2, 8, 13, 16, 19–22). Over the past decade, the use of statistical methods, such as propensity score weighting and the use of instrumental variables have strengthened the possibility of drawing causal inferences on the long-term cognitive outcomes of breastfeeding.

Another limitation is that only a few studies have adopted a longitudinal design with repeated measures of children's cognitive abilities (13, 16, 20, 21, 24). One longitudinal study found that having been breastfed (yes/no) was associated with a small but significant advantage in IQ at age 2 in girls but was not associated with IQ growth from ages 2 to 16 (21). This study, however, did not include measures of breastfeeding duration or breastfeeding exclusivity and did not control for the selection bias for breastfeeding. Furthermore, few studies have examined outcomes in the preschool and early school years (2, 24). Examining the effect of breastfeeding on child cognitive skills is particularly salient during the transition to school entry, when cognitive skills become important determinants of school readiness and later academic achievement. To our knowledge, only one study conducted in Ireland examined the association between breastfeeding and child cognitive abilities (problem-solving, expressive vocabulary) repeatedly at ages 3 and 5 years while controlling for selection bias (24). However, they did not control for parenting and home environment factors. This study also disentangled the duration of breastfeeding (1 month, 2–6 months, 6 months, or more) and the breastfeeding practices: full (exclusive or almost exclusive) and partial breastfeeding, in comparison to never being breastfed. Interestingly, children who were fully breastfed for 6 months or more had higher problem-solving scores at age 3 years in comparison to children who were never breastfed. However, this association was no longer statistically significant at age 5 and did not remain significant at age 3 after adjustment for multiple testing. These findings warrant replication.

Objectives

This study aims to test the effects of breastfeeding on children's cognitive development (early math skills and memory-span) during the transition to school entry. Specifically, we examine how the duration of breastfeeding and exclusive use of breast milk are longitudinally associated with children's early memory-span and math skills (including problem-solving), while controlling the selection bias for breastfeeding due to child, maternal/family, and demographic confounding variables. By doing so, we attempt to disentangle some of the various mechanisms potentially explaining such association. Infants exclusively breastfed and showing the highest levels of memory-span scores and early math skills would provide support to the nutrient hypothesis. To dismiss the hypothesis that breastfeeding mothers also provide a more cognitively stimulating environment, our analyses were adjusted to account for parenting practices.

Methods

Sample and design

Participants were from the Quebec Longitudinal Study of Child Development (QLSCD), an ongoing longitudinal population-based

study aimed at understanding the impact of early experiences on later school success (25). Families were recruited through the Quebec Master Birth Registry of the Ministry of Health and Social Services to be representative of children born in 1997–98 in Quebec, Canada. For practical reasons, data were not collected on children living on Cree or Inuit territories, in Indian reserves, and in northern Quebec. A three-stage sampling design based on living area and birth rate was used. Territories were first divided into regions, which were then divided into second-stage units composed of one or two county regional municipalities, and then further divided in third-stage units according to the number of births in 1996. All selected infants were born after October 1, 1997 to ensure that they entered school the same year. Families were excluded if mothers could not speak French or English, and if babies were born before 24 weeks or after 42 weeks of gestation. A sample of 2,940 families with newborns was initially identified. Selected families that could be located ($N = 2{,}675$) were approached by mail and phone. Of those, 2,223 families were first visited when the child was 5 months old (83%) and 2,210 were followed longitudinally and were assessed every year up to age 23. Ethics approval was obtained from the Direction Santé Québec of the Institut de la statistique du Québec and the Faculty of Medicine of the Université de Montréal. The respondent provided consent and voluntarily responded to this survey. The analytic sample for this study included families for whom information was available about the duration of breastfeeding and the use of exclusive breast milk when the baby was 5 months old ($N = 2{,}120$).

Measures

Breastfeeding practices

Breastfeeding practices were measured using two items reported by the mother when the baby was age 5 months: "Did you breastfeed your baby? (1 = yes, and I am continuing to do so; 2 = yes, but I have since ceased to do so; 3 = no, I never did). Mothers who reported breastfeeding their child and continuing to do so were considered as breastfeeding for more than 5 months, while mothers reporting that they ceased to breastfeed were grouped as breastfeeding for less than 5 months. Mothers that breastfed their infants for more than 5 months were also asked the following question: "Did your baby drink anything other than just breast milk? (yes/no)." At the 17 months interview, mothers breastfeeding their infants also reported how old was their infant (in months) when they ceased breastfeeding.

Four groups of mothers were derived from these items: (1) non-breastfeeding group (commercial milk only, $n = 600$, 28.3%), (2) non-exclusive breastfeeding for 5 months or less ($n = 809$, 38.2%), (3) non-exclusive breastfeeding for more than 5 months ($n = 356$, 16.8%), and (4) exclusive breastfeeding (breast milk only) for more than 5 months ($n = 355$, 16.7%). None of the mothers breastfeeding for 5 months or less used exclusive breast milk.

Child cognitive abilities

Children's early math skills were measured at ages 4, 5, and 6 years with the Number Knowledge Test (26–29). The Number Knowledge Test was developed to document children's understanding of whole numbers and basic operations, and as a tool for teachers to identify children with mathematic difficulties (30). This test has four levels of complexity (from 0 to 3). Each level of the test reflects a current developmental stage of children's number knowledge comprehension (30, 31). The baseline and first levels of the Number Knowledge Test were administered at ages 4, 5, and 6. Except for the low reliability at age 5 (Cronbach's $\alpha = 0.55$), the internal consistency of the Number Knowledge Test in our sample was found to be adequate ($\alpha = 0.68$ at age 4, 0.92 at age 6); and the test–retest stability was high across all time points (Pearson's $r = 0.74$ between ages 4 and 5 and $r = 0.92$ between ages 5 and 6).

Children were also assessed on their memory-span at ages 4, 5, 6, and 7 years with the Visually Cued Recall task (32), a reliable measure ($\alpha = 0.95$ in our sample) of the child's incremental capacity to encode visual items and to recall the spatial locations of the items after a short delay. In each trial, a research assistant showed a cardboard with pictures of 12–18 objects to the child. The research assistant pointed to a certain number of objects and asked the child to remember them. The research assistant then flipped the cardboard for a short delay. When flipped back, the child was cued to identify the objects pointed to previously. The number of objects to remember increased after each trial, up to 12 different levels of difficulty. The test ended when the child made two errors on two consecutive levels. The final score consists of the highest level reached by the child.

Covariates

Covariates were selected as controls for empirical and theoretical reasons (22–24). When children were 5 months old, the person most knowledgeable about the child (99.0% were mothers) provided data on the household income (<30 K CAD/year vs. higher income), maternal education (university diploma vs. no university diploma), if the mother was an immigrant (yes/no), maternal age, and family composition (single-parent, two-parent, or stepfamily). Birth weight (<2,500 g) and developmental/stunted growth (<10th centile) of the child were derived from the birth medical registry. The mothers also reported if they worked since pregnancy (yes/no).

Maternal smoking during pregnancy was coded present if the mother had smoked at least one cigarette/day while pregnant. Prenatal alcohol exposure was coded as 0 = never, 1 = having drunk alcohol less than 3 times/month. Symptoms of maternal depression in the last week were rated at the 5 months interview with the 12-item version ($\alpha = 0.85$) of the Center for Epidemiologic Studies Depression Scale (33). Item responses ranged from 0 (none) to 3 (all the time), and the total scores were then rescaled on a 10-point scale. Two dimensions of parenting were also reported by the mother in the Parental Cognitions and Conduct toward the Infant Scale (34): overprotection (e.g., keeping the child close most of the time; 5 items, $\alpha = 0.68$) and perceived parental impact (e.g., thinks his/her parenting affects the emotional development of the child; 5 items, $\alpha = 0.71$).

Procedure

A trained research assistant administered the Visually Cued Recall task test following a standard procedure in a face-to-face interview. The Number Knowledge Test was orally administered one-on-one by a trained research assistant at school or at home. Breastfeeding practices and the various child, maternal/family, and demographic

confounding variables were reported by the mothers at the 5-months interview.

Analytical strategy

We performed covariate balancing propensity score (CBPS) weighting in R to increase comparability across the four groups of mothers (35). Indeed, breastfeeding does not occur at random, since mothers breastfeeding their babies are not similar to non-breastfeeding mothers regarding important covariates. This procedure reduces the selection bias for breastfeeding. CBPS performed multinomial regression to estimate the associations between the covariates and the four groups of mothers and generates a propensity score for each observation. The propensity score estimates the predicted probability of group membership from all observed covariates. Once estimated, we conducted a balancing test to ensure the quality of weighting (36). The balancing test showed that all covariates had a standardized mean difference less than |0.10| after CBPS, indicating that group differences were minimal (37) (see Figure 1).

After applying the CBPS, we conducted Latent Growth Modeling (LGM) to investigate how the groups based on the duration of breastfeeding and exclusive breast milk were longitudinally associated with changes in children's math ability and memory-span across time. LGM is a special class of Confirmatory Factor Analysis that estimate systematic change or growth over a period of time (also called trajectory), and the inter-individual variability in this change (38). The trajectory can be of various shapes (linear, quadratic, cubic). First, for each child's cognitive outcome, an unconditional (baseline) model was estimated to determine the average trajectory of early math skills and memory-span, using the maximum-likelihood technique for continuous and normally distributed data. Second, conditional growth models (i.e., including predictors) were performed to predict the developmental trajectory for each child outcome from groups of breastfeeding mothers. The goodness of fit of these models were determined with a root means square error of approximation (RMSEA) < 0.08 (39), a comparative fit index (CFI) > 0.90 (40), and a value of chi-square small enough not to reach the significance threshold (41).

The LGM was performed in Mplus (42) using the full information maximum likelihood to handle missing outcome data. The analyses were adjusted for the propensity scores and for sample weight from the QLSCD, which ensures that the sample remains representative of the Quebec population. It was also adjusted for the covariates as additional controls. This allowed us to eliminate the selection bias for breastfeeding while also removing the contribution of these covariates to the predicted outcomes (43). Code for the analysis is available by emailing the corresponding author.

Pattern of missing data

The average proportion of missing data across covariates was 1.5%. Considering the low proportion of missing data, missing data were replaced by the mean for continuous variables, the median for ordinal variables, or the mode for categorical variables. The proportion of missing data on child early math skills and memory-span was on average 12.0 and 7.4% per year, respectively. According to Little's test, the overall pattern of missingness significantly deviates from a pattern of data that is missing completely at random ($\chi^2 = 244.42$, df = 172, $p = 0.000$). The pattern of missingness was most likely at random. A series of t-tests and chi-square revealed that children with missing scores on the number knowledge and memory-span tests tended to be from a lower socioeconomic background, from immigrant and single mothers, younger than 20 years old, with no university diploma, and smoking and drinking during pregnancy. We statistically controlled for these variables in our analyses.

Results

Mothers who never breastfed used commercial milk exclusively. Mothers breastfeeding for 5 months or less stopped breastfeeding when their babies were on average 2.1 months (SD = 1.68). The group of mothers with non-exclusive breastfeeding for more than 5 months introduced commercial milk when their babies were 2.9 months (SD = 0.46) and stopped breastfeeding when their babies were on average 6.8 months old (SD = 1.83). The group of mothers with exclusive breastfeeding for more than 5 months stopped breastfeeding when their infants were 9.2 months on average (SD = 2.73). Descriptive statistics of confounding variables and child cognitive abilities prior to applying CBPS are shown for each group in Table 1.

Early math skills and memory-span developmental growth

The unconditional LGM models yield significant intercept and slope for both outcomes, indicating progressive growth over time in early math skills and memory-span. Early math skills revealed a linear growth while the memory-span trajectory had a quadratic shape. Developmental trajectories of early math skills and memory-span are displayed in Supplementary Figures S1, S2. The unconditional LGM for early math skills had an acceptable fit as evidenced by the non-significant chi-square ($\chi^2 = 0.538$, $p = 0.463$), the RMSEA = 0.000 [0.000; 0.055], and the CFI = 1.00. The unconditional LGM for memory-span also had an adequate fit ($\chi^2 = 3.88$, $p = 0.143$; RMSEA = 0.022 [0.000; 0.055]; CFI = 0.989).

Associations of breastfeeding with early math skills and memory-span

We next examined the extent to which the groups of maternal breastfeeding predicted the initial level and the growth in early math skills and memory-span, once adjusted for the propensity scores and for sample weight, and while controlling for birth weights, stunted growth, smoking and alcohol drinking during pregnancy, maternal depression, household income, maternal age, maternal education, immigrant status, family composition, maternal perception of impact and overprotection, and working since pregnancy. Despite the adequate fit of the conditional model ($\chi^2 = 16.87$, $p = 0.462$; RMSEA = 0.000 [0.000; 0.021]; CFI = 1.00), our findings revealed no significant associations between the groups of breastfeeding mothers (vs. non-breastfeeding group) and children's early math skills' intercept and slope. Results are shown in Table 2.

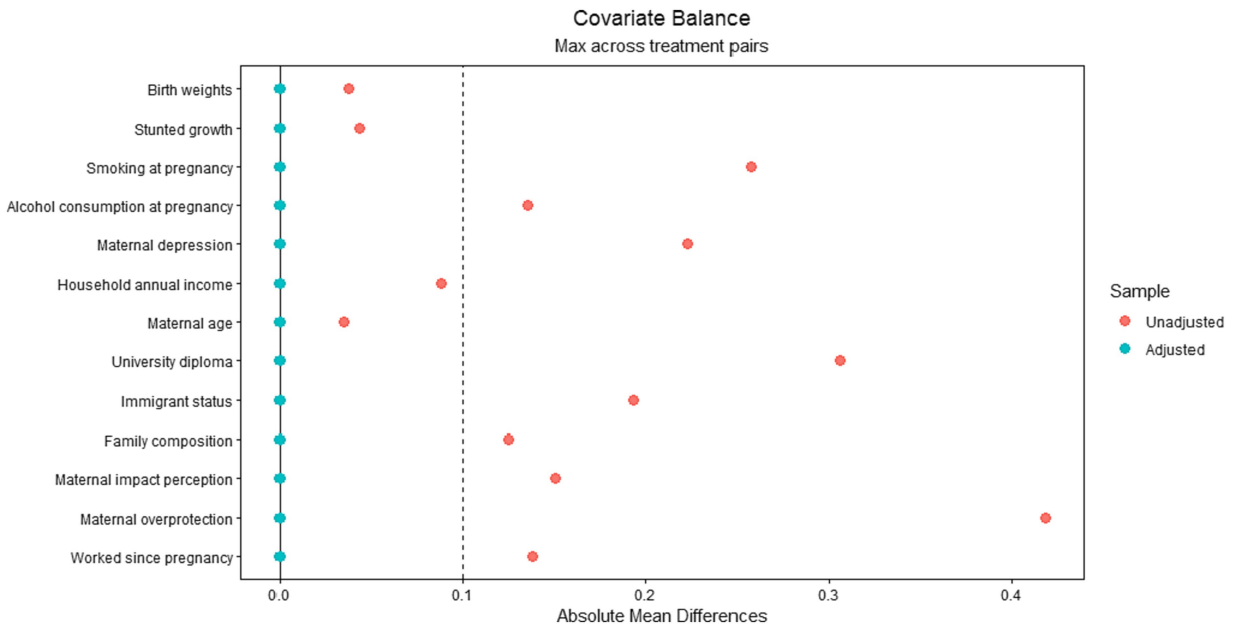

FIGURE 1
The figure displays the average standardized mean difference between groups for all the covariates used in the estimation of the propensity score weights. The orange dots represent the differences among groups on each covariate before applying the propensity score weights, and the blue dots represents the differences after application of the propensity score weights.

TABLE 1 Child, maternal and family-wide factors associated with breastfeeding when the child was 5 months-old ($N = 2,120$).

	Breastfeeding > 5 months		Breastfeeding < or = 5 months	No breastfeeding
	Exclusive breast milk ($n = 355$, 16.7%)	Non-exclusive breast milk ($n = 356$, 16.8%)	Non-exclusive breast milk ($n = 809$, 38.2%)	Exclusive formula ($n = 600$, 28.3%)
Child characteristics				
At birth				
Sex of the child (female, $n = 1,040$)	49.9%	47.8%	49.4%	48.8%
Low birth weight (<2,500 g, $n = 73$)	2.8%	3.9%	2.3%	5.0%
Stunted growth (yes = 172)	7.0%	5.6%	8.5%	9.7%
Maternal characteristics				
At birth				
Maternal age (< 20 years old, $n = 59$)	1.4%	0.8%	3.7%	3.5%
Immigrant status (yes, $n = 253$)	18.3%	19.1%	10.6%	5.7%
University diploma (yes, $n = 566$)	38.0%	44.1%	25.2%	11.7%
Alcohol consumption at pregnancy (yes, $n = 752$)	42.5%	38.8%	38.3%	25.5%
Ever smoked during pregnancy (yes, $n = 533$)	15.2%	12.6%	25.8%	37.5%
At 5 months				
Maternal depression score†	1.27 (1.26)	1.22 (1.29)	1.44 (1.32)	1.53 (1.42)
Maternal impact perception score†	8.46 (1.85)	8.56 (1.71)	8.38 (1.84)	8.17 (1.90)
Maternal overprotection score†	5.35 (2.30)	4.78 (2.23)	4.37 (2.10)	4.82 (2.15)
Worked since pregnancy (yes, $n = 422$)	9.9%	16.6%	24.9%	21.0%
Family-wide factors				
At 5 months				
Household annual income (< 30 K, $n = 483$)	25.0%	17.1%	20.4%	28.0%
Family composition (single parent, $n = 406$)	17.2%	13.8%	17.6%	25.7%

Data are courtesy of the Quebec Institute of Statistics.
†Indicates continuous variables.

TABLE 2 Association between breastfeeding and early math skills (standardized estimates).

	Intercept (R^2 = 0.152)					Slope (R^2 = 0.039)				
	Estimate	SE	CI	*p*	Beta	Estimate	SE	CI	*p*	Beta
Mean	4.85	0.675	3.53; 6.17	0.000		3.16	0.428	2.32; 4.00	0.000	
Variance	7.17	1.02	5.15; 9.18	0.000		2.20	0.407	1.40; 3.00	0.000	
Predictors										
Breastfeeding <5 months	−0.137	0.271	−0.67; 0.39	0.613	−0.020	0.051	0.162	−0.26; 0.36	0.750	0.015
Non-exclusive breastfeeding >5 months[a]	0.539	0.321	−0.91; 1.17	0.094	0.082	−0.046	0.192	−0.42; 0.33	0.811	−0.013
Exclusive breastfeeding >5 months[a]	0.049	0.330	−0.59; 0.69	0.881	0.007	0.067	0.210	−0.34; 0.47	0.748	0.019
Covariates										
Birth weights	0.794	0.784	−0.74; 2.33	0.311	0.052	−0.243	0.540	−1.30; 0.81	0.653	−0.031
Stunted growth	−0.733	0.403	−1.52; 0.05	0.069	−0.071	−0.458	0.305	−1.05; 0.14	0.133	−0.085
Smoking during pregnancy	−0.152	0.266	−0.67; 0.37	0.569	−0.023	0.258	0.167	−0.06; 0.58	0.122	0.074
Alcohol during pregnancy	−0.130	0.232	−0.58; 0.32	0.576	−0.022	−0.020	0.139	−0.29; 0.25	0.883	−0.007
Maternal depression	0.088	0.085	−0.07; 0.25	0.297	0.042	−0.028	0.060	−0.14; 0.08	0.636	−0.026
Household income	**−0.726**	**0.291**	**−1.29; 0.16**	**0.012**	**−0.101**	−0.115	0.181	−0.46; 0.23	0.523	−0.031
Maternal age	−0.817	0.670	−2.13; 0.49	0.223	−0.045	0.840	0.469	−0.08; 1.76	0.074	0.089
University degree	**1.602**	**0.269**	**1.07; 2.13**	**0.000**	**0.246**	−0.262	0.161	−0.57; 0.05	0.104	−0.077
Immigrant status	0.337	0.487	−0.62; 1.29	0.489	0.034	−0.041	0.290	−0.61; 0.52	0.887	−0.008
Intact family	**−0.734**	**0.275**	**−1.27; −0.19**	**0.008**	**−0.097**	0.138	0.179	−0.21; 0.49	0.441	0.035
Perception of impact	0.114	0.059	−0.002; 0.22	0.054	0.069	0.066	0.039	−0.00; 0.14	0.086	0.077
Overprotection	−0.065	0.052	−0.17; 0.03	0.210	−0.049	0.025	0.034	−0.04; 0.09	0.456	0.036
Worked since pregnancy	**−0.553**	**0.271**	**−1.09; −0.02**	**0.041**	**−0.077**	−0.043	0.171	−0.37; 0.29	0.803	−0.011

SE: standard error; bold values denote statistical significance based on the confidence interval (CI). This analysis was adjusted for the propensity scores and for sample weight from the QLSCD. As indicated in the table, it was also adjusted for the following covariates: birth weights, stunted growth, smoking and alcohol during pregnancy, maternal depression, household income, maternal age, maternal education, immigrant status, family composition, maternal perception of impact and overprotection, and working since pregnancy.
[a]In comparison to the non-breastfeeding group (commercial milk only).

The conditional model also revealed an acceptable fit in predicting memory-span (χ^2 = 23.58, p = 0.169; RMSEA = 0.013 [0.000; 0.025]; CFI = 0.981). Results are shown in Table 3. In comparison to the non-breastfeeding group, the group of mothers non-exclusively breastfeeding for more than 5 months was significantly associated with the initial level in child memory-span. Children who were non-exclusively breastfed for more than 5 months had higher levels of memory-span at age 4 than children who were never breastfed. This small (beta = 0.08) but significant difference between the two groups was maintained over time up to age 7.

Discussion

This study investigated the putative associations between mothers' use of exclusive breast milk and the duration of breastfeeding with children's early memory-span and math skills. Similar to previous studies (8, 22–24), our findings revealed little to no significant differences between children being breastfed and those fed with formula (non-breastfed infants) on their early math skills and memory-span. No significant differences were found between non-breastfed children and those being non-exclusively breastfed for 5 months or less, and with children being exclusively breastfed for more than 5 months. Interestingly, children being non-exclusively breastfed for more than 5 months showed a slightly higher levels of memory-span that lasted over time compared to the non-breastfed group.

One possible explanation is that mothers non-exclusively breastfeeding for more than 5 months may benefit from greater marital support and/or from an extend social network, where the mother breastfeed her infant every time she can but the father (and/or grand-parents, educators) also have the chance to bottle-fed the infant. In turn, this social network may provide different and various stimulating interactions to the child, ensuing greater memory-span skills during the preschool years. For children being non-exclusively breastfed for more than 5 months, this small (almost negligible) but significant advantage in memory-span during early childhood could still be translated into later gains in other cognitive components (ex., executive functions) or academic skills.

This finding partially supports the need to keep breastfeeding for more than 5 months. This group of mothers stopped breastfeeding when their babies were on average 6.8 months old. However, contrary to the WHO guidelines (2003) that recommend exclusive breast milk for the first 6 months and to continue breastfeeding with complementary foods until 2 years or beyond, our results rather show that it is a mix of breast milk and formula that confer benefits on memory-span. In this study, we did not distinguish between breastfeeding and breast milk that was bottled-fed to infants. However, one recent study revealed that among infants exclusively fed with breast milk, those fed directly from the mother scored higher on several memory tasks compared to children bottle-fed of breast milk

TABLE 3 Association between breastfeeding and memory-span (standardized estimates).

	Intercept (R^2 = 0.095)					Slope (R^2 = 0.071)					Quadratic (R^2 = 0.209)				
	Estimate	SE	CI	*p*	Beta	Estimate	SE	CI	*p*	Beta	Estimate	SE	CI	*p*	Beta
Mean	2.90	0.349	2.21; 3.58	0.000		1.03	0.559	−0.06; 2.12	0.065		−0.063	0.187	−0.42; 0.30	0.734	
Variance	3.65	0.735	2.21; 5.09	0.000		3.00	0.665	1.70; 4.31	0.000		0.100	n/a	n/a	n/a	
Predictors															
Breastfeeding <5 months[a]	0.028	0.144	−0.25; 0.31	0.847	0.006	−0.020	0.243	−0.49; 0.45	0.934	−0.005	0.003	0.079	−0.15; 0.16	0.969	0.004
Non-exclusive breastfeeding >5 months[a]	**0.367**	**0.184**	**0.01; 0.73**	**0.046**	**0.080**	−0.463	0.285	−1.02; 0.09	0.104	−0.113	0.109	0.095	−0.07; 0.29	0.250	0.134
Exclusive breastfeeding >5 months[a]	−0.254	0.171	−0.59; 0.08	0.137	−0.054	−0.222	0.307	−0.82; 0.38	0.470	−0.053	0.090	0.100	−0.10; 0.28	0.367	0.108
Covariates															
Birth weights	−0.295	0.352	−0.98; 0.39	0.402	−0.028	0.278	0.619	−0.93; 1.49	0.653	0.029	−0.066	0.204	−0.46; 0.33	0.748	−0.035
Stunted growth	**−0.512**	**0.213**	**−0.93; −0.09**	**0.016**	**−0.071**	0.104	0.343	−0.56; 0.77	0.762	0.016	0.009	0.137	−0.25; 0.27	0.948	0.007
Smoking during pregnancy	−0.151	0.141	−0.42; 0.12	0.285	−0.033	−0.190	0.260	−0.70; 0.31	0.464	−0.046	0.097	0.087	−0.07; 0.26	0.265	0.119
Alcohol during pregnancy	0.146	0.133	−0.11; 0.40	0.272	0.035	0.301	0.220	−0.13; 0.73	0.171	0.081	−0.105	0.072	−0.24; 0.03	0.148	−0.142
Mother depression	**−0.091**	**0.040**	**−0.17; −0.01**	**0.023**	**−0.062**	−0.003	0.075	−0.14; 0.14	0.972	−0.002	0.011	0.025	−0.03; 0.06	0.653	0.043
Household income	**−0.489**	**0.146**	**−0.77; −0.20**	**0.001**	**−0.099**	0.275	0.268	−0.25; 0.80	0.304	0.062	−0.055	0.092	−0.23; 0.12	0.552	−0.063
Maternal age	−0.429	0.339	−1.09; 0.23	0.207	−0.035	0.058	0.510	−0.94; 1.05	0.909	0.005	−0.052	0.160	−0.36; 0.26	0.747	−0.024
University degree	**0.687**	**0.153**	**0.38; 0.98**	**0.000**	**0.152**	**−0.505**	**0.252**	**−0.99; −0.01**	**0.045**	**−0.125**	0.111	0.081	−0.04; 0.27	0.172	0.139
Immigrant status	−0.148	0.180	−0.50; 0.20	0.411	−0.022	0.071	0.393	−0.70; 0.84	0.856	0.012	0.021	0.131	−0.23; 0.27	0.871	0.018
Intact family	0.104	0.158	−0.20; 0.41	0.509	0.020	**−0.558**	**0.279**	**−1.1; −0.01**	**0.045**	**−0.120**	**0.225**	**0.095**	**0.04; 0.41**	**0.017**	**0.245**
Perception of impact	0.033	0.031	−0.02; 0.09	0.288	0.029	**0.181**	**0.052**	**0.07; 0.28**	**0.001**	**0.177**	**−0.059**	**0.017**	**−0.09; −0.02**	**0.001**	**−0.290**
Overprotection	−0.003	0.032	−0.06; 0.06	0.927	−0.003	−0.008	0.049	−0.10; 0.08	0.872	−0.010	0.000	0.016	−0.03; 0.03	0.990	0.001
Worked since pregnancy	0.343	0.177	−0.00; 0.69	0.052	0.069	−0.116	0.287	−0.67; 0.44	0.687	−0.026	0.014	0.095	−0.17; 0.20	0.879	0.016

SE: standard error; bold values denote statistical significance based on confidence interval (CI). n/a indicates not available. This analysis was adjusted for the propensity scores and for sample weight from the QLSCD. As indicated in the table, it was also adjusted for the following covariates: birth weights, stunted growth, smoking and alcohol during pregnancy, maternal depression, household income, maternal age, maternal education, immigrant status, family composition, maternal perception of impact and overprotection, and working since pregnancy.

[a]In comparison to the non-breastfeeding group (commercial milk only).

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(44), suggesting that nursing infants directly at the breast may impact memory.

As we found no significant difference between non-breastfed infants and those being exclusively breastfed for at least 5 months (who kept breastfeeding up to age 9.2 months on average), our findings did not support the nutrient hypothesis in promoting child cognitive development. This hypothesis postulates that the nutrients found in breast milk (e.g., DHA, arachidonic acid) facilitate neural maturation and the development of the nervous system (4–6), improving children's cognitive growth. Similarly, this result did not support the early skin-to-skin contact to be the main mechanism in promoting the development of cognitive abilities during breastfeeding. If so, we would have found a significant difference in children's cognitive development between children being breastfed and those from the non-breastfed group. This study, however, cannot rule out the role of these mechanisms in promoting cognitive development, as we did not directly compare the breast milk composition with infant formulas, and we did not measure the frequency of the skin-to-skin contact nor the mother–child bonding.

A key strength of this study is that we controlled for parenting practices involved in child cognitive development. Specifically, the perception of parental impact from the mother was significantly associated with growth in memory-span. Future studies should test parenting practices as potential mediating mechanisms (8, 45).

Although not entirely supporting the WHO guidelines for promoting child cognitive development, our findings do not contradict the many health benefits afforded to infants as a result of breastfeeding (46, 47). Previous studies have shown that breastfeeding decreases the risk of being overweight during infancy (46) but not in adolescence (48), and reduced the risk of chronic diseases such as allergies and asthma (49, 50). Several studies showing the benefits of breastfeeding on child cognitive outcomes were also conducted on preterm or very low-weight infants (1, 2), suggesting that poor fetal growth moderates this association. Future studies should further explore how other perinatal risk factors, such as delivery complications, parental mental health problems, and socioeconomic adversity, may moderate the association between breastfeeding and child cognitive development. Nevertheless, our results revealed that, at the population level, exclusive breastfeeding for more than 5 months (9.7 months on average) does not translate into long-term improvement in memory-span and math skills during early childhood.

Limitations

Despite these new insights, results should be interpreted with caution. First, information on breastfeeding was collected retrospectively when infants were 5 and 17-months old. Although the reliability of recall has been established (51), recall bias may still be present, particularly regarding the duration of full breastfeeding. Second, we could not disentangle the effect of direct breastfeeding versus expressed breast milk feeding, limiting our capacity to investigate whether the association with improved memory-span could partly be the result of skin-to-skin contact. Some studies also revealed that feeding bottled breast milk may not be biologically equivalent to direct breastfeeding. Differences have been observed for infant memory-span (44), suggesting a potential negative impact from the process of bottle feeding and/or reduced bioactivity of expressed breast milk. Future research should capture the complexity of modern feeding practices, even among exclusively breast (milk)-fed infants. Similarly, misclassification of breastfeeding exposure is also possible. Here, we considered the duration and exclusivity of breastfeeding. However, recent research suggests also distinguishing nursing at the breast from expressed breast milk, the relative proportion of breast milk from infant formula and variation in the type of formula used, perinatal feeding exposures in hospitals, and introduction of complementary foods (52). Another limitation is that we did not have information about the mother's diet such as the frequency and the quality of the food consumption, including intake of vitamin and/or mineral supplementation. As evidence by recent systematic reviews [e.g. (53, 54)] maternal diet is reflected in the breast milk composition, which might impact the nutritional quality of the breast milk and its contribution to children's cognitive development. Third, despite our conservative approach to address the selection bias to breastfeed and to additionally controlled for several children, maternal/family, and sociodemographic confounders, we cannot rule out the possibility that selection for breastfeeding exposure resulted from confounding variables not considered in our covariate balancing propensity score approach. For instance, we did not control for parenting practices specifically tapping into memory-span or the early math domain (ex., playing with numbers) and these practices were only indirectly linked to cognitive skills (e.g., perception of impact). Similarly, we did not control for maternal IQ as it was not collected in this cohort (8). A few studies suggest that maternal IQ accounts for a large proportion of the association between breastfeeding and cognitive outcomes (17, 23). Fourth, we did not measure every component of cognitive development. For instance, breastfeeding may not be related to early math skills but may be associated with executive functions, which is located in the prefrontal cortex, a brain area imprinted by postnatal experiences (55).

Conclusion

In conclusion, this study found little evidence that breastfeeding is longitudinally associated with early math skills and memory-span, regardless of the duration of breastfeeding and whether it was exclusively breast milk. Breastfeeding has important health and economic benefits, and the encouragement of breastfeeding to promote child cognitive school readiness may, in some case (i.e., non-exclusive breastfeeding for more than 5 months), show a small and long-lasting advantage in early memory-span. This advantage could potentially promote the development of other cognitive skills or still manifest later in life, but this has not been tested in the current study.

Data availability statement

The data analyzed in this study is subject to licenses/restrictions. Requests for data access should be directed to https://www.jesuisjeserai.stat.gouv.qc.ca/informations_chercheurs/acces_an.html.

Ethics statement

The study involving humans were approved by the Direction Santé Québec of the Institut de la statistique du Québec and the Faculty of Medicine of the Université de Montréal. The study were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

GG-C conceived the project, conducted and oversaw all aspects of the analyses, and wrote the paper. GT conducted the analyses, contributed to the interpretation, and reviewed the manuscript. JB, CM-G, ALa, and CF contributed to the interpretation, and reviewed the manuscript. ALe conducted the analyses. 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/fpubh.2023.1225719/full#supplementary-material

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Implementation of the Baby-Friendly Hospital Initiative in Mexico: a systematic literature review using the RE-AIM framework

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The Baby-Friendly Hospital Initiative (BFHI) is a global strategy to encourage health facilities to promote, support, and protect breastfeeding by implementing a package of policies and practices known as the Ten Steps to Successful Breastfeeding. Prior studies have found that implementing the Ten Steps has a positive impact on breastfeeding outcomes. Yet, little is known about the implementation of the Ten Steps in Mexico. The objective of this study was to conduct a systematic review to evaluate the reach, efficacy/effectiveness, adoption, implementation, and maintenance of the Ten Steps in Mexico, using the RE-AIM framework. The systematic literature review included studies published in English or Spanish without date restrictions. Two of the authors coded each of the articles through a harmonized data extraction tool, and group meetings were used to discuss any discrepancies. The reviewed data were managed in the Rayyan platform. The risk of study bias was assessed through the Johanna Briggs Institute critical appraisal checklists. Of the 1,123 articles initially identified, 6 met the review inclusion criteria. None of the articles evaluated the reach and maintenance of the Ten Steps. The articles identified major gaps in the implementation of the Ten Steps. Most of the articles had important limitations in terms of their quality. In Mexico, it is necessary to rethink the BFHI and employ multiple strategies to improve implementation of the Ten Steps, including developing transparent BFHI monitoring mechanisms that produce data on implementation and that are publicly available, as well as investing in implementation research and evaluation to generate strong evidence to support the adoption and efficient maintenance of the Ten Steps in health facilities in Mexico. When properly implemented, BFHI becomes central to promote, protect, and support breastfeeding. Therefore, it is essential for Mexico to position BFHI as a top priority of the country's public policy agenda.

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KEYWORDS

breastfeeding, Ten Steps, BFHI, RE-AIM, Mexico, implementation research

1 Introduction

The first 1000 days of life, from conception to the first 2 years, constitute a critical stage for healthy growth and development, in which breastfeeding (BF) plays a crucial role (1). Recent evidence has shown that suboptimal BF costs the world close to 1 billion dollars per day in lost productivity (2, 3). Similarly, according to the WHO, investing in promoting optimal BF practices, including initiation within the first hour of life, exclusive (EBF) for 6 months and continuing breastfeeding until the child is at least 2 years old, once nutrient-dense complementary foods get introduced at 6 months (2), could globally prevent the deaths of 820,000 children per year (2, 3).

BF is a personal maternal decision but that is bounded by multiple societal pressures and expectations that limit mothers' and caregivers' infant feeding decisions (4). From a socioecological perspective, such pressures and expectations are expressed through social, political, economic, organizational, and individual determinants (5–7). From this socioecological perspective, one of the many actors that influence mothers and caregivers' infant feeding decisions are health providers and the healthcare systems where they work. There is clear evidence that health providers need to be strongly engaged in BF protection, promotion, and support for BF programs to be effective (8). Given that health providers operate within healthcare systems, the standard operations procedures guiding the continuum of BF care in hospital and community environments, and the coordination between the two, are of the utmost importance for improving BF outcomes. This is because these standard operation procedures strongly influence the practice of health providers as well as breastfeeding decisions among mothers and their support networks (3, 9). At the end of the day, mothers need support and guidance in initiating, implementing, and maintaining optimal BF practices. If healthcare systems do not have skilled BF personnel and counseling programs, then mothers may not have access to the support they need and hence the agency to strengthen their BF self-efficacy, confidence, and motivation (9, 10). For this reason, the WHO and the United Nations Children's Fund (UNICEF) launched the Baby Friendly Hospital Initiative (BFHI) in 1989 based on the Ten Steps to Support Breastfeeding in Maternity Facilities and subsequent support and care at the community level (9, 11).

In many ways, the BFHI is a quality control system that allows maternity facilities to effectively support BF. Each facility that complies with the Ten Steps can eventually become accredited or certified as a "Baby Friendly Hospital" if they meet strict criteria including passing an external evaluation. Indeed, the BFHI provides an evidence-based accreditation program that promotes a series of steps aimed at: (i) planning BF during the prenatal stage, (ii) timely starting of BF in the perinatal period, and (iii) sustaining the exclusivity and duration of BF in the postnatal stage. To achieve this, the Ten Steps must be followed (Table 1) and should be aligned with trained health personnel and adequate hospital pre-, peri-, and postnatal practices. For the postnatal period, it is important to highlight that the tenth Step of the BFHI provides the extension to the Baby-Friendly Community Initiative (BFCI), which focuses on the community-based support needed after discharge. Despite its relevance, there is less evidence about the implementation of BFCI (12).

In 2018, the BFHI steps, and especially the guidance on accreditation, underwent some adjustments to provide flexibility to countries on how best to implement the BFHI accreditation processes in their local contexts, but without sacrificing the reach and quality of implementation of each of the Ten Steps (13). Regarding the steps, an important modification was the need to specifically align maternity facilities with the WHO International Code of Marketing of Breastmilk Substitutes and the World Health Assembly-related resolutions (14). It also implicitly underlines the need to have well-documented standard operation procedures of the internal management information system to monitor the implementation of the Ten Steps in the facility.

The modifications made to BFHI in 2018 recognized the need for flexibility as the accreditation process works differently across countries. In some countries, such as the United States, it depends on a private institution (i.e., Baby-Friendly USA), but in other countries like Brazil and Mexico, the accreditation process is run by the government. The Ten Steps are evidence-based, as when properly implemented they have been shown to improve BF outcomes across the world region (15).

Nevertheless, implementation challenges still need to be better understood and addressed (16, 17). For example, even though the initiative is now over 25 years old, its coverage, measured as the proportion of children born in a BFHI-accredited hospital, remains very low (18, 19). In 2017, only 10% of newborns worldwide were delivered in BFHI-accredited hospitals (19). Previous studies have documented that countries have encountered difficulties in sustaining the BFHI because of financial and human resources considerations (14). It has also been noted that its successful implementation requires political commitment (12). Additionally, successfully implementing the Ten Steps can be challenging due to the lack of robust internal monitoring and evaluation systems at maternity facilities that can support quality assurance efforts related to the Ten Steps (14), including the training of health personnel (20, 21).

According to the experiences of some countries where the implementation of the BFHI has been relatively more successful, the BFHI requires adequate financing and flexibility to support its adoption, expansion, and maintenance at the national level (22). Consistently, these countries have identified the cost-effective training of health providers as being crucial for the success of BFHI rollout on a large scale (23), together with the internal monitoring and evaluation system mentioned above (9, 22).

This study aimed to conduct a systematic literature review of the BFHI in Mexico using the RE-AIM framework to organize the findings from the review (24). The RE-AIM is an implementation science framework that provides a structure for evaluating implementation (25). While all frameworks have limitations, they also provide the foundation for drawing from and developing a cumulative, evidence-informed science (26). In this sense, the RE-AIM allows to better understand how the BFHI has been adopted, implemented, and sustained, while considering its reach and effectiveness in improving breastfeeding outcomes. In fact, the RE-AIM has already been used to assess the BFHI in the United States and Brazil (17). Using the same framework to assess

TABLE 1 Ten Steps to successful breastfeeding (BFHI), 1989 and 2018 versions.

Step	Original version (1989)	Revised version (2018)
1	Have a written breastfeeding policy that is routinely communicated to all healthcare staff.	(a) Fully comply with the International Code of Marketing of Breast-milk Substitutes and relevant World Health Assembly resolutions.
		(b) Have a written infant feeding policy that is routinely communicated to staff and parents.
		(c) Establish ongoing monitoring and data management systems.
2	Train all healthcare staff in the skills needed to implement the breastfeeding policy.	Ensure that all staff has sufficient knowledge, competencies, and skills to support breastfeeding.
3	Inform all pregnant women about the benefits and management of breastfeeding.	Discuss the importance and management of breastfeeding with pregnant women and their families.
4	Help mothers initiate breastfeeding within a half-hour of birth.	Facilitate immediate and uninterrupted skin-to-skin contact and support mothers to initiate breastfeeding as soon as possible after birth.
5	Show mothers how to breastfeed and how to maintain lactation even if they should be separated from their infants.	Support mothers to initiate and maintain breastfeeding as well as to manage common difficulties.
6	Give newborn infants no food or drink other than breastmilk, unless medically indicated.	Do not provide breastfed newborn infants any foods or fluids other than breastmilk, unless medically indicated.
7	Practice rooming-in, allowing mothers and infants to remain together 24 h a day.	Enable mothers and infants to remain together and to practice rooming in 24 h a day.
8	Encourage breastfeeding on demand.	Support mothers to recognize and respond to their infant's cues for feeding.
9	Give no artificial teats or pacifiers (also called dummies or soothers) to breastfeeding infants.	Counsel mothers on the use and risks of feeding bottles, teats, and pacifiers.
10	Foster the establishment of breastfeeding support groups and refer mothers to them on discharge from the hospital or clinic.	Coordinate discharge so that parents and their infants have timely access to ongoing support and care.

Sources: World Health Organization/United Nations Children's Fund Ten Steps to Successful Breastfeeding (original version: 1989 and revised version: 2018) (2).

the same global initiative from an implementation science can lead to important cumulative lessons and may allow for comparisons to be made between studies (27). Hence, we expect that findings from this review can help inform Mexico and other countries about the major gaps in existing knowledge that need to be addressed to help guide the future implementation and scaling up of the BFHI at a national level in a way that is cost-effective and equitable.

1.1 The BFHI in the Mexican context

This systematic literature review focuses on the implementation of the BFHI in Mexico, as it is a good example of a country where the BFHI implementation has not gone according to plan. This in spite that in 1991 Mexico adopted the commitments of the World Summit for Children as part of the BFHI, and a national program called *Hospital Amigo del Niño y la Madre* (HANyM) was created, which incorporated the Ten Steps to improve BF indicators in the country (11). In 1993, maternity hospitals began to be certified at the national level through a government-run program. Between 1993 and 1999, 377 hospitals achieved the BFHI certification, but fewer than 42% (158) were recertified during that same period (28). Mexico faced several challenges with the implementation of the Initiative, including the lack of dissemination, monitoring, and maintenance plan. This led to a voltage drop; that is, the momentum was not maintained leading to a lack of coordination for the sustainability of a program. For example, during this period, Mexico experienced a deterioration in political will and support for BF promotion and protection, which was reflected in the lack of financing, intersectoral coordination, and relevant legislation to scale up and sustain the BFHI in the country over time (20, 29).

One of the objectives of the National Breastfeeding Strategy (ENLM, by its acronym in Spanish) 2014–2018 was to improve institutional competencies to support BF. The strategy proposed to increase the number of hospitals accredited as BFHI by at least 30% at a country level and obtain at least 180 Baby-Friendly Units at the first level of care (i.e., BFCI), but there is no public information to corroborate the achievement of these goals (30).

This deterioration process coincided with a period in which BF practices decreased in Mexico; between 2006 and 2012, there was a decrease in EBF from 22.3% to 14.4% at the national level (29). Due to multisectoral efforts put in place to address these declines in EBF, improvements in BF outcomes were reported by 2018–19, when EBF increased to 28.8% (31). Despite this improvement, Mexico is still far from the EBF goal established by the World Health Assembly for the year 2030 of 70% (32). The BFHI has not been systemically reactivated in Mexico, and considering the global evidence (22, 33), its reactivation is needed to continue improving BF outcomes in the country.

2 Methods

A systematic literature review (34) was carried out based on the Preferred Reporting Items for Systematic Reviews and

Meta-Analyses (PRISMA) (35). The protocol was registered in PROSPERO before starting the search and analysis (N° CRD42021248118).

This review was guided by the RE-AIM framework, which includes five dimensions: (i) reach, which is defined as the number, proportion, and representativeness of individuals who are willing to participate in an intervention, (ii) efficacy or effectiveness of the intervention, (iii) adoption, which refers to the absolute number, proportion, and representativeness of settings and people who deliver the intervention who initiate an intervention, (iv) implementation, which focuses on fidelity to the intervention, its adaptations, and costs, and (v) maintenance, understood as the continuous implementation of the program at the setting level (i.e. sustainability of the Ten Steps) (24).

Guided by the RE-AIM, the review focused on two levels of results: implementation, and effectiveness and efficacy. Within the implementation results, we sought to identify the processes through which hospitals (or health subsystems) decide to adopt the Ten Steps, the barriers and facilitators to implementation, and the level of maintenance of the Initiative. In relation to effectiveness and efficacy, the review sought to identify the proportion of BFHI hospitals, the proportion of births that occurred in BFHI hospitals, and the differences in BF practices, skin-to-skin contact practices, knowledge about the Code, and BF training for health providers in BFHI vs. non-BFHI hospitals.

TABLE 2 MeSH terms used in the systematic review.

MeSH terms used in English
"((Baby Friendly OR BFHI OR Ten Steps OR 10 Steps)) AND (Breast Fe OR Breastfe or Exp Breast Feeding))"
MeSH terms used in Spanish
"((Hospital Amigo OR IHAN OR Diez Pasos OR 10 Pasos)) AND (Lactancia OR Amamantar OR Extracción))"

2.1 Search strategy

Systematic searches were carried out in four databases (Ovid MEDLINE, PubMed, Scopus, and Scielo), and in the UNICEF, WHO, Association of Certified Consultants in Breastfeeding (ACCLAM), and the National Institute of Public Health (INSP) websites. Additionally, a cross-referencing strategy was employed, which involved backward citation. To validate the search results, two local experts in the field were contacted via Zoom after the initial search to ensure no key documents had been omitted.

The search considered concepts related to the BFHI (baby friendly, BFHI), its Ten Steps (ten steps, 10 steps), and breastfeeding, in Spanish, English, and Portuguese. The complete search strategy can be found in Table 2. The country was not specified in the search, so as not to lose global studies that had data from the BFHI in Mexico.

2.2 Inclusion and exclusion criteria

Articles were included if they focused on one or more of the RE-AIM framework dimensions and if they were focused on public or private hospitals in Mexico with obstetric care that were either accredited or in the process of obtaining the accreditation of the BFHI (i.e., when they did not yet have the BFHI, but adopted it later) and were published in English, Spanish, or Portuguese up until February 2021. The review included quantitative or qualitative scientific papers and gray literature focusing on (i) people who had given birth to babies without medical conditions that could prevent initiation of BF, (ii) babies with information about their hospital of birth, or (iii) health professionals involved on the birth and perinatal services (see Table 3).

Articles without information on hospital accreditation status (including whether they focused on mothers, births, or health providers) and studies focused solely or mostly centered on preterm infants or on mothers with complications that limited initiation of BF were excluded. Reviews and meta-analyses were also excluded.

TABLE 3 Inclusion and exclusion criteria used in the systematic review.

Inclusion criteria
Studies were included if they focused on processes and impacts of the implementation of the BFHI in Mexico and if they met the following criteria:
(a) Any Mexican public or private hospital providing obstetric care, that was either in the process of obtaining the accreditation of the BFHI (that is, when they did not yet have the BFHI, but adopted it later) or were already BFHI-accredited
(b) Mothers who had given birth to babies without medical conditions that could prevent initiation of BF
(c) Babies with information about their hospital of birth
(d) Health professionals linked to neonatal hospital services
(e) Studies published in English, Spanish, or Portuguese up until February 2021
(f) Quantitative or qualitative indexed scientific papers and gray literature
Exclusion criteria
(a) Studies without information on the hospital's BFHI accreditation status (including whether they focused on mothers, births, or health providers)
(b) Studies focused solely or mostly centered on preterm infants, or on mothers with complications that limited initiation of BF
(c) Reviews and meta-analyses

TABLE 4 Data extraction guide.

Data synthesis
City/Subsystem (i.e., IMSS and ISSTE)
Sample size/population (i.e., medical doctors, nurses, and mothers)
BFHI steps assessed
Methodology (i.e., qualitative, quantitative, or mixed)
• Design
• Data collection mechanisms
•Aim/research question
•Type of analysis
Main findings
Quality assessment (JBI)

2.3 Selection of articles and data extraction

Rayyan Systems (36) and Excel were used to perform the SLR. Studies and documents identified in databases and websites were initially imported into Excel to identify and remove duplicates. The remaining articles were then exported to Rayyan Systems (36). Three of the authors (AB, NR-V, and VC-V) screened the same first 20 articles and compared their screening decisions; if agreement was not reached or questions emerged, help from one of the senior authors (MV-C) was considered. Subsequently, they independently reviewed the titles and abstracts of articles to select which ones would be reviewed extensively (i.e., full text). Full texts were reviewed by two reviewers, and their inclusion in the SLR was determined by consensus.

2.3.1 Quality evaluation

For the quality assessment, the checklists of the Joanna Briggs Institute (JBI) (34) were used because they have a wide variety of checklists according to the study designs, including one for cross-sectional studies.

2.3.2 Data extraction

The results of the articles and documents selected for inclusion after full-text review were organized in a standardized data extraction table, which included the main characteristics of the documents (see Table 4), as well as information based on the dimensions of the RE-AIM (24) and the quality assessments as per the JBI quality assessment checklists (34).

3 Results

3.1 Study characteristics

Figure 1 summarizes the search results. Before starting the review, duplicate articles ($n = 13$) were eliminated, and then, the titles and abstracts were screened ($n = 1{,}123$), of which 1,094 were excluded, mainly because they presented findings from studies not conducted in Mexico. The authors reviewed the full text of 29 articles and eliminated 23. The reasons for exclusion were as follows: studies carried out in countries other than Mexico ($n = 8$), studies carried out in hospitals without the BFHI accreditation ($n = 4$), studies not related to BFHI ($n = 3$), non-scientific articles ($n = 3$), studies that were systematic reviews of topics related to BFHI ($n = 2$), studies carried out with a population of premature babies ($n = 1$), conference abstracts ($n = 1$), and studies not found ($n = 1$).

The six articles that were included in the review were divided into studies carried out in hospitals before obtaining the BFHI accreditation (pre-accreditation) ($n = 3$) and studies carried out in hospitals that already had the BFHI accreditation (post-accreditation) ($n = 3$).

3.1.1 Pre-accreditation studies

Table 5 summarizes the information of the three pre-accreditation studies (37–39), all of which were conducted in hospitals in Mexico City. Two of the articles presented findings from studies that were carried out at the Luis Castelazo Hospital (37, 38), a tertiary care hospital for obstetrics and gynecology that belongs to the Mexican Institute of Social Security (IMSS). The third article focused on a study conducted at the Hospital General de México (39), which is a public tertiary referral hospital that offers obstetric care. These studies used different quasi-experimental study designs, including pre-post interventions (38, 39) and a cohort study with a comparison group (37).

The pre-accreditation studies focused on research on steps 3 and 7 (37–39) of the BFHI, which focus on providing BF information and support to pregnant women and rooming-in post-delivery, respectively. Similarly, two of the articles addressed step 2 (38, 39), which focuses on ensuring that health personnel are trained to support and promote BF. Step 1, which is linked to the Code, was indirectly addressed in one of the articles (38) that reported findings from infant daily feeding records to register violations of the Code involving the use and promotion of breastmilk substitutes. Finally, one of the articles included step 10 (37), which focuses on postpartum follow-up of mothers and their children, that is, BFCI; specifically, this study addressed continuing care for mothers and their children on days 15, 30, 60, and 120 post-partum.

3.1.2 Post-accreditation studies

The three post-accreditation studies (11, 40, 41) are summarized in Table 6. Two of them were carried out in Mexico City (11, 40), one at the Hospital General de Zona 1 "A," belonging to the IMSS (41), and the other one at the Hospital General de Mexico (11). The third study was conducted at the IMSS Hospital General de Zona IV No. 8 (41) in Ensenada, Baja California.

No pattern was observed in the Ten Steps that were addressed. The study carried out at the Hospital General de Zona I "A" IMSS evaluated step 6 (40), which is related to not providing any food or liquid to breastfed newborns, unless it is medically

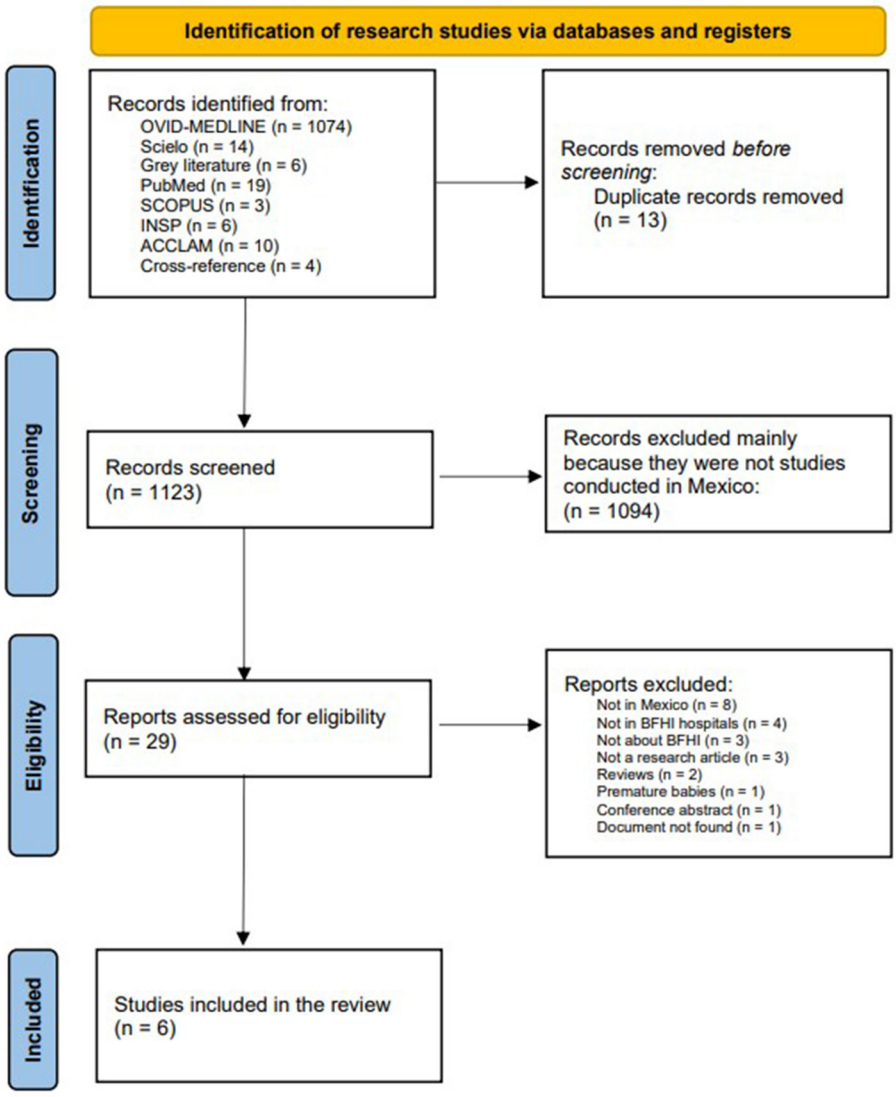

FIGURE 1
PRISMA for the identification of research studies.

indicated. On the other hand, the study carried out at the Hospital General in Ensenada considered steps 3 and 10 (41), linked to providing information on good management of BF to pregnant women and to postpartum follow-up for mothers and their children. The study carried out at the Hospital General de Mexico analyzed step 2 (11), which emphasizes the importance of health personnel knowledge and skills to support BF.

3.1.3 Pre- and post-accreditation studies from the perspective of the RE-AIM

Figure 2 shows the distribution of studies according to the RE-AIM analyzed dimensions (24) and underlines the lack of literature around the reach dimension. As such, no assessment of the proportion of accredited hospitals nor the proportion of births occurring in these hospitals have been published in the peer-reviewed literature.

Studies focused on the efficacy ($n = 4$) of the BFHI in Mexico reported positive impacts including a reduction in the average time of separation of the mother–child from 1.6 h to 1.3 h (33), higher frequency of EBF due to rooming-in (37), and a reduction of hospital costs linked to purchasing breastmilk substitutes (40).

Adoption of the Ten Steps was only analyzed in pre-accredited hospitals ($n = 3$). These articles focused on documenting new practices, such as rooming in and giving information to pregnant women about the importance of BF (37, 39). However, they did not delve into the management aspects that facilitated or led to the adoption of the Ten Steps.

Among the articles that documented the implementation of the Ten Steps ($n = 3$) (11, 38, 39), it was emphasized that such Steps helped in improving hospital routines and in identifying areas for

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TABLE 5 Studies conducted in hospitals before obtaining the BFHI accreditation (pre-accreditation).

Author, year, city	Subsystem/ population	Design/main findings associated with BFHI steps	BFHI steps	RE-AIM components evaluated	Results related to BFHI steps
Cisneros-Silva et al. (38), Mexico City	Luis Castelazo Ayala (IMSS) Obstetrics and Gynecology Hospital/Healthy binomials rooming-in, in a tertiary care hospital/250 without cesarean section and 250 with cesarean section ($n = 500$)	One-group pilot intervention/association between rooming in and initiation of BF	1, 2, 3, and 7	Adoption; Implementation	Rooming-in allowed ↑ consumption of human milk, 100% of newborns with rooming-in in the study were discharged after being breastfed. The children without rooming-in were discharged with a breast-milk substitute.
Vandale et al. (39), Mexico City	General Hospital of Mexico (HGM) / Training for pediatric and obstetric professionals ($n = 110$); BF sessions for primigravida women in the last trimester ($n = 347$); session on breast-feeding techniques for primiparous women + rooming-in ($n = 423$)	Quasi-experimental study with control group/initiation and duration of EBF	2, 3, 5, and 7	Efficacy/effectiveness; adoption; implementation	↑ knowledge in BF after training (≤ 12 h) (F+20.9267; $p < 0.001$ in ANOVA test); ↓ binomial separation time from 1.6 h to 1.3 h, ↑ number of children breastfed, 77.1% to 78.1%, ↑ number of times the child was breastfed, from 1.5 to 1.9 times; ↑ EBF from 52.4 to 54.9% and significant difference in age at full weaning, 12 weeks in control group and >17 weeks in intervention group.
Flores-Huerta and Cisneros-Silva (37), Mexico City	Luis Castelazo Ayala (IMSS) Obstetrics and Gynecology Hospital/Healthy binomials with term infants rooming-in ($n = 29$ born by cesarean section; $n = 61$ born by delivery) and without rooming-in ($n = 31$ born by cesarean section; $n = 57$ born by delivery) ($n = 178$)	Cohort/frequency of exclusive or partial breastfeeding	3, 5, 7, and 10	Efficacy/effectiveness; adoption	Regardless of the form of birth, rooming-in is the factor that influences the frequency of EBF the most. During the first month EBF was ↑ in the group rooming-in (61% vs 42%, $p < 0.05$); RR of EBF at 15 days: total rooming-in (1.62 [1.13–2.32]), births by delivery rooming-in (1.66 [1.04–2.66]); at 30 days: total rooming in (1.49 [1.09–2.04])

[1](BFHI) Ten steps, Step 1. Fully comply with the International Code of Marketing of Breast-milk Substitutes; step 2. Ensure that all staff have sufficient knowledge, competencies, and skills to support breastfeeding; step 3. Discuss the importance and management of breastfeeding with pregnant women and their families; step 4. Facilitate immediate and uninterrupted skin-to-skin contact and support mothers to initiate breastfeeding as soon as possible after birth; step 5: Support mothers to initiate and maintain breastfeeding, as well as to manage common difficulties; step 6. Do not provide breastfed newborn infants any foods or fluids other than breastmilk, unless medically indicated; step 7. Enable mothers and infants to remain together and to practice rooming in 24 h a day; step 8. Support mothers to recognize and respond to their infant's cues for feeding; step 9. Counsel mothers on the use and risks of feeding bottles, teats, and pacifiers; step 10. Coordinate discharge so that parents and their infants have timely access to ongoing support and care. [2] BF Breastfeeding, [3] EBF Exclusive Breastfeeding, [4] RR Relative risk.

improvement. For example, the Hospital Luis Castelazo worked to establish rooming-in, even though it is a high-risk hospital (37, 38). It began by training gynecology and obstetrics health personnel to increase decision-making skills with greater precision regarding when rooming-in should be indicated, continued, or suspended. In addition, the Hospital implemented a program to motivate the staff to acknowledge the importance of BF for both the mother and the child's health (38). The Hospital General de Mexico also provided training to the nursing staff of different shifts and services, either by indication of their immediate superior or out of personal interest. It established courses of a total duration of 18 h with 6 h of supervised clinical practice and followed guidelines established by the Ministry of Health and UNICEF (11).

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TABLE 6 Studies conducted in hospitals after obtaining the BFHI accreditation (post-accreditation).

Author, year, city	Subsystem/ population	Design/main findings associated with BFHI steps	BFHI steps assessed	RE-AIM components evaluated	Results related to BFHI steps
Thompson-Chagoyán et al. (40), Mexico City	IMSS Area 1 "A" General Hospital/Review of reports of consumption of breastmilk substitutes, months before the start of the BFHI program (period A), and months after (period B) ($n = 22$ months)	Cross-sectional/consumption of breastmilk substitutes	6	Efficacy/effectiveness	Significant differences ($p < 0.001$); reduction in the number of containers, kilograms, costs, and liters of breastmilk substitutes offered, as well as in costs per child in period B
Navarro-Estrella et al. (41), Ensenada, Baja California	IMSS Area IV General Hospital No. 8/healthy working mothers, beneficiaries of this hospital, with healthy single babies with gestational age ≥37 weeks ($n = 265$)	Cross-sectional/early abandonment of BF	3 and 10	Efficacy/effectiveness	Group I: mothers with early abandonment in BF; group II: mothers who prolonged BF for more than 3 months. 42.3% ($n = 112$) of the mothers abandoned BF early; the risk factors for early abandonment were: wrong knowledge of BF (OR 5.97, CI 1.67–20.67); not having breastfed before (OR 2.98, CI 1.66–5.36); previous BF planning for only 0–3 months (OR 16.24, CI 5.37–49.12); lack of facilities in the work environment (OR 1.99, CI 1.12–3.56)
Hernández-Garduño and Rosa-Ruiz (11), Ciudad de México	General Hospital of Mexico (HGM)/educational intervention on BF, with initial and final evaluations, in nursing staff; attendance to the course was by direct indication or personal interest ($n = 152$)	Pre- vs. post-evaluation, one-group pilot intervention/changes in knowledge on BF	2	Implementation	Significant results comparing the knowledge evaluations before and after the training on BF ($p < 0.001$) in all levels of professional training. The training lasted 18 h, including 6 h of clinical practice; the thematic content was supported by educational material on BF developed by the Ministry of Health and UNICEF.

[1] (BFHI) Ten steps, step 1. Fully comply with the International Code of Marketing of Breast-milk Substitutes; step 2. Ensure that all staff have sufficient knowledge, competencies, and skills to support breastfeeding; step 3. Discuss the importance and management of breastfeeding with pregnant women and their families; step 4. Facilitate immediate and uninterrupted skin-to-skin contact, and support mothers to initiate breastfeeding as soon as possible after birth; step 5. Support mothers to initiate and maintain breastfeeding as well as to manage common difficulties; step 6. Do not provide breastfed newborn infants any foods or fluids other than breastmilk, unless medically indicated; step 7. Enable mothers and infants to remain together and to practice rooming-in 24 h a day; step 8. Support mothers to recognize and respond to their infant's cues for feeding; step 9. Counsel mothers on the use and risks of feeding bottles, teats, and pacifiers; step 10. Coordinate discharge so that parents and their infants have timely access to ongoing support and care. [2] BF, breastfeeding; [3] OR, odds ratio; [4] CI, confidence Interval.

3.2 Evaluation of the quality of the studies

This SLR identified that the quality of the evidence could be improved. Two cross-sectional studies were analyzed. One study evaluated the effect of a program on the consumption of breastmilk substitutes at a hospital (34). According to the JBI checklist (34), this study did not meet any of the established criteria, for which it was determined as a very low-quality study. There was no clear description of the inclusion and exclusion criteria of the sample, no confounding factors were identified, and the data collection process was not explained, which affects the validity and reliability of the study. On the other hand, another study at the Hospital of Ensenada, Baja California (41) was considered of acceptable quality despite the risk of incurring recall bias by applying a retrospective questionnaire during the postpartum stage about which were the mothers' feeding plans while pregnant.

Three studies were quasi-experimental. One evaluated BF training for nursing staff (11), another one referred to rooming-in and BF initiation in a tertiary care hospital (38), and another one assessed a BF promotion program at the HGM (39). These were considered to be of low quality. None had a control group, limiting the validity of causal inferences.

Finally, a cohort study evaluating rooming-in and EBF (37) showed confusing criteria. Data such as exposure measurements,

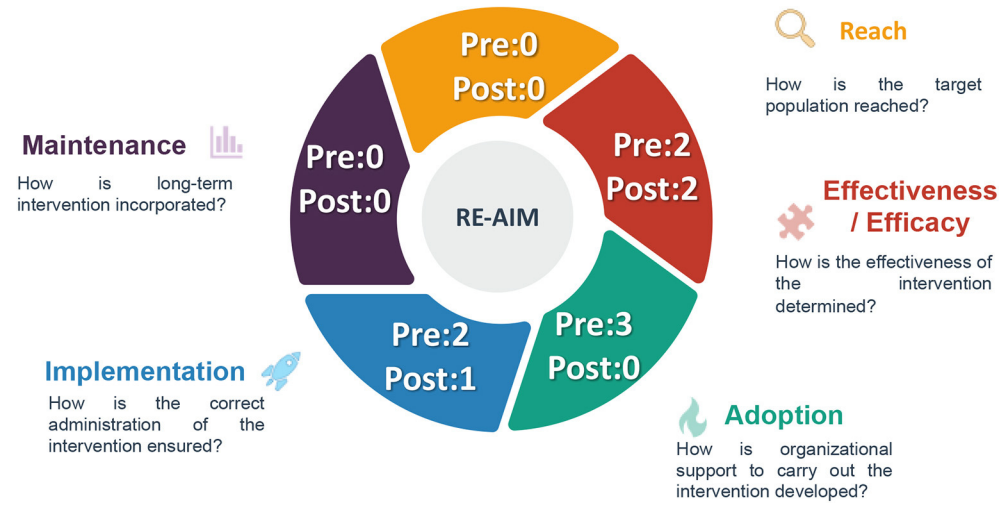

FIGURE 2
Frequency of the RE-AIM model components in the pre–post-accreditation studies.

allocation of exposed and unexposed groups, and confounding factors were not specified. Additionally, the study incurred in loss to follow-up of ∼20%, compromising the internal and external validity of the study. For these reasons, this study was deemed to be of low quality.

4 Discussion

This systematic literature review, based on the RE-AIM framework, provided a structured approach toward understanding BFHI gaps in Mexico. Through its orientation in process results and impact, it showed which barriers and facilitators were contributing to the progress of the implementation of Ten Steps in Mexico as well as the knowledge gaps with respect to the Initiative. Ultimately, it showed the need to have consistent methods to investigate, evaluate, and follow up on BF and BFHI indicators that allow for maximizing the benefits of the Initiative in the country.

Globally, there is sufficient evidence of the positive impact of the Ten Steps on BF outcomes, including the tenth step, which refers to the community-level follow-up and support, i.e., BFCI (15). However, it has also been highlighted that implementing such Steps can be challenging and implementation science can contribute to making sense of when, where, and why the Ten Steps are being implemented or not, and to help better realizing the impact of such evidence-based intervention. Given that in Mexico there have been challenges with hospitals sustaining the Ten Steps over time, this SLR sought to document the existing scientific evidence around the implementation of the BFHI and its Ten Steps. A substantial lack of evidence was found. Only six studies were identified, which reveals there is very little information about the BFHI in Mexico. Moreover, the quality of the published studies was, on average, low. Regarding the tenth step, while the BFCI has been recognized as a relevant practice by the Mexican Ministry of Health, there is a profound lack of evidence about its adoption and implementation. While in Mexico most deliveries happen within a medical context in which the BFHI is fundamental (42), the postpartum follow-up takes place at the primary level and the community in which the adequate implementation of the BFCI is crucial. The community approach needs to be embraced as infant feeding decisions depend on multiple determinants and actors (43, 44).

In Mexico, there is no information indicating the processes by which hospitals or health subsystems decide to adopt or implement the BFHI and BFCI. These are relevant data to make contextual adaptations, scale up good practices, follow up to monitor progress, and identify strategies to improve implementation of the Ten Steps. In the current review, no study in Mexico with a focus on long-term results was found. Therefore, the continuity of the Initiative and its review and control processes are unknown. There are scarce published data regarding the number of accredited hospitals. While some rates are cited in prior reports (28), no official source specifying the status of the implementation of the Ten Steps was found, precluding the establishment of areas of opportunity to strengthen the program.

Mexico could benefit from practices implemented in other countries. For example, in Brazil, the Ministry of Health established a monitoring tool that allows access to information such as data, evaluations, and results of all hospitals. This monitoring tool allows for evaluating what is being implemented. In addition, hospitals that have the BFHI accreditation operate a self-management process carried out by their own health personnel (17, 22). In the United States, the BFHI is supervised by Baby-Friendly USA, an independent accreditation body that monitors the number of babies born in hospitals that have adopted the Ten Steps. In addition, the US Centers for Disease Control and Prevention (CDC) has provided financial support to health departments to increase the adoption of the Ten Steps in hospitals across the

country (21, 45). They also conducted a survey on maternity, nutrition, and childcare practices (mPINC) (45), and a national census of maternity practices in order to identify areas of opportunity to improve the implementation of the Ten Steps and increase BF rates (45). The experience of countries like Kenya in the implementation of the BFCI can also help in understanding the relevance and implementation strategy to care for mothers and their infants after birth, from the health facility to the community where community health volunteers are fundamental to support and improve breastfeeding (46).

During the last 20 years, the ENSANUT has documented the national rates of BF in Mexico. A critical next step is to close information gaps around the implementation of the Ten Steps (47), including the compliance with the Code of Marketing of Breastmilk Substitutes, which could not be really assessed as all the studies were prior to the 2018 modification of the Ten Steps. The Becoming Breastfeeding Friendly Index Committee in Mexico (BBF-Mexico) (47, 48) has tried to obtain information about the number of births that occur in accredited hospitals, but there is no data on how many children have benefited from the Initiative, limiting the assessment of the reach of the program. BBF-Mexico has further underscored the absence of public data on the number of accredited hospitals, which makes it difficult to assess the maintenance of the Initiative (47) and coincides with the SLR findings from a RE-AIM perspective.

According to the BF gear model (BFMG) (20, 49), a model that identifies eight "gears" (i.e., legislation, advocacy, research, funding, promotion, training, political will, and coordination) that must work in harmony for effective support and promotion of BF, Mexico has some important gaps. Recently, the BBF-Mexico Committee warned that several of these gears are not working correctly (44, 49), including hospital practices and BF training for the health workforce (50). Previous studies in Mexico have also found that knowledge of the Code of Marketing of Breastmilk Substitutes among health professionals is severely lacking (18, 51). This is worrisome as large violations of the Code have been documented in Mexico, and health professionals have been found to play a role in these violations (51–53). BFHI steps 1 and 2 represent an opportunity to address these issues and therefore help women be better informed about BF through extensively trained staff.

Because there is no publicly available data on the BFHI in Mexico, transparency regarding the implementation of the Ten Steps is extremely limited. The implementation of the BFHI depends on its nomination granted by IMSS or the Health Ministry (SSA), which is similar to the Brazilian model (22); however, the designation and re-evaluation system is not public and, thus, difficult to follow. Based on data obtained by formal request in 2019 to the Ministry of Health, <11% of maternity hospitals at the national level had been certified in the previous 5 years. There were only 121 baby-friendly hospitals nationwide, of which 85 were accredited at the time of data collection (49).

It is known that the BFHI represents more work for health personnel, who are often already overextended. Therefore, it is necessary to generate incentives to encourage accreditation, maintain it, and rethink the accreditation mechanism (54). For example, the health system of Vietnam established Hospital Quality Assessment Criteria (54), which works by establishing points at the national level that seek to improve the quality satisfaction and safety of patients. Criteria include BF communication, training, and practices. This model implies the strengthening of internal monitoring systems that are targeted at helping hospitals and their staff improve internal management, processes, and practices.

While a potential limitation of this systematic review is its narrow geographic focus, it also contributes to the broader literature on the implementation of the BFHI and BFCI through the RE-AIM framework, which has previously been used in Brazil and the United States. The implementation lens will allow us to document what and how has worked (or not) in scaling and sustaining the Ten Steps.

5 Conclusion

In Mexico, it is necessary to rethink the BFHI. It is fundamental to generate public follow-up and monitoring mechanisms to better understand what the adoption and implementation challenges are. Equally, it is necessary to propose management models that promote the adoption and sustainability of the Ten Steps considering the challenges of the national health system. In Mexico, the BFHI and the BFCI can be key factors in the promotion, protection, and support of BF, but it is necessary to bring the issue forward to the public policy agenda to identify the reasons why the Initiative has not worked and look for effective strategies to improve its implementation, monitoring, and evaluation.

Data availability statement

Since this article is a systematic review, data comes from articles in academic journals that have been published in the public domain. Data sharing is not applicable to this article.

Author contributions

AB conceptualized the systematic review, reviewed abstracts, titles, and manuscripts, participated in synthesis tables, and drafted the full manuscript. MV-C conceptualized the systematic review, developed and tested the search strategy, provided guidance in dissenting and inclusion about specific studies and drafted the full manuscript, and participated in synthesis tables. VC-V drafted the protocol for the systematic review, conducted the search, reviewed abstracts, titles, and manuscripts, and participated in synthesis tables. NR-V conducted the search, reviewed abstracts, titles, and manuscripts, and participated in synthesis tables. ER and RP-E provided a critical review of the review protocol and the full 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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Comparative proteomic analysis of human milk fat globules and paired membranes and mouse milk fat globules identifies core cellular systems contributing to mammary lipid trafficking and secretion

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Introduction: Human milk delivers critical nutritional and immunological support to human infants. Milk fat globules (MFGs) and their associated membranes (MFGMs) contain the majority of milk lipids and many bioactive components that contribute to neonatal development and health, yet their compositions have not been fully defined, and the mechanisms responsible for formation of these structures remain incompletely understood.

Methods: In this study, we used untargeted mass spectrometry to quantitatively profile the protein compositions of freshly obtained MFGs and their paired, physically separated MFGM fractions from 13 human milk samples. We also quantitatively profiled the MFG protein compositions of 9 pooled milk samples from 18 lactating mouse dams.

Results: We identified 2,453 proteins and 2,795 proteins in the majority of human MFG and MFGM samples, respectively, and 1,577 proteins in mouse MFGs. Using paired analyses of protein abundance in MFGMs compared to MFGs (MFGM-MFG; 1% FDR), we identified 699 proteins that were more highly abundant in MFGMs (MFGM-enriched), and 201 proteins that were less abundant in MFGMs (cytoplasmic). MFGM-enriched proteins comprised membrane systems (apical plasma membrane and multiple vesicular membranes) hypothesized to be responsible for lipid and protein secretion and components of membrane transport and signaling systems. Cytoplasmic proteins included ribosomal and proteasomal systems. Comparing abundance between human and mouse MFGs, we found a positive correlation (R^2 = 0.44, $p < 0.0001$) in the relative abundances of 1,279 proteins that were found in common across species.

Discussion: Comparative pathway enrichment analyses between human and mouse samples reveal similarities in membrane trafficking and signaling

pathways involved in milk fat secretion and identify potentially novel immunological components of MFGs. Our results advance knowledge of the composition and relative quantities of proteins in human and mouse MFGs in greater detail, provide a quantitative profile of specifically enriched human MFGM proteins, and identify core cellular systems involved in milk lipid secretion.

KEYWORDS

molecular regulation of human milk secretion, milk fat globule (MFG), milk fat globule membrane (MFGM), mass spectrometry, comparative proteomics

Introduction

Human milk is well appreciated to be the gold standard of nutrition for human infants (Victora et al., 2016). Currently, there is rapidly expanding interest in more fully elucidating how the interactions between individual milk components may make human milk greater than the sum of its parts (Christian et al., 2021; Raiten et al., 2023; Smilowitz et al., 2023). The broader "milk matrix" in which these individual milk components exist is thought to potentially contribute to both infant and maternal health. Many factors are expected to affect the milk matrix, including behavioral, environmental, structural, and organizational influences. Milk fat globules (MFG) are major structural, and organizationally complex, components of milk, and may be important contributors to the effects of the milk matrix. MFGs form a specialized lipid delivery system, providing nutritional components to support infant growth and development as well as immunological protection against disease. Their surrounding phospholipid trilayer membrane (milk fat globule membrane; MFGM) is unique to milk; infant formulas have traditionally provided lipids in the form of vegetable oil. Recent efforts have focused on adding MFGMs to infant formulas in recognition of the immunological and neurodevelopmental benefits associated with the protein, carbohydrate, and lipid constituents of MFGMs (Brink and Lonnerdal, 2020).

Data in laboratory and dairy animals have shown that MFG production begins with triacylglycerol synthesis in the endoplasmic reticulum (ER) (Stein and Stein, 1967; Kassan et al., 2013). Accumulating triacylglycerols are released into the cytoplasm where they are surrounded by an ER-derived phospholipid monolayer containing numerous attached or embedded proteins, resulting in formation of organelle structures referred to as cytoplasmic lipid droplets (CLD) (Dylewski et al., 1984; Walther and Farese, 2012). CLDs in mammary epithelial cells are coated with perilipin 2 (Plin2), which is thought to confer stability by protecting CLDs from lipolysis (Listenberger et al., 2007; Russell et al., 2011). CLDs can fuse to form larger CLDs in a process that is thought to be facilitated by cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (Cidea), while concurrently trafficking toward the apical surface (Wooding, 1971; Stemberger and Patton, 1981; Wang et al., 2012; Wu et al., 2014; Barneda et al., 2015; Monks et al., 2016; Masedunskas et al., 2017) of the polarized luminal mammary epithelial cell (MEC). While moving intracellularly, CLDs can interact with other organelles, including the Golgi apparatus, mitochondria and casein-containing secretory vesicles (Wooding, 1971; Wooding, 1973; Stemberger et al., 1984; Wu et al., 2000; Mather et al., 2001; Honvo-Houéto et al., 2016). When they arrive at the apical cytoplasm, CLDs form contacts with the apical plasma membrane via interactions between CLD-coating Plin2, cytoplasmic xanthine dehydrogenase (Xdh; also known as xanthine oxidoreductase, Xor) and the transmembrane plasma membrane protein butyrophilin, subfamily 1, member A1 (Btn1a1) (Ishii et al., 1995; Keenan et al., 1995; Mather and Keenan, 1998; McManaman et al., 2002; Vorbach et al., 2002; Ogg et al., 2004a; Robenek et al., 2006; Jeong et al., 2009; Monks et al., 2016; Jeong et al., 2021). These proteins and their interactions allow for tight tethering, or docking, of CLDs to the apical plasma membrane, where they can continue to grow by fusion and protrude into the alveolar lumen (Dylewski et al., 1984; Monks et al., 2016; Masedunskas et al., 2017; Monks et al., 2022). Oxytocin release from the pituitary, which is driven by nipple stimulation and/or conditioned release in women (McNeilly et al., 1983), drives contraction of the surrounding myoepithelial cells which is proposed to cause membrane-tethered CLDs to be secreted into the alveolar lumen as MFGs by an apocrine mechanism (Kurosumi et al., 1968; Mather and Keenan, 1998; Mather et al., 2001; Masedunskas et al., 2017) that is incompletely understood. Cellular and biochemical studies indicate that the secretion process incorporates portions of the apical plasma membrane including proteins that form the CLD docking complex, parts of the cytosol, membrane elements of the endoplasmic reticulum, secretory and Golgi vesicles, and organellar transport machinery (Kurosumi et al., 1968; Wu et al., 2000; Honvo-Houéto et al., 2016; Wooding, 2023). Molecular details about how these cellular elements are integrated into MFGs and the precise role they play in the secretion process are limited. However, studies in mice indicate that formation of the CLD docking complex limits the amount of cytoplasm included in MFGs and enhances the efficiency of lipid secretion (Oftedal, 2012; Monks et al., 2016). Further functions of the CLD docking complex remain to be explored, and we anticipate that it may act as an intracellular scaffold and/or signaling hub to regulate overall milk production and secretion.

Milk fat secretion is critical to lactation success, as demonstrated in rodent models. Genetic disruption of the CLD synthetic machinery and docking complex components leads to poor offspring growth or starvation and death due to low milk consumption (Vorbach et al., 2002; Ogg et al., 2004b; Russell et al., 2011; Wang et al., 2012; Monks et al., 2016; Zhao et al., 2020; Jeong et al., 2021). Models targeting the machinery regulating triacylglycerol synthesis and CLD assembly impair glandular development and drive low milk fat production and secretion, decreasing milk caloric content (Smith et al., 2000; Beigneux et al., 2006; Russell et al., 2011; Wang et al., 2012; Suburu et al., 2014), and models targeting the MFG secretion machinery drive the production of extremely large and unstable MFGs, interfering with

overall milk secretion (Vorbach et al., 2002; Ogg et al., 2004b; Monks et al., 2016; Jeong et al., 2021). Many of the mechanistic details of milk fat secretion have been worked out in dairy animals and/or model organisms by electron microscopy, immunohistochemistry and fluorescence microscopy in conjunction with genetic models and most recently, by elegant intravital imaging of glandular tissue (Wooding, 1971; Mather and Keenan, 1998; Monks et al., 2016; Masedunskas et al., 2017; Mather et al., 2019; Monks et al., 2020; Monks et al., 2022). Mammary tissue is difficult to obtain from humans for use with these methods, however, and our understanding of the regulation of human milk lipid production and secretion is therefore limited. As the milk lipid biosynthetic and secretory machinery are known to be retained on MFGs, we aimed to expand our understanding of human milk fat synthesis and secretion using a quantitative untargeted proteomic approach in MFGs. We also aimed to directly compare our findings from human samples to murine samples to identify how well the MFG production machinery is conserved between species, and therefore, how representative experimental murine models are to this process in humans. This is a particularly relevant question because the wide disparity between milk fat content in humans (3%–4%) (Ballard and Morrow, 2013) and mice (>20%) (Görs et al., 2009) could indicate divergent mechanisms of milk fat secretion.

Previous efforts to define the human MFGM proteome have identified the presence of MFG synthesis and docking complex protein homologs, including CIDEA, PLIN2, XDH/XOR and multiple BTN family members (Cavaletto et al., 2002; Fortunato et al., 2003; Liao et al., 2011; Spertino et al., 2012; Yang et al., 2015; Lu et al., 2016; Yang et al., 2016; Juvarajah et al., 2018; Zhang et al., 2021), in addition to over 400 other proteins. These studies have considered all proteins associated with MFGs to be membrane proteins. However, due to the apocrine mechanism of milk lipid secretion, MFGs also contain variable amounts of protein from cytoplasmic compartments (Patton and Huston, 1988). Distinguishing these from true membrane proteins can clarify which are required for CLD docking, envelopment and MFG secretion. We therefore directly compared the MFG proteome and the fractionated MFGM proteome from 13 women across early to mid-lactation. To isolate MFGMs, we utilized physical disruption (Mather, 2000; Reinhardt and Lippolis, 2008) rather than detergent-based disruption, as others have used for human MFGM analysis, to isolate membranes by centrifugation and limit the solubilization and loss of individual proteins from membrane complexes. Advancements in proteomics technology have allowed us to identify a far greater number of MFG and MFGM proteins than previously known, and our pathway analyses point toward potential regulators of milk fat synthesis and secretion.

Materials and methods

Human and mouse milk collection

Human milk (<1oz) was collected after an overnight fast in postpartum women, as part of a randomized controlled trial of diet composition in the control of gestational diabetes (Clinical Trials #NCT02244814). Milk collection protocols were approved by the Colorado Multiple Institutional Review Board (protocol #14–1358) as previously described (Hernandez et al., 2014; Hernandez et al., 2016; Martin Carli et al., 2020; Hernandez et al., 2023), and all participants gave their informed consent. Participants visits occurred at 2 weeks (5 samples), 2 months (3 samples) or 4–5 months postpartum (5 samples), following term deliveries (≥37 weeks). Milk was collected from a total of 9 participants. Two provided samples at both the 2 weeks and 4–5 months timepoints, and one participant provided milk at all three timepoints. Milk collections were not standardized with respect to the time of infant feeding or pumping. Samples were placed on ice in a cooler and transported to a study visit at the Clinical Translational Research Center at the University of Colorado Anschutz Medical Campus and then brought to the laboratory.

Mouse milk was collected from primiparous CD1 females from breeding colonies maintained in the AAALAC-Accredited (#00235) Center for Comparative Medicine at the University of Colorado Anschutz Medical Campus. The colony was housed under 14:10 h light:dark cycle at a temperature of 72 ± 2°F, humidity of 40% ± 10% and fed standard chow (Teklad/Envigo 2920X) and hyperchlorinated (2–5 ppm) water. Females were mated with CD1 males and then housed individually prior to parturition. The day a litter was first seen was counted as lactation day 1 (L1). Dams were allowed to nurse their natural born litter (litters were not standardized, avg: 12 ± 2 pups/litter). Milk samples were collected from 18 dams at L9-11, after 3 h of separation from pups, as previously described (Monks et al., 2022). Briefly, intraperitoneal (IP) xylazine was given at a dose of 8 mg/kg. When the mouse was relaxed enough to have ceased ambulation around the cage (about 5 min), the milking procedure was initiated. The mouse was picked up, and with gentle hand-restraint, a single dose of oxytocin (0.25 IU, 0.12 mL in sterile saline) was administered IP. Milk let-down occurred within 1 min and milk removal was started. Our standard milking apparatus, attached to house vacuum, was used. Hand restraint was used throughout the milking procedure. Milk was collected and processed at room temperature to avoid changes in protein segregation between phases. All animal experiments and procedures were approved by the University of Colorado Anschutz Medical Campus' Institutional Animal Care and Use Committee on protocol 00985 (PI: McManaman).

MFG and MFGM isolation

Intact MFGs from fresh human and murine milk samples were isolated according to procedures previously described (Monks et al., 2022) which were informed by established methods (Patton and Huston, 1986; Wu et al., 2000; Monks et al., 2016). Milk was protected from freezing to avoid damaging membranes. Briefly, whole milk samples were gently combined with ~10 volumes of PBS and centrifuged at 1,500 xg at room temperature for 10 min to float MFGs as described by Patton and Huston (Patton and Huston, 1986). To isolate MFGs from small volumes of highly viscous mouse milk, samples were mixed 1:1 with 10% sucrose and layered under PBS for this first centrifugation wash, which minimized adhesion to tubes and pipet tips, and subsequently allowed the lower density MFGs to float to the top. Two human samples were treated this way, however this appeared to contribute to sample loss, so the remaining human samples were not mixed 1:1 with 10% sucrose. Floated MFGs

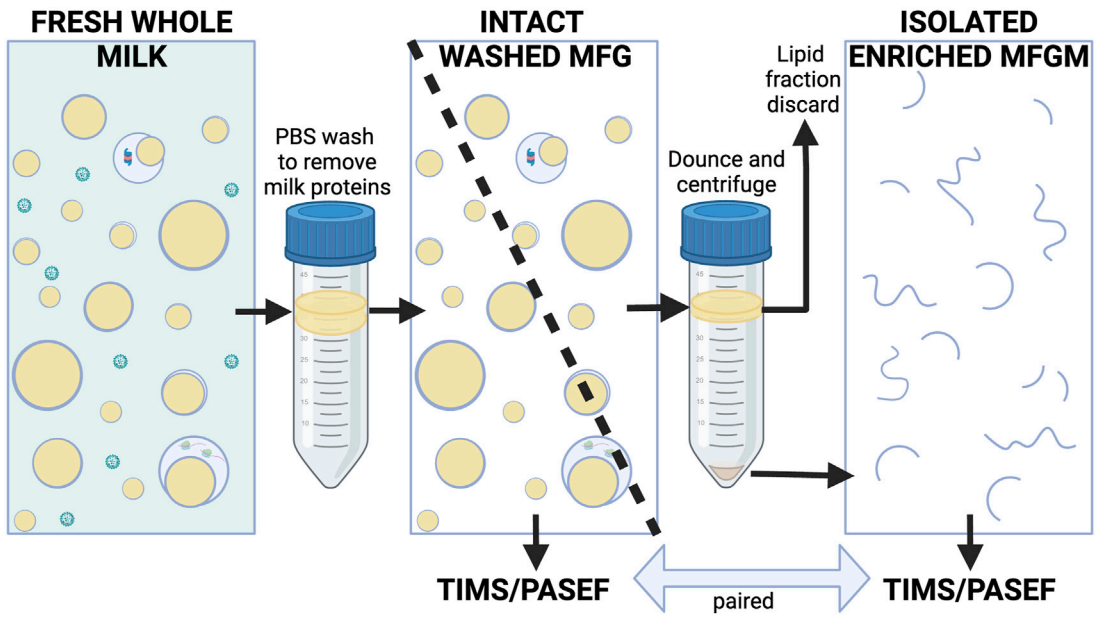

FIGURE 1
Simplified conceptual diagram of milk fractionation for proteomic analysis of human MFG fractions. Freshly collected and floated MFGs were gently washed of milk proteins with PBS. Intact, floated washed MFG from each subject were divided into two aliquots. One aliquot of each sample was used for proteomic analysis of whole MFGs, the other aliquot of each sample was Dounce-homogenized and centrifuged to pellet the enriched MFGM fraction. The MFGM fraction was sent for proteomic analysis along with its paired washed MFG fraction (see Methods for details). Whey proteins and casein micelles are shown in green. Lipids are yellow. Membranes are blue. MFGM fractions from some species are known to contain variable quantities of cytoplasmic components (Wooding and Kemp, 1975), however they are presented in a simplified manner here. Created with BioRender.com.

from individual women were collected, gently washed and refloated twice by mixing with 14 mLs of PBS and centrifuging at 1,500 xg, at room temperature for 10 min. Washed intact human MFGs were divided into two aliquots and then frozen at −80°C. One aliquot was processed directly for mass spectrometry analysis without further fractionation. The second aliquot was used to prepare MFGMs using procedures previously established for isolating bovine MFGMs (Reinhardt and Lippolis, 2008). Briefly, frozen MFGs were thawed on ice, Dounce homogenized (100 strokes) and centrifuged at 22,000 xg for 20 min at 4°C. Pelleted membranes (MFGMs) were stored at −80°C prior to proteomic analysis. We did not obtain enough starting material to efficiently isolate MFGMs from murine milk samples. This process is illustrated in Figure 1.

Liquid chromatography—tandem mass spectrometry (LC-MS/MS)

Washed MFG or isolated MFGM samples were precipitated with 10% trichloroacetic acid for 2 h at −20°C. The precipitated protein samples were pelleted by centrifugation at 14,000 xg for 20 min at 4°C, rinsed in ice-cold acetone and centrifuged again. The pellet was air-dried and solubilized in 5% SDS, 100 mM DTT in 100 TEAB. The samples were digested using the S-Trap filter (Protifi, Huntington, NY) according to the manufacturer's procedure. Briefly, samples were reduced with 10 mM DTT at 55°C for 30 min, cooled to room temperature, and then alkylated with 25 mM iodoacetamide in the dark for 30 min. Next, a final concentration of 1.2% phosphoric acid and then six volumes of binding buffer (90% methanol; 100 mM triethylammonium bicarbonate, TEAB; pH 7.1) were added to each sample. After gently mixing, the protein solution was loaded to a S-Trap filter, spun at 1,000 xg for 1 min, and the flow-through collected and reloaded onto the filter. This step was repeated three times, and then the filter was washed with 200 µL of binding buffer 3 times. Finally, 1 µg of sequencing-grade trypsin (Promega) in 150 µL of digestion buffer (50 mM TEAB) were added onto the filter and digestion was carried out at 37°C for 6 h. To elute peptides, three stepwise buffers were applied, with 100 µL of each with one more repeat, including 50 mM TEAB, 0.2% formic acid in H_2O, and 50% acetonitrile and 0.2% formic acid in H_2O. The peptide solutions were pooled, lyophilized and resuspended in 100 µL of 0.1% FA.

20 µL of each sample was loaded onto individual Evotips for desalting and then washed with 20 µL 0.1% FA followed by the addition of 100 µL storage solvent (0.1% FA) to keep the Evotips wet until analysis. The Evosep One system (Evosep, Odense, Denmark) was used to separate peptides on a Pepsep column, (150 um inter diameter, 15 cm) packed with ReproSil C18 1.9 um, 120A resin. The system was coupled to the timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) via the nano-electrospray ion source (Captive Spray, Bruker Daltonics). The mass spectrometer was operated in PASEF mode (TIMS/PASEF). The ramp time was set to 100 ms and 10 PASEF MS/MS scans per topN acquisition cycle were acquired. MS and MS/MS spectra were recorded from m/z 100 to 1700. The ion mobility was scanned from 0.7 to 1.50 Vs/cm^2. Precursors for data-dependent acquisition were isolated within ±1 Th and fragmented with an ion mobility-dependent collision energy, which was linearly increased from 20 to 59 eV

in positive mode. Low-abundance precursor ions with an intensity above a threshold of 500 counts but below a target value of 20,000 counts were repeatedly scheduled and otherwise dynamically excluded for 0.4 min.

Database searching and protein identification

MS/MS spectra were extracted from raw data files and converted into .mgf files using MS Convert (ProteoWizard, Ver. 3.0). Peptide spectral matching was performed with Mascot (Ver. 2.6) against the Uniprot human and mouse databases. Mass tolerances were ±15 ppm for parent ions, and ±35 ppm for fragment ions. Trypsin specificity was used, allowing for one missed cleavage. Met oxidation, protein N-terminal acetylation and peptide N-terminal pyroglutamic acid formation were set as variable modifications with Cys carbamidomethylation set as a fixed modification.

Scaffold (version 5.0, Proteome Software, Portland, OR, United States) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified unique peptides. Proteins are identified in the text by their official gene names and symbols, as these were utilized for pathway analyses described below. In instances where there are multiple protein products encoded by a single gene, the major product is specified.

Statistical analyses

Milk collections were treated as independent samples, even though a subset of participants provided repeated samples. This is because milk fat composition is largely affected by diet and collection variables that we could not account for, such as whether foremilk or hindmilk was collected, or time since last breast emptying. Statistical analyses were conducted as described using Graphpad Prism 9.5.1 and Metaboanalyst 5.0 (https://www.metaboanalyst.ca/home.xhtml). GO cellular component analysis was conducted using DAVID (https://david.ncifcrf.gov/home.jsp) from NIAID/NIH (Huang et al., 2009; Sherman et al., 2022).

Pathway analyses

Gene symbols of the proteins detected in ≥50% or 100% of MFG or MFGM samples, for human, or ≥50% or 100% of murine MFG samples were utilized for pathway enrichment analysis using Metascape (https://metascape.org/gp/index.html#/main/step1). This tool queries multiple different ontology sources, and minimizes redundancy by clustering related pathway terms (Zhou et al., 2019). First, all statistically enriched terms were identified (can be GO/KEGG terms, canonical pathways, hall mark gene sets, etc., based on the default choices under Express Analysis), accumulative hypergeometric *p*-values and enrichment factors were calculated and used for filtering. Remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships. Then 0.3 kappa score was applied as the threshold to cast the tree into term clusters. To visualize our results in the context of cellular mechanisms, we utilized Reactome's (Fabregat et al., 2018) pathway diagram viewer (https://reactome.org/, version 3.7) with our gene symbol lists.

Data availability

Proteomics datasets were uploaded into the MassIVE Center for Computational Mass Spectrometry entitled "Proteomic Analysis of Paired Human Milk Fat Globules and Milk Fat Globule Membranes." (MassIVE MSV000092892) and "Proteomic Analysis of Mouse Milk Fat Globules" (MassIVE MSV000092915).

Results

In this study, we defined quantitative proteomic profiles of MFG and MFGM pairs from 13 women across early (2 weeks postpartum, $n = 5$) to mature lactation (2 months postpartum, $n = 3$, and 4–5 months postpartum, $n = 5$) by LC-MS/MS (Figure 1). The sum of the total spectral counts was not different between MFG and MFGM proteins, indicating effective sample preparation and similar loading between sample types (Figure 2A). We included in our analyses the 2,933 proteins which were detected in ≥50% of replicates in one or both groups to identify low abundance proteins which might provide important biological data in aggregate. Using a threshold of proteins detected in 100% of replicates in one or both sample types, we identified 1,812 proteins. We first considered the possibility that MFG and/or MFGM proteins from late-transitional/early mature milk at 2 weeks postpartum could differ from those found in mature milk from 2 to 5 months postpartum, especially as bovine MFGM proteins have been shown to change from colostrum to mature milk (Reinhardt and Lippolis, 2008). Using unbiased principal components analysis (PCA), we found that MFG proteins (Figure 2B) could not be distinguished across timepoints by the first five principal components (79.7% of total variance). However, MFGM proteins at transitional vs. mature timepoints were separated across PC2 (14.0%; Figure 2C). BTN1A1 was the biggest driver of this separation, with a loading score of −0.23 (Figure 2D). The abundance of BTN1A1 increased from 0.027 (0.026–0.028) normalized spectral abundance factor [med (IQR); NSAF] in early lactation to 0.034 (0.031–0.037) NSAF, in mature lactation (adj. $p < 0.01$; Figure 2E). Other components of the CLD docking complex, XDH/XOR, PLIN2 and CIDEA, which are increased in the transition from bovine colostrum to mature milk (Reinhardt and Lippolis, 2008), were not increased during the transition from early to mature human lactation (Figure 2E). When the entire proteomic dataset was considered, we did not find statistically significant differential abundances between early and mature lactation (Supplementary Figure S1). Many factors, including time since last feeding and time respective to the feeding/pumping bout are expected to affect CLD docking and

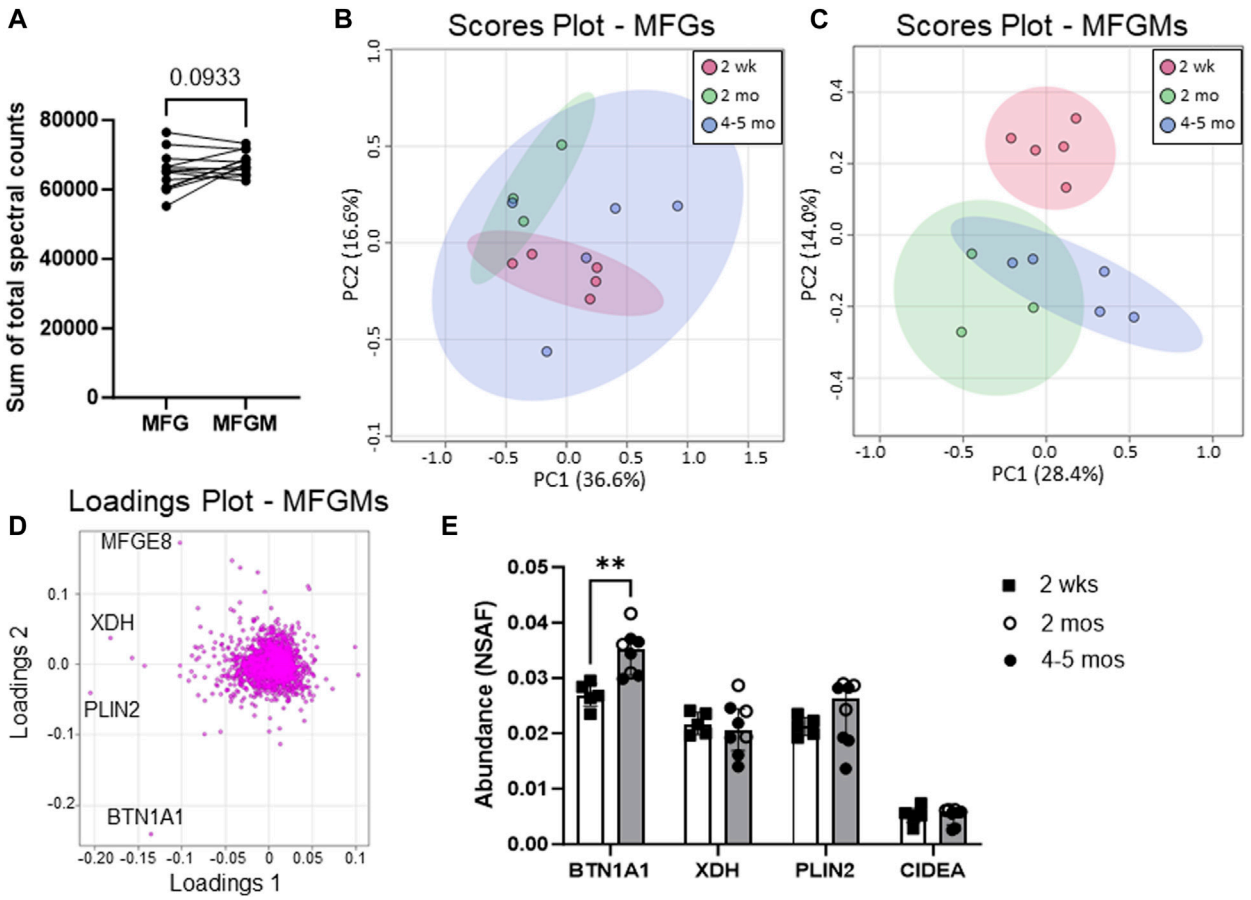

FIGURE 2
Human MFG and MFGM protein abundance over time. Sum of human MFG and MFGM spectral counts by LC-MS/MS **(A)**. Principal components analysis of 2,453 MFG-associated **(B)** and 2,795 MFGM-associated **(C)** proteins separated by time postpartum: red = 2 weeks, green = 2 months, blue = 4–5 months. Pareto scaling was used to limit the effects of large fold changes found in low abundance proteins. Loadings of MFGM-associated protein PCA **(D)**. Abundance of known CLD docking proteins BTN1A1, XDH, PLIN2 and CIDEA **(E)**. Open bars = 2 weeks (n = 5), gray bars = 2–5 months, open circles = 2 months (n = 3), closed circles = 4–5 months (n = 5). Multiple nonparametric (Mann-Whitney), unpaired t-tests, adjusted for multiple testing with the Holm-Šídák method. $^{**}p < 0.01$.

envelopment (Masedunskas et al., 2017; Mather et al., 2019) and therefore, protein composition of the MFGM. We are unable to account for these collection details in the current study, which did not standardize these factors, and we do not have access to samples from the colostrum phase. As we were underpowered to investigate temporal changes across lactation, we combined all timepoints for subsequent analyses to maximize our statistical power.

We identified 2,315 proteins that were common between MFGs and their membrane fractions, as well as 138 unique proteins in the MFG samples and 480 unique proteins in the MFGM samples. (Figure 3A). A heatmap of the 20 most abundant proteins across both sample types is shown in Figure 3B and values are listed in Table 1. Values for all proteins detected are listed in Supplementary Table S1. Although differences in sequence coverage, due to variation in tryptic sites and ionization efficiency, can limit stoichiometric analysis of proteomic data, we obtained high coverage (>60%) of most major proteins in the MFG and MFGM fractions (Supplementary Figure S2A). The abundances of BTN1A1, PLIN2 and XDH/XOR were greater than other MFGM proteins with the exception of fatty acid binding protein 3 (FABP3), which had a relative abundance similar to that of BTN1A1. In the MFG fraction, FABP3 and milk fat globule EGF and factor V/VIII domain containing (MFGE8, major protein product: lactadherin) were the most abundant proteins, followed by BTN1A1, PLIN2, lactotransferrin (LTF), XDH/XOR and perilipin 3 (PLIN3). We found that of previously described CLD docking proteins, both in MFG samples and isolated MFGM proteins, BTN1A1 was the most abundant protein, followed by PLIN2 and XDH/XOR. These three proteins were more highly abundant than CIDEA, which is also implicated in CLD docking (Monks et al., 2016). In aggregate, these four CLD docking complex proteins represented 6.8% of spectra detected across all 2,933 proteins, which is consistent with their proposed structural role in mediating CLD-apical plasma membrane contacts that facilitate milk lipid secretion (Monks et al., 2022).

We next compared MFG and MFGM proteins by unbiased PCA and identified distinctly different proteomes in these fractions (Figure 3C). As expected, the loadings contributing to this distinction primarily consisted of proteins implicated in CLD docking; XDH/XOR, BTN1A1, PLIN2 and CIDEA (Figure 3D).

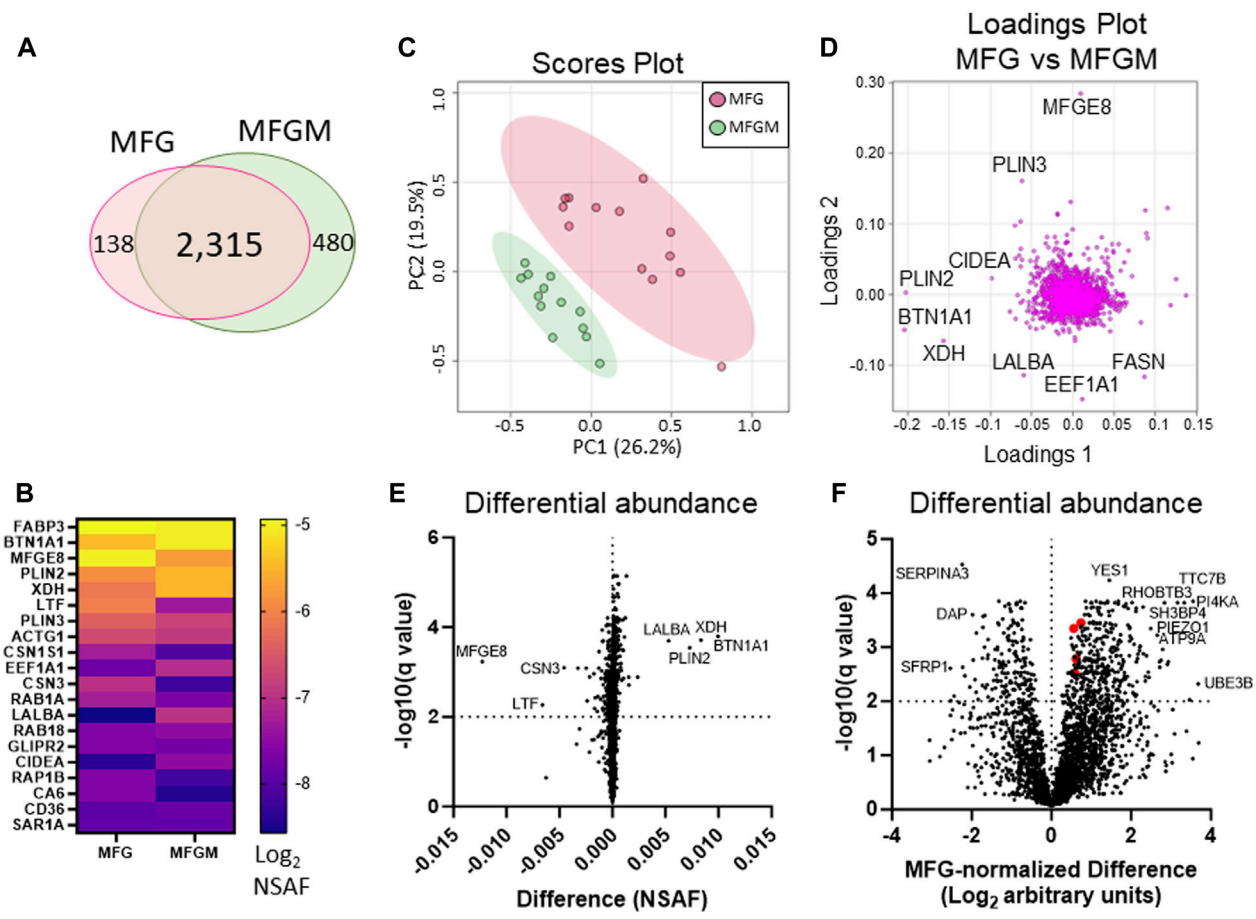

FIGURE 3
Differential protein abundance between human MFGs and MFGMs. Venn diagram of shared and distinct human MFG- and MFGM-associated proteins **(A)**. Abundance of the top 20 human MFG- and MFGM-associated proteins [**(B)**, Log_2 NSAF], sorted by means of MFG and MFGM values combined. Principal components analysis of MFG-associated (red) and MFGM-associated (green) proteins **(C)**. Pareto scaling was used to limit the effects of large fold changes found in low abundance proteins. Loadings of MFG- and MFGM-associated protein PCA **(D)**. Multiple paired t-tests of MFG-vs MFGM-associated proteins, calculated by absolute difference [MFGM-MFG, **(E)**] or by differences in Log_2-transformed, MFG-normalized values, [Log_2(MFGM/AvgMFG)-Log_2(MFG/AvgMFG); **(F)**] with FDR correction for multiple comparisons (1%). BTN1A1, XDH, PLIN2 and CIDEA are labeled red in **(F)**.

To identify possible additional components of the human CLD docking complex, we calculated differential protein abundances between sample types by volcano plots, comparing differences in both absolute NSAF values (Figure 3E) to assess highly abundant proteins, and values normalized to average MFG values (MFG-normalized; Figure 3F), to assess proteins with low abundance. We considered proteins that were more highly abundant in the MFGM fraction to be MFGM-enriched and proteins more highly abundant in the whole MFG proteome to be cytoplasmic. The top 10 proteins with the largest absolute and MFG-normalized differences between sample types, both MFGM-enriched and cytoplasmic, are listed in Table 2. All differentially abundant proteins by absolute and MFG-normalized differences are listed in Supplementary Tables S2, S3, respectively. We identified 900 proteins which were statistically significant by absolute difference (699 MFGM-enriched and 201 cytoplasmic) and 537 by MFG-normalized difference (371 MFGM-enriched and 166 cytoplasmic).

To identify possible functional properties of MFGM-enriched proteins, we conducted unbiased pathway analysis using Metascape (Zhou et al., 2019), which comprehensively utilizes KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM, WikiPathways, and PANTHER Pathway as ontology sources. We report pathways identified using the threshold of proteins present in 50% of samples, and in Supplementary Figures S3–S8 show the top 100 pathways identified using a threshold of proteins present in 100% of samples, to eliminate pathways identified due to low abundance and/or low confidence proteins. Differentially abundant proteins identified in Figure 3E were utilized to avoid losing datapoints undetected in MFGs, and therefore with a denominator of 0 when conducting normalization to MFG values. Using this approach, we found that human MFGM-enriched proteins (Figure 4A—top 20 pathway clusters, Supplementary Figure S3—top 100 pathway clusters and Supplementary Table S4—all pathways) identified highly enriched ($-\log_{10} p < 14$) pathway clusters related to lipid metabolism and localization (lipid biosynthetic process and lipid localization), endoplasmic reticulum (protein N-linked glycosylation) and intracellular trafficking, as expected. Intracellular trafficking-related pathway clusters include membrane organization, membrane trafficking, import into cell, transport of small

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TABLE 1 Top 20 most abundant MFG and MFGM-associated proteins.

Identified protein	Gene symbol	MFG NSAF Avg (SD)	MFGM NSAF Avg (SD)	All NSAF Avg (SD)
Fatty acid-binding protein, heart	FABP3	0.038 (0.0176)	0.0317 (0.0102)	0.0348 (0.0145)
Butyrophilin subfamily 1 member A1	BTN1A1	0.0217 (0.0042)	0.0317 (0.0051)	0.0267 (0.0069)
Lactadherin	MFGE8	0.0308 (0.0096)	0.0185 (0.004)	0.0247 (0.0095)
Perilipin-2	PLIN2	0.0155 (0.005)	0.0228 (0.0047)	0.0192 (0.006)
Xanthine dehydrogenase/oxidase	XDH	0.0129 (0.0031)	0.0213 (0.0039)	0.0171 (0.0055)
Lactotransferrin	LTF	0.0137 (0.0082)	0.007 (0.003)	0.0103 (0.0069)
Perilipin-3	PLIN3	0.0109 (0.0036)	0.009 (0.0032)	0.0099 (0.0035)
Actin, cytoplasmic 2	ACTG1	0.0096 (0.0012)	0.0082 (0.0019)	0.0089 (0.0017)
Alpha-S1-casein	CSN1S1	0.0075 (0.0046)	0.0053 (0.0033)	0.0064 (0.0041)
Elongation factor 1-alpha 1	EEF1A1	0.005 (0.0021)	0.0074 (0.0016)	0.0062 (0.0022)
Kappa-casein	CSN3	0.0083 (0.004)	0.0038 (0.002)	0.006 (0.0039)
Ras-related protein Rab-1A	RAB1A	0.0064 (0.0017)	0.0054 (0.0013)	0.0059 (0.0016)
Alpha-lactalbumin	LALBA	0.0032 (0.0016)	0.0085 (0.0035)	0.0059 (0.0038)
Ras-related protein Rab-18	RAB18	0.0051 (0.0014)	0.0054 (0.0012)	0.0052 (0.0013)
Golgi-associated plant pathogenesis-related protein 1	GLIPR2	0.0049 (0.0021)	0.0043 (0.0011)	0.0046 (0.0016)
Cell death activator CIDE-A	CIDEA	0.0036 (0.0016)	0.0052 (0.0015)	0.0044 (0.0017)
Ras-related protein Rap-1b	RAP1B	0.0052 (0.0018)	0.0036 (0.0009)	0.0044 (0.0017)
Carbonic anhydrase 6	CA6	0.0055 (0.0034)	0.0031 (0.0018)	0.0043 (0.0029)
Platelet glycoprotein 4	CD36	0.0041 (0.0016)	0.0045 (0.0011)	0.0043 (0.0014)
GTP-binding protein SAR1a	SAR1A	0.0041 (0.0018)	0.0043 (0.0011)	0.0042 (0.0015)

Abundance of the top 20 human MFG- and MFGM-associated proteins, sorted by means of MFG and MFGM values combined, displayed as a heatmap in Figure 2B.

molecules, intracellular protein transport, regulation of vesicle-mediated transport, regulation of secretion, positive regulation of protein localization and localization within membrane. Interestingly, this analysis also identified neutrophil degranulation and VEGFA-VEGFR2 signaling pathway clusters. Using GO cellular component analyses, we find that MFGM-enriched proteins correspond to significant contributions from the cell membrane and organelle sub-compartments, including the ER, Golgi apparatus and endosome (Figure 4B).

Not all proteins in the MFGM fraction were significantly elevated in comparison to the whole MFG, due to similar distribution across membrane and cytoplasmic compartments. We considered the possibility that the totality of proteins (n = 2,795) associated with the MFGM may reflect the cellular process involved in MFG formation and secretion. When all proteins in the MFGM fraction (MFGM-associated proteins) were included in pathway analysis, we identified highly enriched clusters ($-\log_{10} p < 50$) related to pathways and processes corresponding to metabolism of lipids, intracellular trafficking and organization (intracellular protein transport, membrane trafficking, membrane organization, regulation of vesicle-mediated transport, localization within membrane, fatty acids and lipoproteins transport in hepatocytes, vesicle organization and endocytosis) and the immune system (viral infection pathways, neutrophil degranulation, adaptive immune system and *salmonella* infection). VEGF-VEGFR2 and Rho and Miro GTPases and RHOBTB3 signaling, protein catabolic process, and monocarboxylic acid metabolic process were also present (Figure 4C–top 20 pathway clusters, Supplementary Figure S4—top 100 pathway clusters and Supplementary Table S5—all pathways). GO cellular component analyses of MFGM-associated proteins show significant contributions of membrane and cytoplasmic proteins as well as those from the ER (Figure 4D). Collectively, these observations are consistent with the initial proteomic studies of mouse MFGM which suggest that MFGM proteins are derived in part from the ER (Wu et al., 2000), and further indicate that proteins within specific ER sub-compartments, secretory granules and vesicular membranes are major contributors to the human MFGM proteome.

Although the MFG and MFGM proteomes were distinct by principal components analysis, we aimed to determine which pathways were found in common between MFG-associated and MFGM-associated proteins and which were unique. Using all MFG-associated proteins (n = 2,453; Figure 5A—top 20 pathway clusters, Supplementary Figure S5—top 100 pathway clusters and Supplementary Table S6—all pathways) for pathway analysis, we identified pathway clusters which we identified using the MFGM proteome, including neutrophil degranulation, vesicle-mediated transport, VEGFA-VEGFR2 signaling, and Rho GTPases, Miro GTPases and RHOBTB3, in addition to lipid metabolism,

TABLE 2 Most differentially abundant proteins in human MFG and MFGM samples.

A. Absolute differences					
Enrichment	Gene Symbol	MFGM Mean (SD) (NSAF)	MFG Mean (SD) (NSAF)	Difference (SE) (NSAF)	q value
MFGM-enriched	BTN1A1	0.0317 (0.0051)	0.0217 (0.0042)	0.01 (0.0013)	0.00016
	XDH	0.0213 (0.0039)	0.0129 (0.0031)	0.0083 (0.0011)	0.00020
	PLIN2	0.0228 (0.0047)	0.0155 (0.005)	0.0073 (0.0011)	0.00029
	LALBA	0.0085 (0.0035)	0.0032 (0.0016)	0.0053 (0.0007)	0.00020
	EEF1A1	0.0074 (0.0016)	0.005 (0.0021)	0.0024 (0.0004)	0.00130
	CIDEA	0.0052 (0.0015)	0.0036 (0.0016)	0.0016 (0.0003)	0.00193
	ABCG2	0.0027 (0.0012)	0.0012 (0.0005)	0.0015 (0.0003)	0.00130
	CYB5A	0.0025 (0.0005)	0.0012 (0.0005)	0.0013 (0.0001)	0.00001
	S100A1	0.0023 (0.0005)	0.001 (0.0004)	0.0012 (0.0001)	0.00006
	TLR2	0.0021 (0.0005)	0.001 (0.0003)	0.0011 (0.0001)	0.00017
Cytoplasmic	MFGE8	0.0185 (0.004)	0.0308 (0.0096)	−0.0123 (0.002)	0.00058
	LTF	0.007 (0.003)	0.0137 (0.0082)	−0.0066 (0.0017)	0.00541
	CSN3	0.0038 (0.002)	0.0083 (0.004)	−0.0046 (0.0008)	0.00080
	ENO1	0.0021 (0.0013)	0.0054 (0.0029)	−0.0033 (0.0006)	0.00082
	RALB	0.002 (0.0008)	0.0044 (0.002)	−0.0025 (0.0004)	0.00082
	CA6	0.0031 (0.0018)	0.0055 (0.0034)	−0.0024 (0.0006)	0.00439
	GAPDH	0.0024 (0.0012)	0.0046 (0.002)	−0.0022 (0.0004)	0.00129
	CDC42	0.0025 (0.0006)	0.0043 (0.0011)	−0.0018 (0.0003)	0.00109
	CEL	0.0025 (0.0006)	0.0043 (0.0011)	−0.0017 (0.0004)	0.00649
	RAP1B	0.0036 (0.0009)	0.0052 (0.0018)	−0.0016 (0.0004)	0.00373
B. MFG-normalized differences					
	Gene Symbol	MFG-normalized MFGMMean (SD) (AU)	MFG-normalized MFGMean (SD) (AU)	Difference of Log2 MFG normalized values Mean (SE) (AU)	q value
MFGM-enriched	UBE3B	33.73 (16.9)	1 (1.56)	3.68 (0.27)	0.00476
	TTC7B	13.76 (6.17)	1 (0.69)	3.55 (0.3)	0.00014
	NBAS	10.87 (7.53)	1 (1.82)	3.47 (0.59)	0.00936
	PI4KA	9.35 (4.17)	1 (0.88)	3.33 (0.35)	0.00015
	SH3BP4	6.79 (2.33)	1 (0.85)	3.16 (0.34)	0.00015
	TRPM4	10.49 (6.56)	1 (1.61)	2.93 (0.3)	0.00185
	ITPR1	4.57 (2.01)	1 (1.35)	2.91 (0.52)	0.00205
	RHOBTB3	7.13 (2.23)	1 (0.8)	2.83 (0.27)	0.00015
	WFS1	7.45 (4.05)	1 (1.15)	2.79 (0.41)	0.00109
	SPTBN1	4.58 (1.79)	1 (1.08)	2.75 (0.51)	0.00173
Cytoplasmic	SFRP1	0.19 (0.33)	1 (1.18)	−2.54 (0.34)	0.00246
	MRAS	0.16 (0.27)	1 (0.65)	−2.53 (0.44)	0.00977
	C9	0.16 (0.2)	1 (1.02)	−2.45 (0.33)	0.00797
	SERPINA3	0.19 (0.17)	1 (0.91)	−2.24 (0.15)	0.00003

(Continued on following page)

TABLE 2 (*Continued*) Most differentially abundant proteins in human MFG and MFGM samples.

B. MFG-normalized differences					
	Gene Symbol	MFG-normalized MFGMMean (SD) (AU)	MFG-normalized MFGMean (SD) (AU)	Difference of Log2 MFG normalized values Mean (SE) (AU)	q value
	CA2	0.22 (0.28)	1 (0.62)	−2.23 (0.36)	0.00233
	GSS	0.21 (0.24)	1 (0.89)	−2.17 (0.39)	0.00337
	GCHFR	0.3 (0.47)	1 (0.75)	−2.05 (0.38)	0.00542
	DAP	0.16 (0.23)	1 (0.94)	−1.98 (0.1)	0.00025
	IGHG2	0.15 (0.1)	1 (1.86)	−1.97 (0.34)	0.00172
	PPT1	0.23 (0.32)	1 (1.26)	−1.92 (0.25)	0.00395

Top 10 most differentially abundant proteins in the MFGM (upper values) and cytoplasmic fraction of the MFG (lower values) calculated by absolute difference (A) or MFG-normalized differences (B).

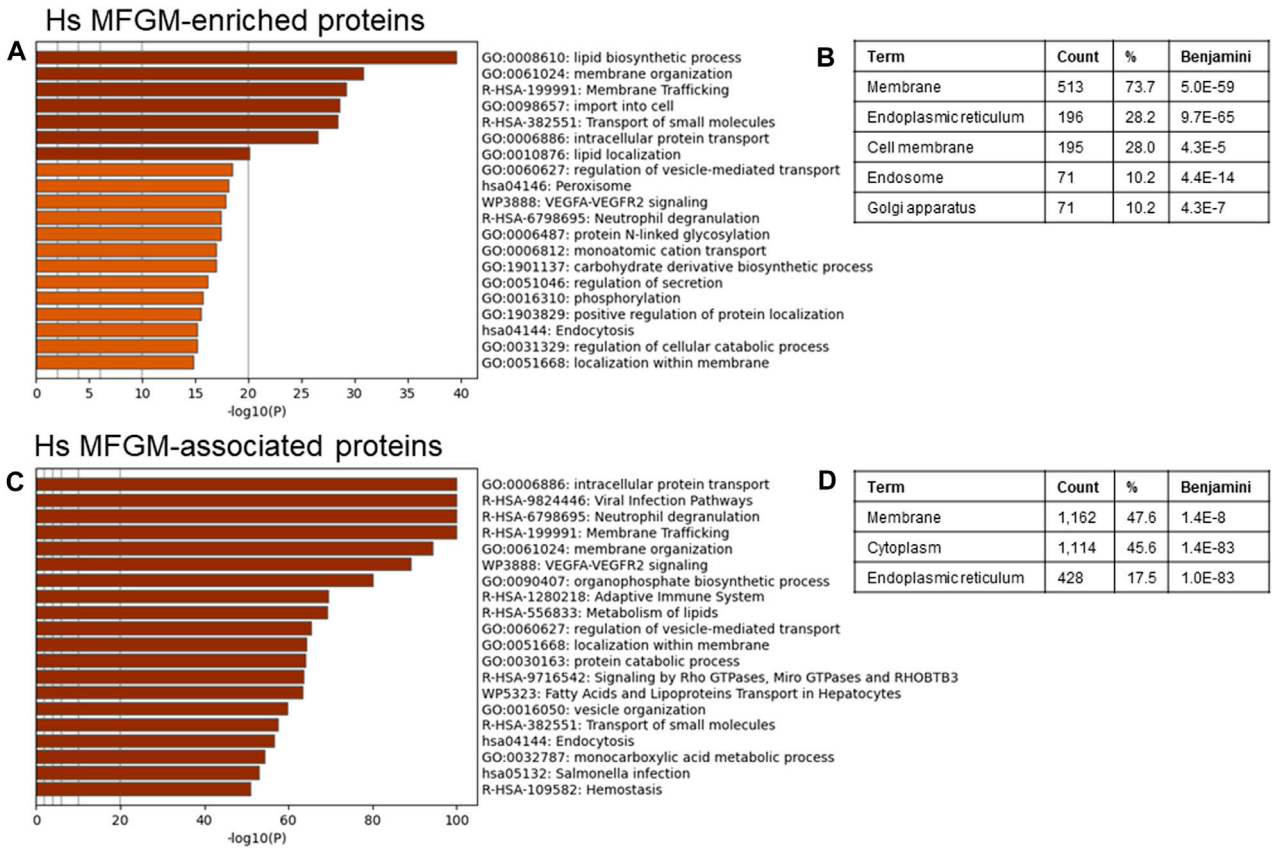

FIGURE 4
Pathway analyses of human MFGM-enriched and MFGM-associated proteins. Metascape pathway enrichment analysis **(A)** and GO Cellular components analysis **(B)** of MFGM-enriched proteins, calculated by difference. Top 20 pathway clusters are shown in **(A)**. Metascape pathway enrichment analysis **(C)** and GO Cellular components analysis **(D)** of all MFGM-associated proteins. Top 20 pathway clusters are shown in **(C)**.

endoplasmic reticulum and intracellular trafficking-related pathway clusters. Therefore, pathway analysis of MFG-associated pathways identifies pathways active both in the endoplasmic reticulum and apical plasma membranes as well as the encapsulated cytoplasm.

To obtain a clearer understanding of proteins found exclusively in the encapsulated cytoplasm, we also conducted pathway analysis of the 201 proteins which were differentially more abundant in the MFG compared to the MFGM (Figure 5B–top 20 pathway clusters, Supplementary Figure S6—top 100 pathway clusters and Supplementary Table S7—all pathways). Translational pathways were heavily represented in the resulting pathway clusters identified (60S ribosomal subunit, cytoplasmic, ribosome

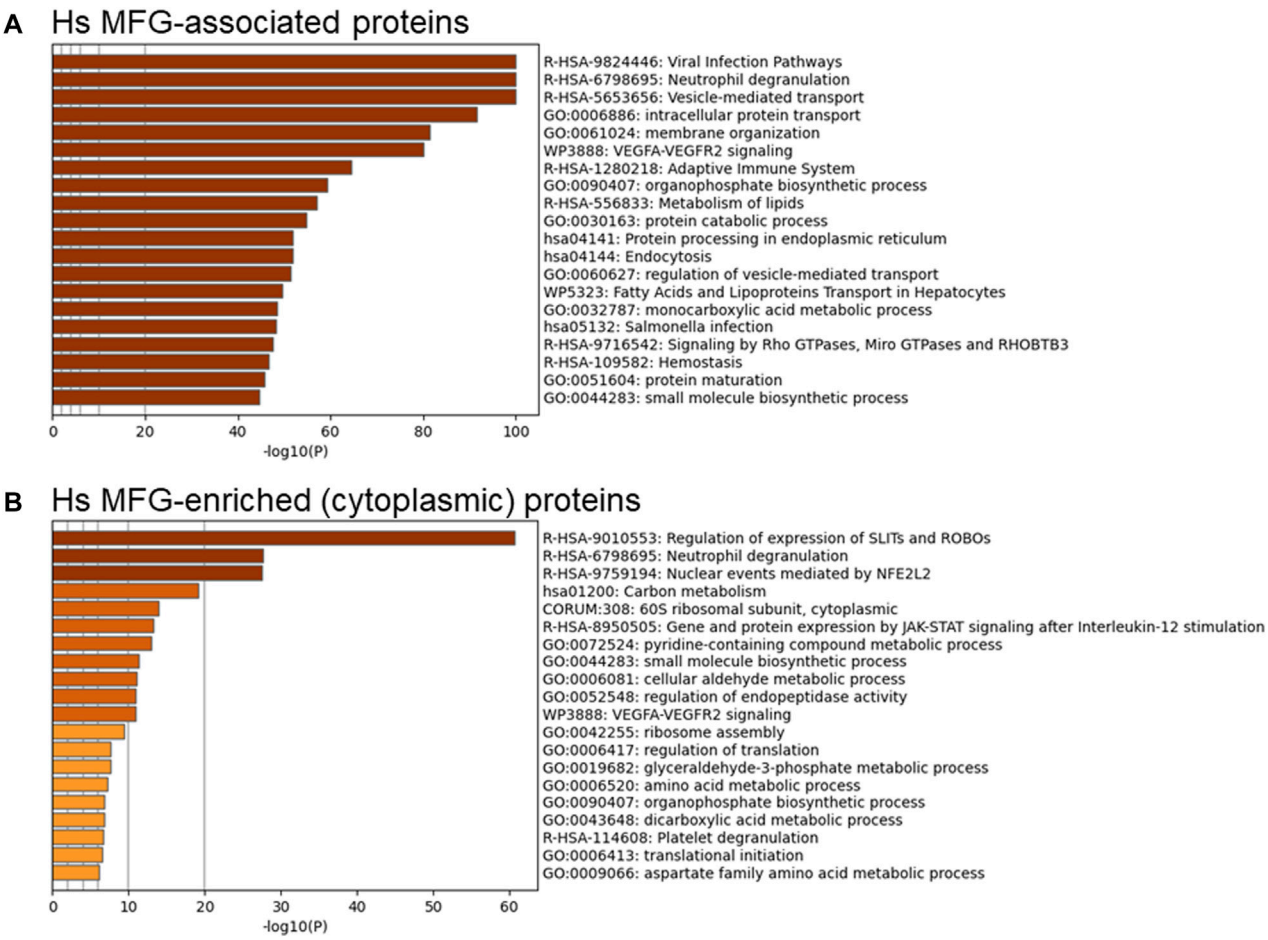

FIGURE 5
Pathway analyses of human MFG-associated proteins. Metascape pathway enrichment analysis of MFG-associated proteins **(A)** and MFG-enriched (cytoplasmic) proteins **(B)**. Top 20 pathway clusters are shown.

assembly, regulation of translation and translational initiation). Other pathway clusters indicate overrepresentation of ribosomal proteins and also suggest the presence of proteasomal proteins. SLITs and ROBOs have been implicated in development of both the normal mammary gland and breast cancer (Marlow et al., 2010; Macias et al., 2011; Harburg et al., 2014; Ballard Mimmi et al., 2015; Zhao et al., 2016) and ROBO1 signaling has been shown to mediate differentiation for milk secretion (Cazares et al., 2021). However, the proteins in this pathway which are present in our MFG-enriched list include 16 proteins which are components of the proteasome, 30 proteins which are ribosomal subunits and none which suggest that MFG secretion regulates specific expression of the SLIT/ROBO pathway. Similarly, the identification of the pathway cluster, "nuclear events mediated by NFE2L2" appears to be driven by the same 16 proteasomal proteins. It is not clear if loss of these cellular components in cytoplasmic crescents during MFG secretion affects the function of the milk secreting cell.

As we and others utilize murine models to investigate mechanisms supporting milk secretion by the mammary gland, we aimed to identify the similarities between mouse and human MFG secretion. A fuller understanding of how well the mouse mammary gland represents the human breast can inform translational research into human lactation. We therefore collected milk from lactating CD1 mouse dams and isolated MFGs. The small milk volumes available precluded us from isolating the MFGM fraction from these samples, and therefore, we were restricted to analyzing the MFG proteome, with the understanding that these proteins comprise a mix of cytoplasmic and membrane proteins. We identified 1,577 proteins present in ≥50% of samples and 1,007 proteins present in 100% of samples. A heatmap of the 20 most abundant proteins is displayed in Figure 6A, and all proteins are listed in Supplementary Table S8. We obtained similar coverage of the known CLD docking components (mostly >50%–60%; Supplementary Figure S2B). Interestingly, in contrast to human MFGs, Xdh/Xor was more highly abundant than Btn1a1 and Cidea, and these three were more highly abundant than Plin2 in murine MFG. Pathway analyses of murine MFG-associated proteins found in ≥50% of the samples identified similar pathway clusters as the human analysis, with intracellular transport, endoplasmic reticulum and ribosomal pathway clusters being well represented, with translational pathway clusters being more highly represented in the murine MFG dataset than the human MFG dataset (Figure 6B–top 20 pathway clusters, Supplementary Figure

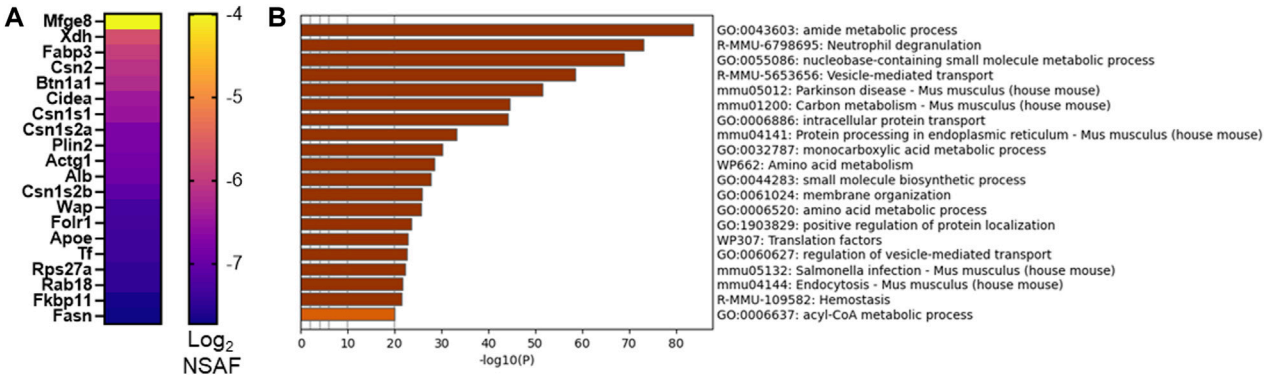

FIGURE 6
CD1 mouse MFG proteome at L10. Abundance of the top 20 MFG-associated proteins [**(A)**, Log_2 NSAF], sorted by median. Metascape pathway enrichment analyses of MFG-associated proteins **(B)**. Top 20 pathway clusters are shown.

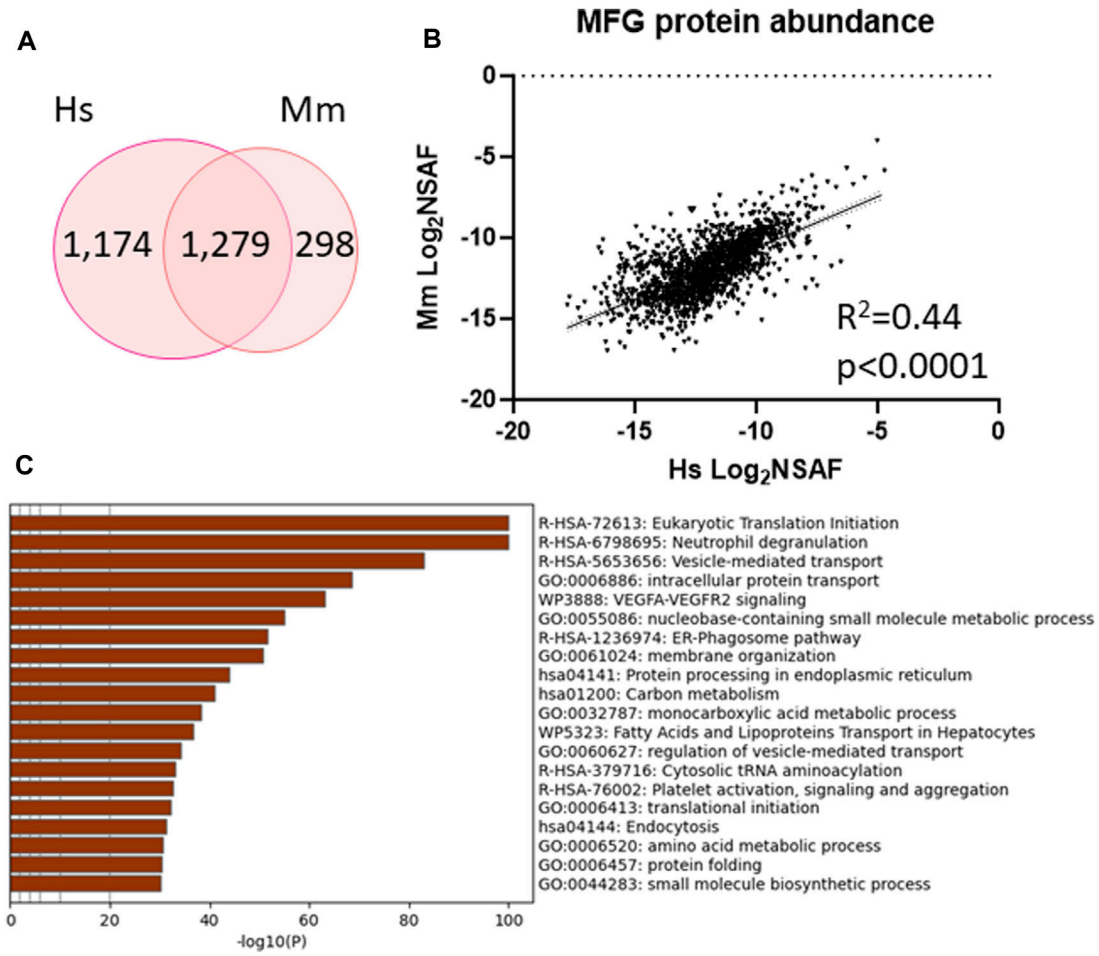

FIGURE 7
Shared protein abundance between human MFG and murine MFG. Venn diagram of shared and distinct human MFG and murine MFG-associated proteins **(A)**. Pearson correlation of abundance between proteins shared across human and murine MFGs **(B)**. Metascape pathway enrichment analyses of shared human and murine MFG-associated proteins **(C)**. Top 20 pathway clusters are shown.

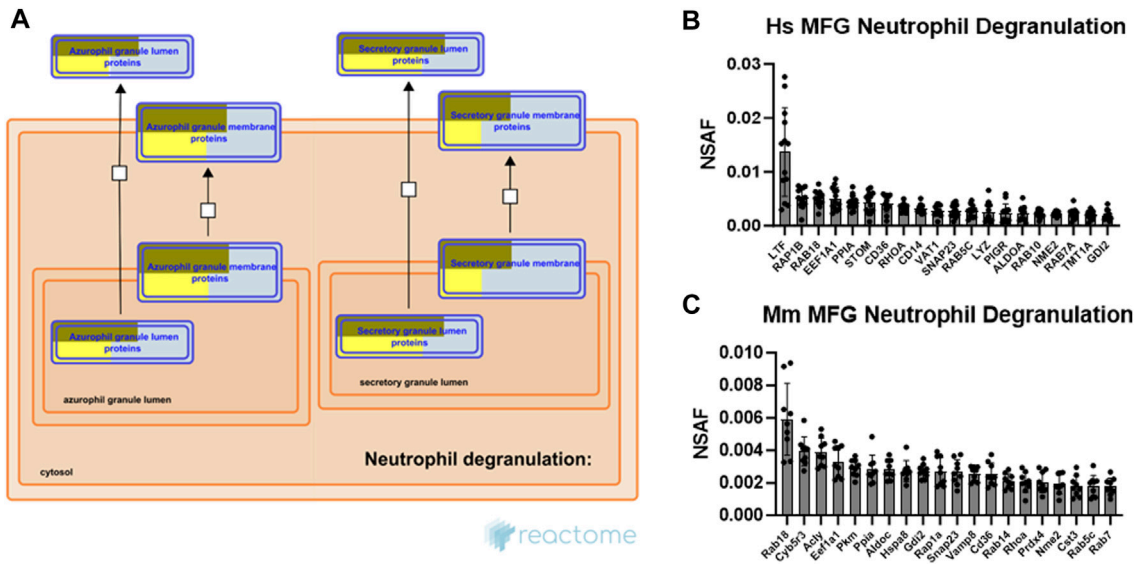

FIGURE 8
Neutrophil degranulation pathway analysis. Reactome pathway diagram with overlaid overrepresentation pathway enrichment analyses of human and mouse MFG-associated proteins in the neutrophil degranulation pathway, including only the primary (azurophil) and secretory granule entities **(A)**. Entities are re-colored (gold for human, yellow for mouse) if they were represented in the submitted data set. The top 20 most highly abundant neutrophil degranulation-related proteins from the human **(B)** and mouse **(C)** MFG proteomes, sorted by mean.

S7—top 100 pathway clusters and Supplementary Table S9—all pathways). Of the notable pathway clusters identified in the human analysis described above, we also identified neutrophil degranulation pathway clusters with murine MFG-associated proteins. Notably missing from the murine top 100 pathway cluster list was VEGFA-VEGFR2 and Rho GTPases, Miro GTPases and RHOBTB3 signaling, which were highly significant in the human pathway cluster analyses, and lipid metabolism pathway clusters were not as highly represented.

To further understand the inter-species similarities, we identified proteins which were unique to either human (1,174 proteins) and mouse (298 proteins) MFG proteomes or detected in both (1,279 proteins, Figure 7A, Supplementary Table S10) and investigated how well correlated the shared proteins are (Figure 7B). We identified moderate correlation across species (R^2 = 0.44, $p < 0.0001$) and utilized this common protein list to conduct further pathway analysis (Figure 7C–top 20 pathway clusters, Supplementary Figure S8—top 100 pathway clusters and Supplementary Table S11—all pathways). Ribosome, endoplasmic reticulum, intracellular transport pathway clusters and neutrophil degranulation clusters were shared across species, indicating that the pathways identified across species largely utilize common sets of proteins within pathways rather than utilizing distinct proteins which function within the same broader pathways. Although not identified in pathway analysis of murine MFG proteins, the VEGF-VEGFR2 signaling pathway cluster was significantly overrepresented in pathway analysis of proteins common to both species.

The neutrophil degranulation pathway was consistently one of the most highly significant pathways identified across species and sample types. As evidence has only recently emerged in mice that secretion of membrane-tethered CLD is regulated, at least in part, by oxytocin-mediated myoepithelial cell contraction (Masedunskas et al., 2017), the processes responsible for this newly identified stimulated form of secretion remain largely unknown. Therefore, commonalities with the carefully controlled neutrophil degranulation process may provide insight into stimulated MFG secretion mechanisms. Neutrophils contain at least 4 distinct types of preformed secretory vesicles known as granules, which are thought to form sequentially during neutrophil differentiation, and which contain distinct effector subsets (Le Cabec et al., 1996). These include primary, secondary, tertiary and secretory granules, with secondary granules containing lactoferrin, an antibacterial protein which is also highly abundant in milk (Kell et al., 2020). Pathway analysis of both human (Supplementary Figure S9A) and mouse (Supplementary Figure S9B) MFG-associated proteins identified the strongest overrepresentation of primary (azurophilic) granule membrane proteins and secretory granule lumen proteins, as illustrated by Reactome (Figure 8A).

Neutrophil degranulation entails cytoskeletal remodeling to allow for granule trafficking to the plasma membrane, granule tethering and docking, granule priming for fusion, and fusion of the granule with the plasma membrane to allow for release of its contents. Small GTPases, which are critical for cytoskeletal remodeling and vesicle trafficking, are known to regulate many of the interactions controlling these processes (Lacy, 2006). The top 20 most abundant neutrophil degranulation pathway proteins identified in human and mouse MFG-associated proteomes are shown in Figures 8B, C, respectively. Common between these top 20 proteins across species are the small GTPases RAB18, member RAS oncogene family (RAB18), which is known in other cell types to regulate lipid droplet growth (Ozeki et al., 2005; Xu et al., 2018; Deng et al., 2021), ras homolog family member A (RHOA), RAB5C, member RAS oncogene family (RAB5C), as well as the GDP dissociation inhibitor 2 (GDI2). Also highly abundant are

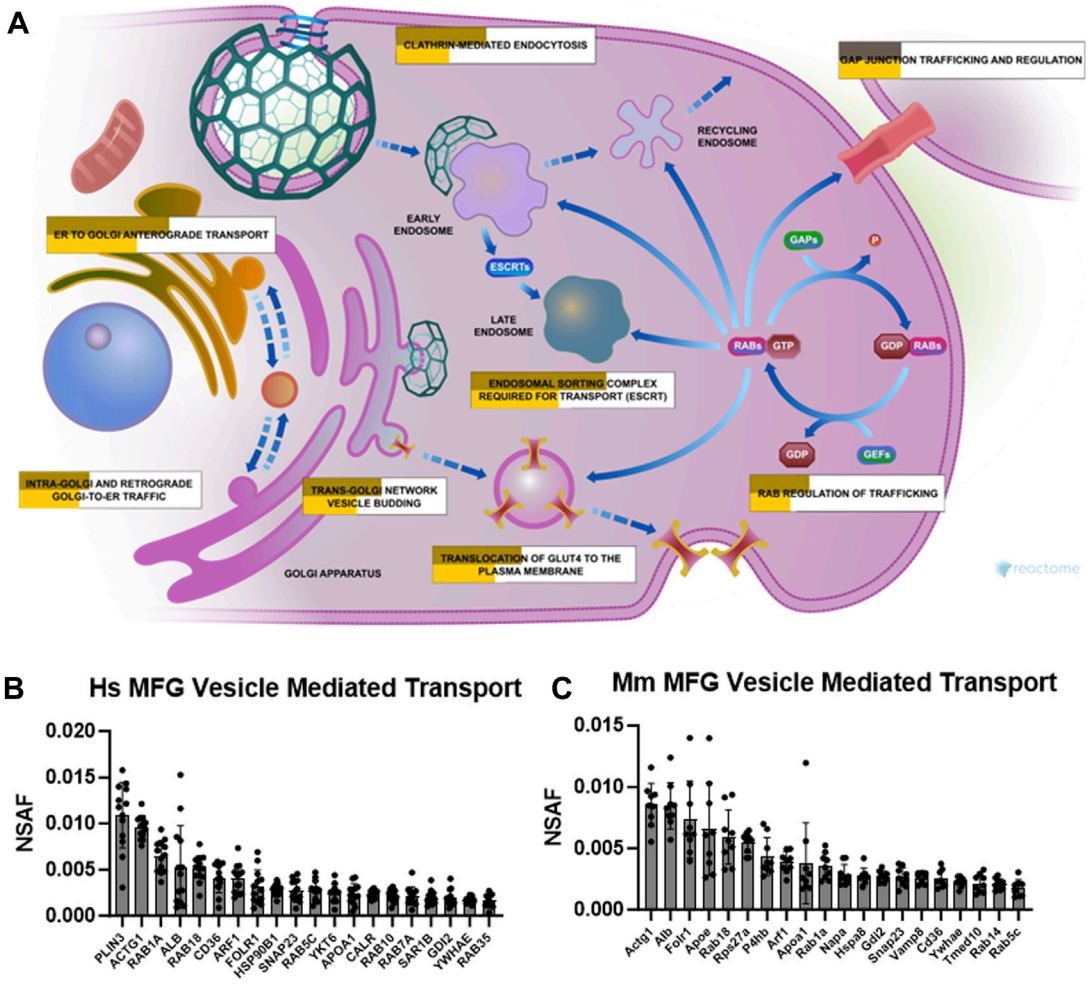

FIGURE 9
Membrane trafficking and vesicle mediated transport pathway enrichment analysis. Reactome pathway diagram with overlaid overrepresentation pathway enrichment analyses of human and mouse MFG-associated proteins in the membrane trafficking pathway **(A)**. Entities are re-colored (gold for human—upper, yellow for mouse—lower, gray if not statistically significant) if they were represented in the submitted data set. The top 20 most highly abundant vesicle mediated transport-related proteins from the human **(B)** and mouse **(C)** MFG proteomes, sorted by mean.

CD36 molecule (CD36), elongation factor 1-alpha 1 (EEF1A1), peptidylprolyl isomerase A (PPIA), NME/NM23 nucleoside diphosphate kinase 2 (NME2) and synaptosome associated protein 23 (SNAP23), which has been shown to form a soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex with vesicle-associated membrane protein 8 (Vamp8) in murine mammary epithelial cells, potentially allowing secretory vesicles to contribute membrane to the MFGM in addition to the apical plasma membrane (Honvo-Houéto et al., 2016).

Related to the specialized trafficking responsible for neutrophil degranulation, we also identified vesicle mediated transport, and its subcluster membrane trafficking, as overrepresented pathway clusters in both human (Supplementary Figure S10) and mouse (Supplementary Figure S11) MFG-associated proteomes. As illustrated by Reactome, multiple pathways were represented by the membrane trafficking pathway cluster (Figure 9A) which were significantly overrepresented in our MFG datasets, including ER to Golgi anterograde transport, intra-Golgi and retrograde Golgi-to-ER traffic, trans-Golgi network vesicle budding, translocation of GLUT4 to the plasma membrane, RAB regulation of trafficking, clathrin mediated endocytosis and endosomal sorting complex required for transport (ESCRT). Gap junction trafficking and regulation was also significantly overrepresented, but only in our murine dataset. The top 20 most abundant vesicle mediated transport pathway proteins identified in human and mouse MFG-associated proteomes are shown in Figures 9B, C, respectively. Common in the top 20 most abundant pathway proteins between species are RAB18, CD36, SNAP23, RAB5C and GDI2, in addition to actin gamma 1 (ACTG1), RAB1A, member RAS oncogene family (RAB1A), albumin (ALB), ADP-ribosylation factor 1 (ARF1), folate receptor alpha (FOLR1), apolipoprotein A1 (APOA1), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE).

RAB18 has been shown to be involved in lipid droplet interactions with other organelles (Li et al., 2017; Zappa et al.,

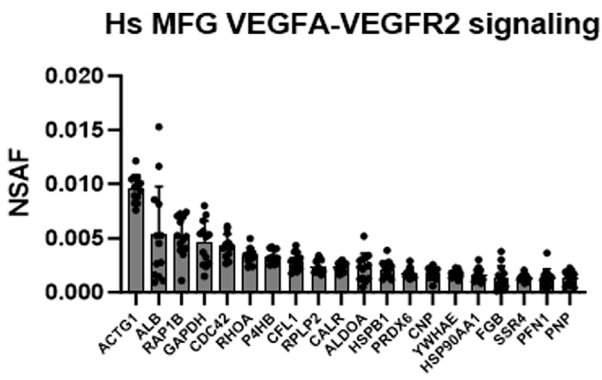

FIGURE 10
VEGFA-VEGFR2 signaling pathway analysis. Top 20 most abundant VEGF-related proteins found in the human MFG proteomes, sorted by MFG mean.

2017). We anticipate that the high abundance of RAB18 found in MFGs from both human and mice indicates a critical role for this GTPase in regulating MFG secretion.

Intriguingly, the VEGFA-VEGFR2 signaling pathway cluster was identified as highly significant in Metascape pathway analyses of the human MFG proteome (Figure 5), and in the shared human and murine MFG proteome (Figure 7). We illustrate this pathway using Reactome in Supplementary Figure S12, with the top 20 most abundant proteins in this pathway identified in the human MFG displayed in Figure 10. As vascular endothelial growth factor (VEGF) is best studied for its effects on endothelial cells, potential direct effects on epithelial cells are unexpected. We did not identify any VEGF receptors in either the human MFG or MFGM proteomes, indicating that if VEGF signaling is occurring in the mammary epithelial cells which secrete MFGs, it is not occurring on the apical surface, and is likely occurring across the basal membrane. Non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1) and subunits of Protein Kinase C, protein kinase C delta (PRKCD) and protein kinase C beta (PRKCB), appear to be main factors driving the identification of the VEGF signaling pathway. These signal transduction molecules are not specific to the VEGF signaling pathway, and we expect that their function in other signaling pathways active in the lactating mammary epithelial cells may be more relevant for understanding MFG synthesis and secretion.

Discussion

MFGs are unique, nutritionally important, membrane enveloped structures that are the primary source of neonatal calories and fat-soluble vitamins, fatty acids and lipid signaling molecules implicated in neonatal development (Oftedal, 1984). We used TIMS/PASEF proteomics to quantify relative abundances of proteins in freshly isolated human MFGs and MFGM fractions and to compare mouse and human MFG proteomic profiles. Ours is the first study to comprehensively quantify the protein compositions of human MFG and their paired, mechanically isolated membrane fractions to identify proteins specifically enriched on human MFGM and sequestered in the cytoplasm. In conjunction with pathway enrichment analyses, our results advance knowledge of the composition and relative quantities of proteins in human and mouse MFG in greater detail, provide a quantitative profile of specifically enriched human MFGM proteins, and identify core cellular systems involved in forming MFGs and MFGMs.

Due to the unique apocrine mechanism of milk lipid secretion, in which portions of the apical plasma membrane, CLD, and apically targeted cellular elements, including Golgi and secretory vesicles, are released from secretory epithelial cells (McManaman, 2012; Honvo-Houéto et al., 2016; Farkaš et al., 2020), the protein composition of MFGs is predicted to be an aggregate of a select set of proteins captured from the plasma membrane, CLD, and cytoplasmic fractions of the cell during MFG secretion. Prior studies of human MFGs have largely focused on the putative MFGM fractions of these structures (Liao et al., 2011; Yang et al., 2016), and few studies have been directed at defining MFGM protein composition relative to intact MFGs and identifying the cellular systems involved in the formation of these structures. Using MS/MS analysis of human MFG proteins separated by 1D SDS-PAGE electrophoresis, Spertino et al. (2012), identified 13 of the most abundant proteins in human MFG and using LC-MS/MS of iTRAC-labeled proteins, Yang et al. (2015) identified 520 proteins from human MFG. In contrast, using TIMS/PASEF analysis, we reproducibly quantified relative abundances of 2,453 proteins in intact, freshly isolated, human MFGs and 1,577 proteins in mouse MFGs, which significantly increases the depth of knowledge about the protein composition of these structures and enhances identification of cellular pathways that contribute to their formation. For intact, freshly isolated human and mouse MFGs, we found a positive correlation in the relative abundances of 1,279 proteins that were found in common, which indicates that similar cellular systems may contribute to MFG formation in both species. Additionally, we found that the most abundant proteins in human and mouse MFG were either involved in regulating CLD-membrane interactions during MFG secretion (BTN1A1, XDH/XOR and PLIN2) (Ogg et al., 2004b; Jeong et al., 2013; Monks et al., 2016; Monks et al., 2022), were cytoplasmic or cytoplasmic secretory vesicle components MFGE8, FABP3 and caseins [casein alpha s1 (CSN1S1) and casein kappa (CSN3) in human, casein beta (Csn2), Csn1s1, casein alpha s2-like A (Csn1s2a) and casein alpha s2-like B (Csn1s2b) in mouse)], or were implicated as regulators of membrane processes and vesicle trafficking [Rab proteins (RAB18) and ACTG1 in both human and mouse]. Pathway enrichment analysis of human and mouse MFG proteomes also revealed similarities in their corresponding biological pathways. The common, most significantly enriched pathways in MFG from both species were related to vesicular transport, and membrane organization, suggesting that they may represent core cellular processes that contribute to MFG formation.

Nevertheless, we also found marked differences between human and mouse MFGs in the relative abundances of specific proteins, which demonstrates differences in their expression and/or incorporation into MFGs and in the relative contributions of certain biological processes to MFG formation. For example, LTF, which is a secreted protein found in cytoplasmic vesicles, is 5th in abundance in human MFGs versus 106th in mouse; perilipin 3 (PLIN3), which is a CLD-associated protein that is also implicated in

endosomal trafficking (Bickel et al., 2009), is 7th in abundance in human MFGs versus 616th in mouse; and CIDEA, which is a CLD-associated protein in the mouse mammary gland that is implicated as a regulator of milk lipid secretion (Wang et al., 2012) is 31st in abundance in human MFG versus 6th in mouse MFGs.

We identified proteins specifically associated with human MFGMs by comparing the relative abundances of proteins found in intact MFGs from individual subjects with their relative abundances in corresponding MFGM fractions in a single LC-MS/MS run. This approach allowed us to directly compare relative protein abundances in MFGs and MFGMs from individual subjects, which eliminates the potential for inter-run variability and improves the power and quantitative rigor of MFGM protein enrichment analysis over prior studies of human MFGM proteins. These analyzed pooled samples and did not directly compare protein abundances in MFG and MFGM fractions (Liao et al., 2011; Yang et al., 2015; Yang et al., 2016; Zhang et al., 2021). Using differences in relative abundances between MFGs and isolated MFGMs, we found 699 proteins whose relative abundances were significantly enriched in MFGMs after correcting for multiple comparisons. The top 20 MFGM-enriched proteins included those demonstrated to contribute to CLD docking in mouse models of milk lipid secretion - BTN1A1 (#1), XDH/XOR (#2), PLIN2 (#3) and CIDEA (#6) (Monks et al., 2016; Monks et al., 2022) which have previously been reported to be major MFGM proteins (Reinhardt and Lippolis, 2008; Thum et al., 2022). We also found several known membrane proteins among the top 20 MFGM-enriched proteins, including the ATP binding cassette subfamily G member 2 (ABCG2, #7) that is linked to riboflavin transport in human milk (Golan and Assaraf, 2020), cytochrome B5 type A (CYB5; #8), a redox enzyme previously found on MFGM in humans and other species (Jarasch et al., 1977), toll-like receptor 2 (TLR2, #10), which has been detected on bovine MFGM (Reinhardt and Lippolis, 2008), CD59 molecule (CD59 blood group) (CD59, #11), a complement factor previously identified in human MFG (Hakulinen and Meri, 1995); and calcium and integrin binding 1 (CIB1, #17), a suppressor of integrin activation (Kim et al., 2011) and calcineurin-like EF-hand protein (CHP1, #18), a regulator of endocytosis (Janzen et al., 2018), which have not been reported to be MFGM proteins previously. In addition, lipid synthesis enzymes acetyl CoA synthetase long chain family members 1 and 4 (ACSL1 #12, ACSL4 #13) and lanosterol synthase (LSS, #15) are among the top human MFGM-enriched proteins, ACSL1 and LSS have been detected previously on bovine MFGM (Reinhardt and Lippolis, 2008). Other abundant human MFGM-enriched proteins that have been detected on MFGMs previously are lactalbumin alpha (LALBA, #4) which regulates lactose synthesis and is reported to have antimicrobial properties (Charlwood et al., 2002); eukaryotic translation elongation factor 1 alpha 1 (EEF1A1, #5), an actin-binding protein that contributes to the regulation of epithelial cell junctions (Erasmus et al., 2016); calcium binding proteins S100 calcium binding protein A1(S100A1, #9) and calnexin (CANX, #19) (Reinhardt and Lippolis, 2008; Yang et al., 2016); and syntaxin binding protein 2 (STXBP2, #22) (Reinhardt and Lippolis, 2008). We did not find that 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP, #14), or Ribophorin 1 (RPN1, #16) had been detected on MFGMs previously.

Importantly, we found that the relative abundances of several proteins reported to be major MFGM proteins from humans or other species (Thum et al., 2022) either did not differ significantly between MFG and MFGM fractions or were significantly more abundant in the MFG fraction. The relative abundance of MFGE8, for instance, was significantly greater in human MFG compared to corresponding MFGM fractions, whereas relative abundances of FABP3, mucin1 (MUC1); and CD36 did not differ significantly between human MFG and MFGM. These results provide evidence that for some proteins previously thought to be enriched on MFGMs, their membrane association may be comparatively weak or their abundances in other MFG compartments may be comparable to their membrane abundances.

We found 201 proteins with significantly greater abundances in MFG compared to their corresponding MFGM factions. In addition to MFGE8, LTF and CSN3, which are found in secretory vesicles, we found significant increases in the abundances of several cytoplasmic proteins including enolase 1 (ENO1), RAS like proto-oncogene B (RALB), carbonic anhydrase-6 (CA6), glyceraldehyde-3-phophate dehydrogenase (GAPDH) as well as numerous ribosomal proteins (RPS7, RPS17, RPS15A and RPS15) in MFG relative to MFGM fractions. Notably, pathway analyses of MFG-associated proteins showed greater enrichment of ribosomal proteins in mouse than in human, although these proteins were evident in the human dataset of MFG-enriched, or cytoplasmic, proteins, suggesting the possibility that more or larger cytoplasmic components are captured upon MFG secretion in mice than in humans. Collectively these data are consistent with the apocrine mechanism of lipid secretion, which is proposed to capture soluble and vesicular fractions of the cytoplasm in addition to CLD. However, mouse data have shown that apocrine lipid secretion is facilitated by, but does not require, contact between CLD and the apical plasma membrane (Monks et al., 2016; Monks et al., 2022). Thus, variations in the extent of CLD docking or in maternal physiological processes provide opportunities for variable combinations of plasma, vesicular and organellar membranes, and cytoplasmic components to be included in secreted MFGs.

By PCA, we found differences in relative abundances of human MFGM-associated proteins between transitional vs. mature lactation timepoints that was driven primarily by the significantly increased abundance of BTN1A1 in MFGMs over time. Although previous human and bovine studies have reported that the expression of protein mediators of CLD-membrane interactions including BTN1A1, XDH/XOR, PLIN2 and CIDEA increase between colostral and mature phases of lactation (Reinhardt and Lippolis, 2008; Yang et al., 2016), we did not detect significant differences in the relative abundances of XDH/XOR, PLIN2 or CIDEA in MFGM fractions from transitional and mature milk in our cohort, which suggests that molecular complexes involved in docking CLDs to the apical membrane are largely established in humans by the 2nd week of lactation.

Pathway analysis of the proteins enriched in human MFGMs identified lipid biosynthesis and localization as among the most significantly enriched pathways. In addition, several significantly enriched pathways related to MFG proteins were also found to be significantly enriched in analysis of MFGM-enriched proteins, including vesicle transport and membrane organization. These findings are consistent with the proposal that vesicular

compartments contribute to MFGM formation (Wooding, 1973; Wooding, 2023). We also identified significant enrichment of ER-associated pathways in human MFGM-enriched proteins, including N-linked glycosylation. The enrichment of these pathways in the human MFGM is consistent with studies in mice proposing that ER proteins contribute to MFGM formation (Wu et al., 2000; Honvo-Houéto et al., 2016). This ER-related pathway is related to protein folding, which suggests that discrete ER elements required for correct protein folding and/or protein quality control may be specifically directed to apocrine lipid secretion. Consistent with this mechanism, our proteomics data show that several proteins implicated in tethering the ER to the plasma membrane (Li et al., 2021) are enriched in isolated human MFGM, including VAMP associated protein A (VAPA, #68), extended synaptotagmin proteins ESYT1 (# 199) and ESYT2 (# 31) and oxysterol-binding protein like proteins OSBPL2 (# 233), OSBPL1A (# 318) and OSBPL8 (# 391).

We unexpectedly identified the neutrophil degranulation pathway to be highly enriched for both human MFG and MFGM and mouse MFG protein lists. We anticipate that there may be multiple reasons for this signal. We speculate that the mammary gland has borrowed some of the molecular processes utilized by neutrophil degranulation for stimulated secretion mechanisms and repurposed them for MFG secretion. However, this signal may also be related to known immune functions of the MFG (Brink and Lonnerdal, 2020). Our pathway analyses suggest that in addition to lipid transfer, the structure of MFGs may convey neutrophil-related immunological protection for infants, beyond the previously recognized bactericidal effects of XDH/XOR and LTF. We and others (Lemay et al., 2013) have found that milk secretion pathways are poorly annotated in the databases used for pathway analysis, and improved annotation of these pathways may support research into mechanisms which alter human milk production and/or composition.

Recently, the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) of the National Institutes of Health (NIH) initiated the "Breastmilk Ecology: Genesis of Infant Nutrition (BEGIN)" Project (Raiten et al., 2023), which convened leaders in the field of lactation science to "explore factors influencing the synthesis, composition, and best use of human milk." The BEGIN working groups conceptualized new approaches toward understanding human milk as a complex biological system (Donovan et al., 2023; Krebs et al., 2023; Neville et al., 2023; Nommsen-Rivers et al., 2023; Smilowitz et al., 2023). Indeed, milk fat secretion is thought to be influenced by myriad factors within the context of the mother-milk-infant triad. Milk fat composition has been shown to be linked to mother's diet and metabolism (Bravi et al., 2016; Daniel et al., 2021), to vary across a single feed and the circadian cycle (Neville et al., 1984; Khan et al., 2013). Maternal diet and metabolism have been shown to affect MFG size, and therefore protein content as MFGM surface area is altered (Argov-Argaman, 2019). Additionally, infant-related factors, such as frequency of feeding, are expected to affect the amount of plasma membrane included in MFGs (Masedunskas et al., 2017; Mather et al., 2019). As a secondary analysis, with limited samples, our study was not designed to analyze MFG and MFGM in context with other elements of the mother-milk-infant triad. Rather, we have contributed a method which can be incorporated into the framework of larger future studies. In particular, we propose that freshly collected MFGs should be washed of other milk components prior to freezing, and that mechanical disruption and centrifugation of MFGMs may be utilized when aiming to investigate membrane-specific functions of the MFG, so as to minimize contributions of cytoplasmic proteins. As many different factors, including time postpartum, maternal diet, metabolism, circadian rhythm, drug usage and frequency of milk removal are likely to affect proteins present on MFGs and MFGMs, future research into these factors and their effects on the MFG and MFGM proteome will provide crucial information related to regulation of the core systems driving MFG synthesis and secretion. Here, we report the presence of multiple vesicular transport pathway pathways on the MFGM. These are responsible for secretion of other milk components, and we posit that these interactions contribute to a "system within a system" by which the mammary epithelial cell regulates the balance of milk components within a narrow range of values, potentially by pairing the balance of membrane lost in MFG secretion with membranes from secretory vesicles containing lactose or milk proteins. Our findings underscore the importance of understanding the contributions of MFGs in the larger context of milk composition and infant health and immunity.

In summary, we have used TIMS/PASEF proteomics to identify and define relative abundances of proteins in human MFG and MFGM and mouse MFG in greater detail than previously known. Coupled with bioinformatic pathway analyses, our results provide new information about the protein compositions of human and mouse MFGs and the cellular processes that contribute to their formation. By comparing relative abundances of human MFG and MFGM proteins we were able to identify a set of proteins that are specifically enriched on human MFGMs. Collectively these data provided new insight into the protein compositions of human MFGs and MFGMs and the cellular processes involved in their formation, which we speculate will help to define the importance of these unique structures in infant nutrition.

Data availability statement

The original contributions presented in the study are publicly available. These data can be found here: Center for Computational Mass Spectrometry (CCMS), Mass Spectrometry Interactive Virtual Environment (MassIVE), https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp, MSV000092892 and MSV000092915.

Ethics statement

The studies involving humans were approved by the Colorado Multiple Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by the Center for Comparative Medicine at the University of Colorado Anschutz Medical Campus. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JFMC: Writing–original draft, Writing–review and editing, Investigation, Formal analysis. MD: Writing–original draft, Writing–review and editing, Investigation. TH: Writing–review and editing. JM: Writing–original draft, Writing–review and editing, Conceptualization, Investigation, Formal analysis. JLM: Writing–original draft, Writing–review and editing, Conceptualization, Formal analysis.

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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/fmolb.2023.1259047/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Multiple non-parametric (Mann-Whitney) unpaired t-tests of MFGM-associated proteins between early (2 weeks) and mature (2–5 months) lactation timepoints with FDR correction for multiple comparisons (5%).

SUPPLEMENTARY FIGURE S2
Percent coverage of top 20 human MFG- (dark gray bars) and MFGM-associated (light gray bars) proteins **(A)**. Multiple paired t-tests, adjusted for multiple testing with the Holm-Šídák method. *$p < 0.05$. Percent coverage of top 20 CD1 mouse MFG-associated proteins **(B)**.

SUPPLEMENTARY FIGURE S3
Metascape pathway enrichment analysis of human MFGM-enriched proteins, calculated by difference. Top 100 pathway clusters are shown, using a threshold of proteins found in ≥50% **(A)** or 100% **(B)** of one or both sample types.

SUPPLEMENTARY FIGURE S4
Metascape pathway enrichment analysis of all human MFGM-associated proteins Top 100 pathway clusters are shown, using a threshold of proteins found in ≥50% **(A)** or 100% **(B)** of MFGM replicates.

SUPPLEMENTARY FIGURE S5
Metascape pathway enrichment analysis of all human MFG-associated proteins. Top 100 pathway clusters are shown, using a threshold of proteins found in ≥50% **(A)** or 100% **(B)** of MFG replicates.

SUPPLEMENTARY FIGURE S6
Metascape pathway enrichment analysis of all human MFG-enriched (cytoplasmic) proteins. Top 100 pathway clusters are shown, using a threshold of proteins found in ≥50% **(A)** or 100% **(B)** of one or both sample types.

SUPPLEMENTARY FIGURE S7
Metascape pathway enrichment analyses of mouse MFG-associated proteins. Top 100 pathway clusters are shown, using a threshold of proteins found in ≥50% **(A)** or 100% **(B)** of MFG replicates.

SUPPLEMENTARY FIGURE S8
Metascape pathway enrichment analyses of MFG-associated proteins common to human and mouse. Top 100 pathway clusters are shown, using a threshold of proteins found in ≥50% **(A)** or 100% **(B)** of MFG replicates.

SUPPLEMENTARY FIGURE S9
Neutrophil degranulation pathway analysis. Reactome pathway diagrams with overlaid overrepresentation pathway enrichment analyses of human **(A)** and mouse **(B)** MFG-associated proteins in the neutrophil degranulation pathway. Entities are re-colored (gold for human, yellow for mouse) if they were represented in the submitted data set.

SUPPLEMENTARY FIGURE S10
Reactome pathway diagrams with overlaid overrepresentation pathway enrichment analyses of human MFG-associated proteins in the vesicle mediated transport pathway. Entities are re-colored (yellow if statistically significant, gray if not statistically significant) if they were represented in the submitted data set.

SUPPLEMENTARY FIGURE S11
Reactome pathway diagrams with overlaid overrepresentation pathway enrichment analyses of mouse MFG-associated proteins in the vesicle mediated transport pathway. Entities are re-colored (yellow if statistically significant, gray if not statistically significant) if they were represented in the submitted data set.

SUPPLEMENTARY FIGURE S12
VEGFA-VEGFR2 signaling pathway analysis. Reactome pathway diagram with overlaid overrepresentation pathway enrichment analyses of human MFG-associated proteins in the signaling by VEGF pathway. Entities are re-colored (gold) if they were represented in the submitted data set.

SUPPLEMENTARY TABLE S1
MFG and MFGM-associated proteins identified by LC-MS/MS. Abundance of all human MFG- and MFGM-associated proteins identified with UniProt Accession Numbers, sorted by means of MFG and MFGM values, combined. Average and standard deviations of peptide count and % coverage obtained are provided.

SUPPLEMENTARY TABLE S2
Differentially abundant proteins by absolute difference. Multiple paired t-tests of MFG- vs. MFGM-associated proteins, calculated by absolute difference (MFGM-MFG).

SUPPLEMENTARY TABLE S3
Differentially abundant proteins by MFG-normalized difference. Multiple paired t-tests of MFG- vs MFGM-associated proteins, calculated by

differences in Log2-transformed, MFG-normalized values, [Log2(MFGM/AvgMFG)-Log2(MFG/AvgMFG)].

SUPPLEMENTARY TABLE S4
MFGM-enriched protein pathway enrichment analysis. The terms within each cluster displayed in Figure 3A and Supplementary Figure S3 are listed.

SUPPLEMENTARY TABLE S5
MFGM-associated protein pathway enrichment analysis. The terms within each cluster displayed in Figure 3C and Supplementary Figure S4 are listed.

SUPPLEMENTARY TABLE S6
MFG protein pathway enrichment analysis. The terms within each cluster displayed in Figure 4A and Supplementary Figure S5 are listed.

SUPPLEMENTARY TABLE S7
MFG protein pathway enrichment analysis. The terms within each cluster displayed in Figure 4B and Supplementary Figure S6 are listed.

SUPPLEMENTARY TABLE S8
CD1 mouse MFG proteome at L10 identified by LC-MS/MS. Abundance of all human MFG-associated proteins identified, sorted by MFG medians (normality not assumed with n = 9 samples).

SUPPLEMENTARY TABLE S9
Murine MFG protein pathway enrichment analysis. The terms within each cluster displayed in Figure 5A and Supplementary Figure S7 are listed.

SUPPLEMENTARY TABLE S10
Cross-species MFG similarity. Shared and distinct human MFG and murine MFG-associated proteins are listed. Means are listed for murine samples, despite not assuming normality, to provide metrics comparable to human samples.

SUPPLEMENTARY TABLE S11
Cross species MFG protein pathway enrichment analysis. The terms within each cluster displayed in Figure 6C and Supplementary Figure S8 are listed.

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High-intensity exercise increases breast milk adiponectin concentrations: a randomised cross-over study

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Introduction: Adiponectin plays a role in glucose and fat metabolism and is present in human breast milk. It has been postulated that higher breast milk adiponectin concentrations may prevent rapid weight gain in infancy. Prior research indicates that circulating adiponectin increases acutely after endurance exercise, but no prior research has investigated the effect of exercise on breast milk adiponectin concentrations. The purpose of this randomised, cross-over study was to determine the acute effects of endurance exercise on adiponectin concentrations in human breast milk.

Methods: Participants who were exclusively breastfeeding a 6–12 week-old term infant (N = 20) completed three conditions in the laboratory: (1) Moderate-intensity continuous training (MICT), (2) High-intensity interval training (HIIT), and (3) No activity (REST). At each condition, we collected breast milk at 07:00 h (before exercise/rest), 11:00 h (immediately after exercise/rest), 12:00 h (1 h after exercise/rest), and 15:00 h (4 h after exercise/rest) and determined adiponectin concentrations using enzyme-linked immunosorbent assay. We compared changes in adiponectin concentrations after MICT and HIIT, adjusted for the morning concentration on each test day, with those after REST, using paired t-tests.

Results: Adiponectin concentrations increased 1 h after HIIT, from 4.6 (± 2.2) μg/L in the 07:00 h sample to 5.6 (± 2.6) μg/L. This change was 0.9 μg/L (95% confidence interval 0.3 to 1.5) greater than the change between these two timepoints in the REST condition (p = 0.025). There were no other statistically significant changes in adiponectin concentrations.

Conclusion: HIIT may increase adiponectin concentrations in breast milk acutely after exercise. Further studies should determine the impact of exercise-induced elevations in breast milk adiponectin concentrations on growth and metabolism in infancy.

KEYWORDS

high-intensity interval training, lactation, nutrition, adipokine, running, obesity

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1 Introduction

The World Health Organization (WHO) estimated in 2020 that 39 million children under the age of 5 were overweight/obese, and that the prevalence of overweight/obesity among children and adolescents aged 5–19 years rose from 4% in 1975 to 18% in 2018 (1). One contributing factor for the rapid increase in childhood obesity is early nutritional programming: that nutrition in early life partly determines later health. Indeed, the period from conception to 2 years of age, often referred to as "the first 1000 days," is the most critical period for pathophysiological disorders leading up to childhood and later life obesity (2). The mechanisms behind nutritional programming are not fully understood, but potentially include appetite regulation, epigenetic modifications, and changes in the gut microbiome (3–5). One of the reasons why WHO recommends exclusive breastfeeding for the first 6 months of life is that breastfed children have lower likelihood of becoming overweight/obese compared with bottle-fed children (6). However, recent evidence suggests that breast milk concentrations of nutrients and bio-active molecules vary between mothers with high and low body mass index (BMI), and that differences in breast milk composition may play a role in the mother-to-child transmission of obesity through lactational programming (7). Based on recent evidence, we have postulated that exercise may improve breast milk composition and thereby reduce the intergenerational transmission of obesity (8).

Breast milk contains adiponectin, an adipokine that plays a role in glucose and fat metabolism (9), which can cross the intestinal barrier and may modify infant metabolism (10). Adiponectin is a protein hormone mostly secreted into circulation from adipocytes in white adipose tissue (11). Low levels of circulating adiponectin are associated with insulin resistance and type 2 diabetes (12, 13). In 2006, adiponectin was shown to be present in human milk (14). Given the importance of adiponectin in inflammation, insulin sensitivity, and fatty acid metabolism, later studies have examined whether breast milk adiponectin has a role in infant metabolic development with a postulated protective effect of breast milk adiponectin on rapid weight gain in infancy (15). However, research on the association between breast milk concentration of adiponectin and measures of infant adiposity and weight gain has shown contrasting results, with some evidence suggesting an inverse association between levels of this hormone and infant adiposity measures (15–18), others showing no association (19–22), others yet a positive association (23–25).

Maternal lifestyle, including smoking, BMI, gestational diabetes, and diet, has been shown to affect the composition of breast milk, and this interplay with infant health is an area of research that is gaining traction (26–30). Exercise is a behavioural factor that has received little attention in this context. Aerobic exercise, either as moderate-intensity continuous training (MICT) or high-intensity interval training (HIIT), can increase circulating levels of adiponectin (31, 32). No prior study has investigated the effect of exercise on breast milk concentrations of adiponectin. The aim of this study was to determine the acute effect of one bout of MICT and HIIT on breast milk adiponectin concentrations. We hypothesised that both MICT and HIIT would increase adiponectin concentrations compared with no exercise.

2 Materials and methods

2.1 Study design and participants

This was a randomised cross-over study conducted at the Norwegian University of Science and Technology in Trondheim (NTNU), Norway. The Regional Committee for Medical and Health Research Ethics, Central Norway approved the study (REK-263493). The study was pre-registered in clinicaltrials.gov (NCT05042414, 13/09/2021). We included females aged 18 years or more who had given birth to a singleton, term infant 6–12 weeks ago. To be eligible, they had to exclusively breastfeed, be able to walk or run on a treadmill for at least 50 min, and live in the Trondheim area. Exclusion criteria were known cardiovascular disease or diabetes mellitus type 1 or 2. The participants signed an informed, written consent. In random order, the participants underwent three conditions: REST (sitting), MICT, and HIIT, with minimum 48 h washout between conditions (**Figure 1**). Randomisation was completed on the first test day. We used a computer random number generator developed at the Faculty of Medicine and Health Science, NTNU, to randomise the sequence of the conditions for each participant. The sequence was shown on the screen and sent by e-mail to the investigators. We did not inform the participants about which condition they were going to complete before they attended the laboratory on test days. Participants and investigators were not blinded due to the nature of the intervention (exercise). All methods were performed in accordance with the relevant guidelines and regulations.

2.2 Baseline assessments

The participants came in for assessments of body composition and peak oxygen uptake, on a separate day prior to the three

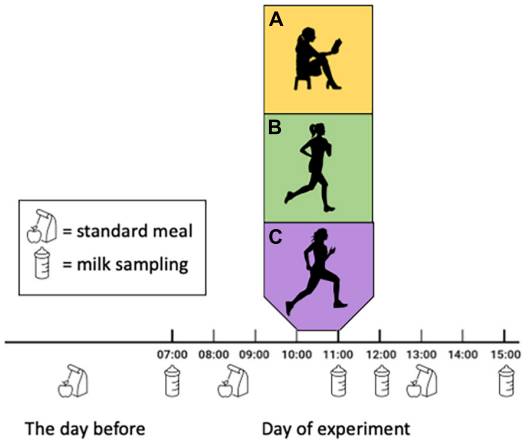

FIGURE 1
Study design. Participants completed three conditions in the laboratory, in random order: **(A)** sitting (REST), **(B)** moderate-intensity continuous training (MICT), and **(C)** high-intensity interval training (HIIT). Breast milk samples were collected at standardised timepoints in all conditions and conditions were separated by >48 h. We did not control for or record breastfeeding on test days.

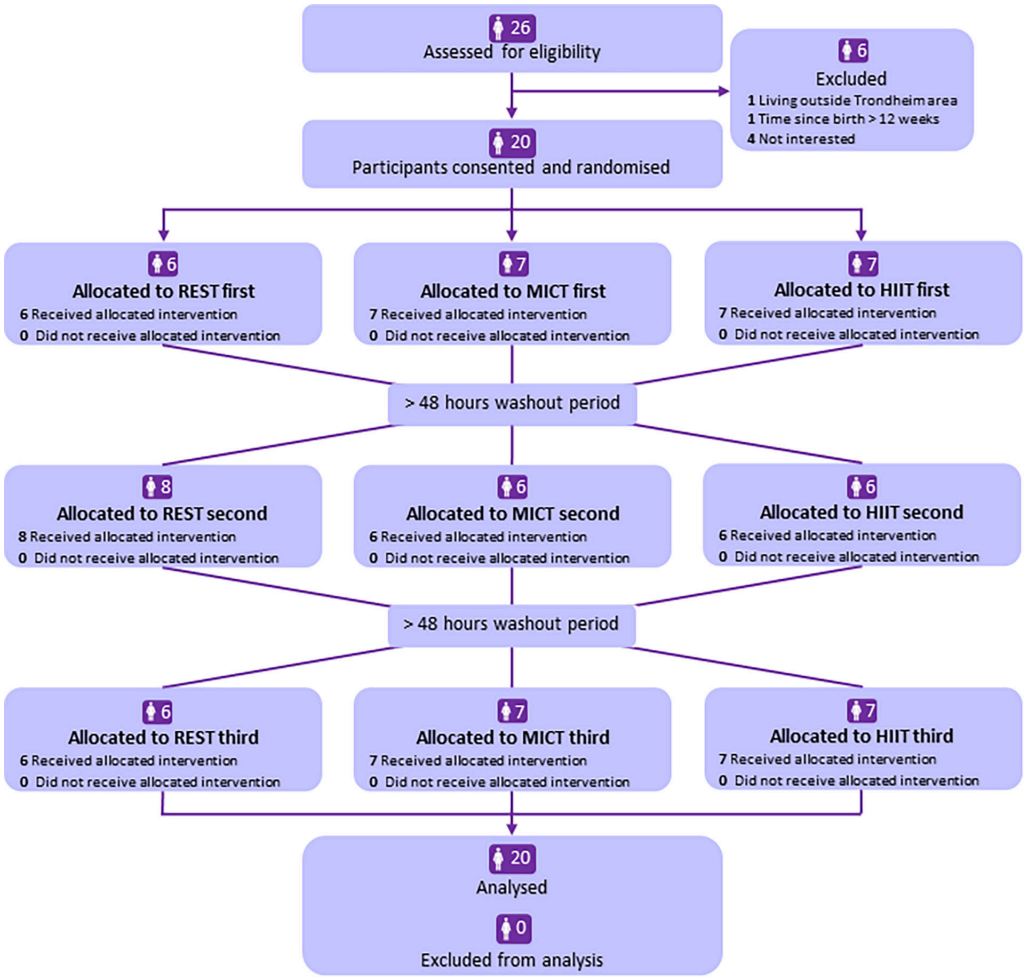

FIGURE 2
Flowchart of participants in the study. REST, resting condition in the laboratory; MICT, moderate-intensity continuous training; HIIT, high-intensity interval training.

conditions. We estimated body composition using bioimpedance (InBody720 Biospace Co., Republic of Korea). The participants completed a maximal effort exercise test on a treadmill, and we measured maximal oxygen uptake using MetaLyzer II with MetaSoft Software (CORTEX Biophysik, Germany). They wore heart rate monitors (Polar, Finland) during the test, and we used the highest heart rate during the test as an estimate of heart rate maximum (33). The participants also filled out questionnaires about background characteristics and physical activity levels (International Physical Activity Questionnaire) on this day.

2.3 Exercise and rest conditions

We requested the participants to abstain from exercise >48 h prior to all assessments in the laboratory. To minimise the effect of diet on breast milk composition, the participants recorded their diet (type of food, amount, and time of consumption) the evening prior to the first test day, as well as on the day of assessments (Figure 1). We requested them to repeat the same dietary intake for the two latter conditions.

For the REST condition, the participants rested in a chair in the laboratory for 45 min. We fitted the participants with heart rate monitors prior to the exercise conditions. Exercise intensity was based on percentage of heart rate maximum and the two protocols are isoenergetic (34). MICT consisted of walking or jogging for 48 min at 70% of heart rate maximum. In the HIIT condition, a 10-min warm-up at moderate intensity was followed by four 4-min work-bouts at 90–95% of heart rate maximum, separated by 3 min of low-to-moderate intensity. To calculate the actual exercise intensity during MICT, we recorded heart rate every fifth minute, whereas we recorded average heart rate in the last 2 min of every work-bout during HIIT.

2.4 Breast milk sampling

We provided the participants with electronic breast milk pumps (Medela Swing Flex, Medela AG, Switzerland). The participants sampled breast milk at four standardised timepoints in each of the three conditions: 07:00 h (before breakfast), 11:00 h (immediately after exercise/rest), 12:00 h (1 h after exercise/rest), and 15:00 h (4 h after exercise/rest) (Figure 1). The participants

sampled from the same breast at each timepoint, and we asked them to provide at least 25 mL each time. We did not ask the participants to pump as much as possible, only to provide minimum 25 mL at each time point, before feeding their infant. The first (07:00 h) and last (15:00 h) samples were stored in the participants home freezer and transported on ice to the laboratory at the next visit, whereas the other samples were collected when the participants were in the laboratory. All samples were stored at −80° until analysis. We did not control for or record breastfeeding of the infant on the test days.

2.5 Adiponectin analysis

After thawing the breast milk at room temperature, we centrifuged samples at 10,000 × *g* for 60 min. The fat layer on the top was carefully removed using tweezers and skimmed milk extracted for analysis. We used enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of adiponectin (IBL International GmBH, Germany, Catalog no: 30126762), using a Dynex DS2 automation system programmed with compatible DS-Matrix software (Montebello Diagnostics AS, Norway). The intra- assay variability for the ELISA kit is <5% and inter-assay variability 7.5%. Undiluted breast milk samples were measured in duplicate wells with samples from the same participant on the same microtitre plate (using the same kit). The coefficient of variability for the duplicates was 2.0 (SD 1.5). Based on our pilot testing of the ELISA kits, we set the time for the final incubation step to 12 min, instead of 15 min as described in the instruction manual, otherwise we followed the manufacturer's instruction. The range of the ELISA assay was 0.27–31000 μg/L and all measurements were obtained in the linear range of the assay. The coefficient of determination for the standard curve was 1.

2.6 Statistical analysis

No formal sample size calculation could be done for this study due to the exploratory nature of the research question. We aimed to recruit 20 participants. Crossover studies allows comparison at the individual rather than the group level and fewer participants are required in a crossover design compared with a parallel group design to obtain the same power for a target effect size and type 1 error rate. We calculated the change in adiponectin concentrations from the morning sample (obtained at 07:00 h on the same day) to each of the post-exercise timepoints (11:00, 12:00, and 15:00 h) on each of the test days and used this difference in the statistical analysis. The data were not normally distributed, and log-transformation did not make them so. We therefore decided to analyse differences at each time-point using bootstrapped *t*-tests. The changes (delta value) at each post-exercise timepoint were compared with the changes in concentrations at the corresponding timepoint after REST using paired-samples *t*-tests. The difference at each timepoint is the mean change after MICT or HIIT, compared with the change in the REST condition, for which we report the estimate, corresponding 95% confidence interval (CI), and *p*-value. Since the data were not normally distributed, we used bootstrap with 3000 samples and bias corrected and accelerated CIs. We consider *p*-values < 0.05 as statistically significant and have made no corrections for multiple comparisons due to the exploratory nature of our research question.

3 Results

3.1 Participants and breast milk adiponectin

Figure 2 shows the flow of participants across the three conditions. All the 20 participants completed all three conditions and provided breast milk at all the timepoints, thus the total number of breast milk samples analysed was 240. Recruitment of participants started in August 2021 and was completed in May 2022. Table 1 shows participants' baseline characteristics, according to which condition they completed first (Supplementary Table 1). There were no adverse events. The trial was ended when we had included 20 participants. We detected adiponectin in all breast milk samples, with an average concentration for all 240 samples of 5.2 (SD 2.5) μg/L (range 1.2–12.7 μg/L). The variation within each person (for the 12 samples obtained from each participant) was smaller, with SD 0.9 μg/L (Supplementary Table 2).

3.2 Acute effects of exercise on breast milk adiponectin concentrations

Table 2 shows the concentrations at each time-point for each condition. There were no statistically significant differences between adiponectin concentrations at the first time-point (07:00 h) on the three test days (REST vs. MOD: $p = 0.143$, REST vs. HIIT: $p = 0.077$, MOD vs. HIIT: $p = 0.831$). Compared with the REST condition, adiponectin tended to increase after exercise, statistically significant so only 1 h after HIIT (Figure 3 and Table 3).

3.3 Implementation and compliance with exercise protocols and breast milk sampling

The minimum washout period between conditions was, per protocol, 48 h, whereas the actual minimum washout period was 4 days (96 h). The average interval between test-days was 7.3 (SD 2.0) days between the first and second condition, and 6.9 (SD 1.4) days between the second and third condition. Most samples were

TABLE 1 Baseline characteristics of participants, by which condition they completed first.

	ALL (*n* = 20)	REST (*n* = 6)	MICT (*n* = 7)	HIIT (*n* = 7)
Age, years	31 (3)	31 (3)	30 (2)	32 (3)
Body mass, kg	72.2 (9.0)	77.8 (9.6)	66.0 (6.2)	73.6 (8.1)
Body mass index, kg/m^2	26.2 (3.6)	28.3 (3.8)	24.3 (3.0)	26.2 (3.3)
Fat mass, kg	23.4 (8.3)	28.6 (9.3)	18.1 (7.3)	24.2 (5.6)
Peak oxygen uptake, mL·kg^{-1}·min^{-1}	39.5 (7.8)	34.7 (5.0)	44.0 (7.9)	38.9 (7.8)
Time since delivery, weeks	8.9 (2.8)	9.1 (2.2)	8.4 (1.9)	9.3 (1.7)
Infant birth weight, g	3652 (422)	3963 (188)	3338 (276)	3700 (493)

Numbers are averages with standard deviations. REST, no exercise; MICT, moderate-intensity continuous training; HIIT, high-intensity interval training.

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TABLE 2 Breast milk adiponectin concentrations at different time points in the three conditions.

		Time point			
Condition	Days postpartum	07:00 h	11:00 h	12:00 h	15:00 h
REST	69 (13)	5.2 (3.0) μg/L	5.2 (2.0) μg/L	5.4 (2.6) μg/L	5.4 (2.4) μg/L
MICT	70 (16)	4.6 (2.3) μg/L	5.3 (2.8) μg/L	5.4 (2.8) μg/L	5.5 (2.7) μg/L
HIIT	70 (14)	4.6 (2.2) μg/L	5.1 (2.0) μg/L	5.6 (2.5) μg/L	5.5 (2.5) μg/L

Observed data are presented as descriptive mean with standard deviation (SD) for 20 participants. REST, no exercise; MICT, moderate-intensity continuous training; HIIT, high-intensity interval training.

obtained at the exact time-point prescribed in the protocol, with an average of 2.5 (SD 10.1) min deviation. The largest deviations from the prescribed time-points were at the sampling at 15:00 h (4.7 min, SD 11.4), whereas the samples obtained in the laboratory (at 11:00 h and 12:00 h) deviated by on average <1 min from the protocol. The exercise intensity was 70% (SD 1) of heart rate maximum during MICT and 96% (SD 2) of heart rate maximum during HIIT.

4 Discussion

There is scarce research on the acute effects of exercise on breast milk composition and currently few exercise guidelines for lactating people. The most recent recommendations on postpartum physical activity from the American College of Obstetricians and Gynaecologists merely states that *regular aerobic exercise in lactating women has been shown to improve maternal cardiovascular fitness without affecting milk production, composition, or infant growth* (35). These recommendations are based on observational studies (36, 37) and one RCT (38) from the 1990s analysing total concentrations of lipids, protein, lactose, and some minerals in breast milk after exercise.

In our study, we found statistically significant increased (~ 22%) concentrations of breast milk adiponectin 1 h after high-intensity training (HIIT) in exclusively breastfeeding people, compared with a day with no exercise (REST), with tendencies of elevated concentrations at all other time-points after both moderate intensity continuous training (MICT) and HIIT. These findings suggest that it may take some time before an exercise-induced increase in breast milk adiponectin concentration is evident, and that HIIT is a more potent stimulus for such increase than MICT. On the day that the participants did not exercise (REST), the adiponectin concentrations were quite stable throughout the day (from 07:00 to 15:00 h), suggesting that there is little chronobiological variations in breast milk adiponectin concentrations within this time frame.

There are some limitations to our study, including the relatively small sample size and no control of breastfeeding on the test days. The interval between test days was 7 days for most participants, with 4 days as the shortest (for one participant) and 14 days as the longest (for one participant). We assumed that there would be no order effect and did not control for this in our analysis. There may be differences in adiponectin concentration between foremilk and hindmilk. We asked the participants to collect the breast milk samples before feeding their infants at the set time points, but did not control for the time since last feed.

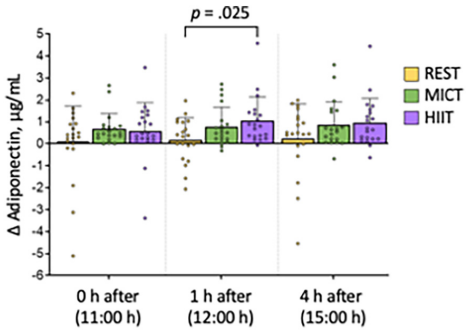

FIGURE 3
Change in breast milk adiponectin concentrations compared with a sample obtained at 07:00 h at each condition. Bars show average concentrations, error bars show standard deviation, and symbols show individual data. *P*-value is from paired-samples *t*-test and shows the difference at 1 h after high-intensity interval training (HIIT) condition compared with 1 h after no exercise (REST). MICT, moderate-intensity continuous training.

There could be an alternative explanation for the presented findings of increased adiponectin concentration 1 h after HIIT. Contrary to our expectation, we observed higher average morning concentration at the day of the REST condition despite randomising the order of HIIT, MICT and REST conditions. Thus, the increase we observed at the MICT and HIIT conditions could have been due to regression to the mean. However, circulating adiponectin concentrations (in serum) have shown to be elevated after exercise. While this is the first study on the acute effects of exercise on adiponectin concentrations in breast milk, breast milk adiponectin concentrations have been reported to correlate with concentrations in blood (10). Presuming that there is a correlation between concentrations in these two body fluids, we may compare our findings to studies on acute effect of endurance training on circulating adiponectin levels.

Elevated blood adiponectin concentrations have been observed after different forms of endurance exercise including rowing, running, cycling, and step-aerobic (39–43). In agreement with our findings, Jürimäe and colleagues found that plasma adiponectin concentrations were unchanged immediately after a maximal 6,000 m rowing ergometer test (lasting on average 20 min) in highly trained male rowers but increased by ~20% after 30 min of recovery (39). In contrast to both Jürimäe and colleagues and our present study, Schön and colleagues reported ~ 10% elevated serum adiponectin concentrations immediately after a 90-min run at 75–80% of heart rate maximum, followed by a return to baseline concentrations after 60 min of recovery in healthy young

TABLE 3 Changes in adiponectin concentrations (in μg/L) compared with a sample obtained at 07:00 hours (h) at each condition at different time points after moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT), compared with no exercise (REST), with mean difference, corresponding 95% confidence interval (CI), and *p*-values from paired samples *t*-tests.

	Immediately after exercise (11:00 h), compared with no exercise			1 h after exercise (12:00 h), compared with no exercise			4 h after exercise (15:00 h), compared with no exercise		
Condition	Mean difference	95% CI	*p*	Mean difference	95% CI	*p*	Mean difference	95% CI	*p*
MICT	0.63	−0.03 to 1.40	0.164	0.60	0.04 to 1.21	0.080	0.64	−0.01 to 1.42	0.158
HIIT	0.55	0.08 to 1.09	0.074	0.89	0.30 to 1.58	0.025	0.75	0.06 to 1.56	0.105

Data are from 20 participants.

individuals (40). There are also studies that suggest no alterations in circulating adiponectin levels acutely after exercise (44–47). The reasons for these diverging findings may include sex-differences, differences in the type of exercise, duration and intensity of exercise, as well as methodological differences in analyses and in time-points of sampling. Our study is the first that has analysed adiponectin concentrations in breast milk after exercise. As studies report that increased concentrations of adiponectin in breast milk may play a role in protection against early rapid weight gain in infancy (15–18), our findings indicate that maternal exercise during lactation can be one of the factors that affect the risk of childhood obesity. If breast milk adiponectin can protect against rapid weight gain in infancy, our results indicate that the best time to breastfeed is around 1 h after high-intensity exercise. Our results should be confirmed in further studies, including sampling of breast milk several hours after an exercise session. We therefore suggest further studies investigating both the immediate influence of a single exercise session on breast milk composition, as well as chronic adaptations with regular exercise training. We also propose that further research should consider the whole breast milk matrix, which is composed of many bio-active components. We envision that more research on the detailed effects of maternal exercise on breast milk composition will provide an evidence-base for more detailed guidelines from the American College of Obstetricians and Gynaecologists and other organisations.

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 author.

Ethics statement

The studies involving humans were approved by the Regionale Komiteer for Medisinsk og Helsefaglig Forskningsetikk (REK). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MH: Data curation, Investigation, Methodology, Writing – original draft, Writing – review and editing. GG: Data curation, Formal analysis, Methodology, Writing – review and editing. TM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review and editing.

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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.2023.1275508/full#supplementary-material

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Barriers to promoting breastfeeding in primary health care in Mexico: a qualitative perspective

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Objective: This article aimed to identify the main barriers related to promoting and counseling breastfeeding (BF) at the Primary Health Care (PHC) in Mexico.

Methodology: A qualitative study with a phenomenological approach was carried out in 88 health centers of the Ministry of Health in the states of Chihuahua, Oaxaca, Chiapas, Veracruz, Mexico, and Yucatan. From September to November 2021, we interviewed 88 key health professionals (HPs) (physicians, nurses, nutritionists, and others) from the PHC and 80 parents of children under 5 years old. In addition, nine focus groups were conducted with parents and caregivers. The data obtained were triangulated with information from focus groups and semi-structured interviews.

Results: Of the total interviews, 43.2% ($n = 38$) were nurses, 29.5% ($n = 26$) were physicians, 19.3% ($n = 17$) were nutritionists, and the rest were other health professionals. In the group of users, 97.6% ($n = 121$) were women. We identified contextual barriers, such as the lack of well-trained health professionals and the scarcest nutrition professionals, as material resources in the health units, without mentioning the low user attendance at their control consultations. Furthermore, we identified barriers related to the orientation and promotion of breastfeeding in health units, including a lack of specific strategies, ineffective communication, and the recommendations of commercial milk formulas.

Conclusion: The results presented reflect the reality of Mexico in relation to BF, making it urgent to take immediate action to improve the quality of nutritional care related to the promotion and orientation of BF at the PHC.

KEYWORDS

breastfeeding, primary health care, child health services, quality of health care, postpartum, infant, prenatal care

1 Introduction

Breastfeeding (BF) is paramount for the optimal development of newborns. Current guidelines on infant feeding practices underscore the importance of initiating breastfeeding within the first hour of life, followed by 6 months of exclusively breastfeeding on demand and continuing breastfeeding for up to 2 years or beyond (1–3). Despite these recommendations, social and cultural stigmas persist, hindering BF's initiation and continuity (4–6).

In response to this problem, the Mexican national health system has designated BF as a priority in national policy (7). Strategies and targeted actions have been implemented to enhance health services and promote infant development by supporting and protecting BF (8). Among these strategies is hospitals' adherence to the Baby-Friendly Hospital Initiative (BFHI) (9, 10), which stands out as a commitment to promoting, protecting, and supporting BF. The BFHI, updated in 2018, emphasizes that healthcare institution personnel must possess the knowledge, competencies, and skills required to ensure BF support (Step 2), among other activities promoting the practice. However, a significant gap is noted as primary-level units, including health centers, are not included in this initiative (9).

Concurrently with the BFHI, the National Breastfeeding Strategy, implemented from 2014 to 2018, emerged as a political instrument to address sustainable development goals, focusing on reducing malnutrition and infant mortality (10). Due to the lack of accreditation of all medical units nationwide, a key objective of this strategy was to increase the number of children fed breast milk from birth to at least 2 years of age (10). However, its evaluation and monitoring yielded null results (11). Additionally, the dissemination and surveillance of compliance with the International Code of Marketing of Breastmilk Substitutes and the correct, rational, and medically indicated use of these products are fundamental actions to prevent discouraging BF practice, with implications for the health and economy of the health sector and families (12).

Mexico also has technical regulations to promote exclusive breastfeeding, breastfeeding techniques, and guidance on common issues through health units that focus on encouraging mothers to breastfeed (1). However, despite efforts and the increase in exclusive breastfeeding (from 14.4% in 2012 to 35.9% in 2021), the current prevalence remains considerably below priority targets for maternal and child health (13–15).

The benefits of BF have been extensively documented, ranging from combating all forms of infant malnutrition to promoting health throughout the life cycle (16). Recognized as the most cost-effective strategy to prevent infant mortality, it is estimated to prevent 13.8% of deaths in children under 2 years in medium- and low-income countries (4, 17). Moreover, BF brings economic benefits to families (18) and the health system, reducing the use of health services by preventing diseases in newborns (19, 20). Studies such as that of Hanieh et al. (20) indicate that infants exclusively breastfed at 6 weeks after birth have lower odds of hospitalization for diarrhea (OR 0.37; 95% CI 0.15, 0.88) and suspected pneumonia (OR 0.39; 95% CI 0.20, 0.75).

In addition to benefits for children, BF also provides multiple advantages for breastfeeding mothers, including contributions to their health and wellbeing, reduced risk of breast and ovarian cancers, decreased risk of type 2 diabetes, increased family and national resources, and environmental respect (21–23).

Despite these benefits and efforts, various reasons, such as the perception of low milk supply, physical issues such as nipple cracks or pain, mastitis, and maternal return to work, lead some mothers to abandon or not initiate breastfeeding (4, 5, 24). Therefore, breastfeeding is an activity that mothers cannot carry out entirely on their own; the intervention of healthcare personnel, providing counseling, group support, and willingness to support the mother are required (25–27).

In this context, it is essential for all women to receive quality care during pregnancy, childbirth, and the postnatal period (2). For this, the definition of quality of care proposed by the World Health Organization is adopted, defining it as "the set of diagnostic and therapeutic services most suitable for optimal healthcare, taking into account all patient and medical service factors and knowledge to achieve a result with the minimum risk of effects and maximum patient satisfaction" (28).

In this regard, healthcare professionals play a crucial role in guiding and promoting BF respectfully and sensitively to individual user preferences and cultural characteristics, providing healthcare services that ensure benefits for all, regardless of factors such as ethnicity, geographic location, and socioeconomic status. Additionally, care must be safe, effective, timely, and efficient, ensuring optimal quality (29).

Despite the importance of studying the provision of quality services to promote this practice from pregnancy onwards, few studies have addressed this problem in the health system. Therefore, this study aimed to identify barriers to promoting and counseling breastfeeding at Primary Health Care (PHC) in Mexico. Results from this study would guide the development of policies and interventions regarding improving the orientation and promotion of BF in first-level healthcare units, which may generate an increase in the prevalence of exclusive and continued BF in this country.

2 Materials and methods

We conducted a cross-sectional study with a mixed approach in the Mexican Secretary of Health first-level healthcare units to assess the quality of nutritional care during preconception, pregnancy, postpartum, childhood, and preschool age. This article presents the findings related to identified barriers to promoting and counseling breastfeeding. Data collection took place from September to November 2021 in centers affiliated with the Mexican Secretariat of Health located in the following states: Chihuahua, Oaxaca, Chiapas, Veracruz, the State of Mexico, and Yucatan.

2.1 Population and study unit

The study population included two groups: (a) healthcare professionals (HPs) (including nursing, medical, and nutrition staff) and (b) users [women in preconception, pregnant,

postpartum, or mothers with infants (0–2 years), preschoolers (3–5), or their partners]. Unit selection for health centers involved random sampling, considering the total number of health centers in the six selected states, with 50% of the medical units having an acceptable rating, a confidence level of 95%, and a precision of 10%, aiming for representation in all six states. A sample of 97 units was estimated. Access to health centers was facilitated through state authorities and, in some cases, with the support of health authorities in the corresponding jurisdictions.

For the selection of user participants or their partners, the criteria included: (a) being a woman in preconception, (b) being pregnant, or (c) being a parent of a child under 5 years. Regarding HP, the criterion was initially to have worked in the health center for at least 2 years. However, due to frequent staff rotation, this criterion was eliminated. In both groups, participants needed to be at least 18 years old and provide signed informed consent to participate in the research. At least one interview with an HP, an interview with a user in each health center, and 30 focus groups (5 in each state) were expected to be conducted.

2.2 Data collection

Two instruments were used for data collection: a semi-structured interview guide and a guide for focus groups. Given the characteristics of the target population, eight semi-structured interview guides were designed: (1) physician staff, (2) nursing staff, (3) nutrition staff, (4) women in preconception, (5) pregnant women, (6) women in postpartum, (7) mothers, fathers, or caregivers of infants aged 0–2 years, and (8) mothers, fathers, or caregivers of children aged 3–5 years. The instruments were developed following a phenomenological approach (30).

For HP, the emphasis was on exploring their medical care practices, barriers, and training in quality nutritional care. In the case of users, the interviews explored perceptions of breastfeeding promotion and counseling received while using public health services. For focus groups, a guide for users was designed, specifying sections to explore and deepen according to the life stage. All instruments were previously tested, adjusted, and validated in a pilot test in five health centers in the State of Mexico.

Three trained and standardized researchers conducted the interviews and focus groups. The HP in charge of the unit received the research team in the health center. Subsequently, the researchers were directed to the HP for the interview. Regarding user interviews, quota sampling (31) was employed, with the researcher directly requesting an interview with a woman in the medical office area. Due to time constraints in the units, priority was given to conducting at least one interview with an HP and another with a user.

Focus groups were convened by health center staff 1 day in advance, requesting the presence of five to six users belonging to the same life stage (preconception, pregnancy, postpartum, and/or mothers with infants or preschoolers) in the units at 8:00 a.m. However, only nine of the expected focus groups were conducted due to low user attendance related to the COVID-19 pandemic. In Estado de México, conducting a focus group was impossible due to difficulties in gathering users.

They were conducted in a private space within the designated areas of the health center during working hours. A private, ventilated space with chairs was provided within the unit for focus groups. Data collection took place in 88 rural and urban health centers across the six states, with the participation of 88 HP and 119 users, including 39 in focus groups. All participants were asked for sociodemographic information, which were recorded with the REDCap software.

2.3 Data analysis

All interviews and focus groups were audio-recorded with participants' informed consent and subsequently transcribed by the nine field researchers. Information management was consistently anonymous and confidential.

Data analysis followed grounded theory principles (32), allowing for the generation of emerging categories (open coding) added to *a priori* categories in a codebook developed by at least two researchers (see Table 1). Representative narratives were selected to achieve category saturation, aiming for a robust theoretical explanation of the phenomenon. Differences and convergences in the data among researchers were discussed. Taguette software supported information analysis (33).

2.4 Ethical considerations

Ethical aspects of the research outlined in the Declaration of Helsinki were considered. The study received approval from the Ethics Committee of the Iberoamerican University (103/2021). The identity of participants remains anonymous, and permission to publish results was obtained through informed consent.

3 Results

A total of 88 interviews with HP were conducted, and 80 interviews and 9 focus groups were conducted with users in different age stages. Due to technical issues, 18 interviews were discarded, 8 from users. The mean age in HP was 40.2 years (SD 9.9). Of the total interviews, 43.2% ($n = 38$) were nurses, 29.5% ($n = 26$) were physicians, 19.3% ($n = 17$) were nutritionists, and the rest were other health professionals. In the group of users, 97.6% ($n = 121$) were women. Most users reported common law and married civil status at 47.6% and 44.4%, respectively. They had completed middle school (46%) and high school (24.2%); only 10.5% had a bachelor's degree. Tables 2 and 3 show the main characteristics of the HP and of the users who participated in the different techniques. The main barriers identified in the research are presented in three sections: contextual barriers, barriers from users' perspectives, and barriers from health professionals.

3.1 Contextual barriers

Low utilization to health centers and incentives to attend. The disappearance of the Social Inclusion Program (PROSPERA, as per

TABLE 1 Breastfeeding codebook.

Actor	Category	Description
User	BF knowledge and information	Overview about the users' knowledge and how they get it
	BF recommendations	Guidance on techniques for effective latching, breast extraction, and breast massage users receive at the health center
	BF length	Period that women say they have given exclusive breastfeeding and/or continued breastfeeding
	BF barriers	Difficulties identified by women to follow the BF recommendations during the postpartum period, the infant, and the preschool child stage
	Formula	Use or introduction of milk formula in babies reported by women
Health professionals	BF promotion	Strategies or actions to promote exclusive breastfeeding and continued breastfeeding carried out by the HP in the health center
	BF guidance	Breastfeeding information HP give to users during control medical consultation
	BF follow-up	Follow-up given to breastfeeding provided by postpartum mothers and up to 2 years of age of the child
	BF barriers	Difficulties identified by HP for women to follow the BF guidance or recommendations

BF, breastfeeding.

the acronym in Spanish) had an impact on attendance at health centers, and it has affected aspects related to BF. For example, women who had a pregnancy while PROSPERA was implemented had to attend workshops where they were provided with guidance on various topics about the stage they were at, including breast milk. Those workshops are no longer implemented. Even when they had difficulties describing the information in detail, they said it was clear and useful, and they also considered that the workshops should return as a BF promotion strategy.

Sanitary emergency: the COVID-19 pandemic in 2020–2021 also affected general attendance, except for pregnant women. So, they got the regular BF's attention. The health emergency also changed the way in which some women would get information, especially after birth, replacing control and postpartum visits to the doctor with social networks or researching on the internet.

3.1.1 Lack of resources to promote BF (materials and humans)

Material resources: a systemic barrier identified was the need for more informational material (such as brochures and posters) on BF, both in Spanish and in indigenous languages. Most interviewed women mentioned that they had never been given any printed material about BF. The HP considers that it would be helpful if women could take this information with them because, they say, sometimes they do not pay attention, forget what they are told, or leave with doubts that they do not clarify. Women think having materials for later consultation at home would be useful.

One pregnant woman from Chihuahua said that "the doctor has only given me the brochures" but no specific guidance on, for example, how long she should exclusively breastfeed. In this case, the material has not made any difference since the interviewee only receives it and does not ask questions. No woman reported having observed the posters placed in some health centers, sometimes as official material from the Ministry of Health and, most of the time, prepared by nursing staff.

Human resources: those who could offer more and better BF nutritional guidance are nutrition professionals, but they are the scarcest personnel in the PHC units. Only 21 of the 95 health centers were evaluated in this study; they were nutritionists with permanent HP. In some cases, nutrition professionals assist weekly or biweekly to offer outpatient services.

Health system organization: another relevant obstacle for the population to receive preventive information from nutrition personnel is that physicians must refer them, but this is only possible when there is already a diagnosis of a poor nutrition condition. The above limits the possibilities of guiding and promoting BF to most of the population.

3.2 BF at primary health care: users' perspective

3.2.1 Lack of promotion at the health center

The stage of life in which the promotion of BF at the health centers most frequently occurred was pregnancy. This general information seems to focus on the baby's benefits. Yucatán has a nutrition office, which seems to contribute to better BF promotion. The preconception women interviewed had not received any BF promotion. Some information was provided in the immediate postpartum and child stages.

> *Well, the nurse just asked me if I was going to breastfeed. I not only said I would but also that I had breastfed my first daughter, so I would give this one, too. And she told me that it was healthier for the baby than formula. I think that's all she told me* (Pregnant woman, Veracruz, rural health center).

> *Yes, they gave me a talk about how to take care of the baby in what position you should put him because there are babies who settle into different positions to drink milk, so they gave us the cradle technique; when they turn upside down, they also told us*

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TABLE 2 Sociodemographic characteristics of health professionals.

	Chiapas *n* = 14	Chihuahua *n* = 13	State of Mexico *n* = 20	Oaxaca *n* = 11	Veracruz *n* = 18	Yucatan *n* = 12	Total *n* = 88
Age (years)							
Media (DE)	40.5 (7.4)	37.4 (7.4)	38.3 (9.6)	41.9 (9.4)	43.6 (8.8)	39.7 (16.1)	40.2 (9.9)
Sex n (%)							
Woman	9 (64.3)	10 (76.9)	16 (80)	9 (81.8)	15 (83.3)	6 (50)	65 (73.9)
Man	5 (35.7)	3 (23.1)	4 (20)	2 (18.2)	3 (16.7)	6 (50)	23 (26.1)
Marital status n (%)							
Single	3 (21.4)	5 (38.4)	5 (25)	4 (36.4)	7 (38.9)	5 (41.7)	29 (33)
Married	10 (71.3)	4 (30.8)	13 (65)	3 (27.3)	6 (33.3)	6 (50)	42 (47.7)
Divorced	0	1 (7.7)	0	0	1 (5.6)	0	2 (2.3)
Common law	1 (7.1)	2 (15.4)	2 (10)	4 (36.4)	4 (22.2)	1 (8.3)	14 (15.9)
Widow	0	1 (7.7)	0	0	0	0	1 (1.1)
Education n (%)							
Elementary school	0	1 (7.7)	0	0	0	0	1 (1.1)
Middle school	0	1 (7.7)	0	0	0	1 (8.3)	2 (2.3)
High school	0	0	3 (15)	0	1 (5.6)	2 (16.7)	6 (6.8)
Bachelor's degree	8 (57.2)	11 (84.6)	15 (75)	9 (81.8)	15 (83.3)	3 (25)	61 (69.3)
Technical major	3 (21.4)	0	0	1 (9.1)	0	0	4 (6.6)
Post graduated	3 (21.4)	0	2 (10)	1 (9.1)	2 (11.1)	6 (50)	14 (15.9)
Position n (%)							
Physician	2 (14.3)	4 (30.8)	4 (20)	3 (27.3)	12 (66.7)	1 (8.3)	26 (29.5)
Nurse	7 (50)	5 (38.5)	11 (55)	7 (63.6)	5 (27.8)	3 (25)	38 (43.2)
Auxiliary nurse	1 (7.1)	0	3 (15)	0	0	0	4 (4.6)
Nutritionist	4 (28.6)	2 (15.4)	2 (10)	1 (9.1)	1 (5.6)	7 (58.3)	17 (19.3)
Social worker	0	0	0	0	0	1 (8.3)	1 (1.1)
No answer	0	2 (15.4)	0	0	0	0	2 (2.3)
Ethnicity n (%)							
No	11 (78.6)	11 (84.6)	17 (85)	8 (72.7)	14 (77.8)	11 (91.7)	72 (81.8)
Yes	3 (21.4)	2 (15.4)	3 (15.0)	3 (27.3)	4 (22.2)	1 (8.3)	16 (18.2)

> *they gave the twins technique, for when moms have twins. How you have to breastfeed them and all that* (Pregnant woman, Yucatán, rural health center).

Most women recognized breast milk as the first form of food that a baby should get, and formula was considered the second-best option. However, in this group of women of all life stages included in this study, the majority have used the formula. The main benefit of breast milk they mention is that "children grow up healthy." The biggest detriment of breast milk substitutes is that "children get fat." In addition, they realize that the formula represents an economic expense for the families. In general, they do not identify benefits for themselves. It is important to mention that it could be obtained by previous experience, through family or the internet, and not at the health center.

Pregnant women who received or found some information about BF are convinced to give breast milk to the expected child due to the benefits it offers. They get the information mainly from either their nurse or physician. The minimum time range that they would like to do it is from 3 to 6 months, but there are also several mentions, such as "*until my baby wants to drink it*" or *"until he/she is 2 years old*." Mothers of children in the infant or preschool stage also refer to having breastfed for 6 months, some of them exclusively and some others using formula too. The reasons to stop doing it were that the baby did not want any more breast milk or that they had to work or study.

> *I did breastfeed her exclusively for 6 months; then I combined it with formula because at work, they almost didn't let me go out to feed her. Now (9 months), I have taken her off breast milk to give her a bottle. I buy the formula myself. It is very expensive. Sometimes, when I don't have money, I make him maseca atole (a corn flour drink) with sugar.* (Mother of a child in infancy stage, Chihuahua, rural CAAPS).
>
> *I read on verified (internet) pages that when breastfeeding, antibodies are passed to the baby that will be of use to them during*

> *their first years of life. I also asked the doctor, but he gave me little information. So, throughout my pregnancy, I was taking vitamins, and then I continued taking them, so that my baby did not lack anything in terms of nutrition and did not get sick all the time (…) I wanted to give him only breastfeeding, but in the end, I had to give him formula because when he was 1 month old, I used to go to school and I would only give him my milk* (Mother of a preschool child, Yucatan, urban health center).

PHC units are not recognized as a source for the promotion of breastfeeding. Most of the interviewees in the postpartum, child, and preschool stages reported that it was at the secondary care hospitals where they gave birth when they were informed about techniques for effective latch-on, but one could detail what she was told. There was only one case about the breast massage technique. Although she was appreciated for getting her mother's main guidance and support.

> *Yes, in the hospital, they told me that I had to breastfeed him and not to give him formula because the breast is better (…); they said to me that my baby had to latch on from the top of the nipple to be able to be suckled well, to make sure that his mouth did not sound and that he was not sucking air* (Postpartum woman, Veracruz, urban health center).

> *Before I was discharged from the hospital, they gave me the information about BF printed on paper, and they explained to me how I should breastfeed my baby, how long, and how often I had to give him my breast milk.* (Mother of infant, Estado de México, rural health center).

> *In the hospital, they gave me a syringe to stimulate my breast, and they barely told me about how to give myself massages. But the one who really supported me in that part was my mother, who was implementing a lot of the breast pump. We implemented the nipple cover to see if it would help him to be suckled a little more, and then we were massaging him, and all that was what helped me little by little so that she could reach the point of … well, yes, adjusting it so that he had a better grip and could suck better* (Postpartum woman, Yucatan, urban health center).

In the few cases in which women go to the nutritionist in childhood, there does not seem to be specific care for BF since the person who has been referred is the child, not the mother. However, the mothers indicated that they received the general recommendation to continue breastfeeding, and if they reported having low milk production, the advice is to drink plenty of water in order to increase it.

An interesting finding was that in rural women's areas, the idea of breast milk losing its nutrition properties after 3 to 6 months seems to persist, so continued BF is a practice that can be jeopardized.

> *I am almost going to take him off breast milk in 2 months, when he is 9 months old, because it is no longer giving him any benefit; I think that at 6 or 7 months, it is no longer beneficial. Then I will start giving him formula milk* (Mother of an infant, Chiapas, rural health center).

> *I am only going to breastfeed him until he is 7 months old because the doctor tells me that at 6 months, he has had enough breast milk, and if I continue to give him milk, I can delay his learning* (Mother of Infant, State of Mexico, rural health center).

In summary, even if women do not explicitly state it, it is clear there is weak BF promotion through all stages (preconception, pregnancy, and postpartum). It seems that pregnancy is the only moment in which BF is promoted, but not enough for this practice to be carried out in later stages.

3.2.2 Lack of follow-up and orientation

The length of BF is related to contextual aspects such as customs and habits or the economy, but mainly to the consultation given at the health center after the birth. Only 25% of the interviewees in the postpartum and child stages said they had exclusively breastfed for the baby's first 6 months. The rest was combined with formula, either since birth or during some of these months. The proportion of those who continued breastfeeding without formula is even lower.

Several of the postpartum, child, and preschool stages interviewees reported using formula at least once because they could not breastfeed immediately due to actual or perceived low production or technique difficulties, so the baby was fed with it at the hospital; fortunately, in most cases, the mothers did not continue with this type of food and breastfed their babies.

> *I am combining breastfeeding with formula because I am not able to get my son satisfied. They haven't told me why I can't fill him up. Still, the doctor has recommended that if I give him one ounce of formula now, I should give him two ounces the following month so he doesn't stay hungry* (Postpartum woman, State of Mexico, rural Advanced Center for Primary Health Care).

> *Yes, they gave my baby formula at the hospital; they offered him formula on one occasion just because he could not suckle my breast well. But now I only give him my milk* (Postpartum Woman, Oaxaca, rural health center).

It was mentioned that pregnant women get basic information about BF. However, when the time to breastfeed comes, if they do not receive guidance in the face of any difficulty or doubt, they will likely stop giving breast milk. None of the women interviewed received guidance on latching techniques, milk extraction, or breast massaging during the puerperium.

As mentioned, postpartum women do not attend check-ups unless they have an alert sign. In this sense, it seems that BF difficulties are not recognized as relevant and require immediate attention. Although attendance is higher in childhood, according to the mothers interviewed, no special attention is given to BF. During control visits, some nurses and doctors ask if they are sharing breast milk. They continue the review without delving into details if the answer is positive. If they comment that they are having difficulties, few nurses offer advice on latching techniques and breast massaging, but doctors directly recommend introducing formula. Women follow this instruction because it relieves their anxiety about not "filling" (satisfying their babies). Although most of them express remorse because they know or

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TABLE 3 Sociodemographic characteristics of users.

	Chiapas *n* = 19	Chihuahua *n* = 22	State of México *n* = 13	Oaxaca *n* = 16	Veracruz *n* = 24	Yucatán *n* = 30	Total *n* = 124
Age (years)							
Media (DE)	29 (6.4)	28.2 (6.1)	25.5 (5.2)	29 (4.8)	28.8 (6.9)	27.3 (8.5)	28.1 (6.7)
Sex n (%)							
Woman	18 (94.7)	22 (100)	12 (92.3)	16 (100)	24 (100)	29 (96.7)	121 (97.6)
Man	1 (5.3)	0	1 (7.7)	0	0	1 (3.3)	3 (2.4)
Civil status n (%)							
Single	0	2 (9.1)	0	1 (6.2)	3 (12.5)	2 (6.7)	8 (5.5)
Married	8 (42.1)	9 (40.9)	3 (23.1)	6 (37.5)	11 (45.8)	18 (60)	55 (44.4)
Divorced	0	1 (4.6)	0	0	0	0	1 (0.8)
Common law	11 (57.9)	10 (45.4)	10 (76.9)	9 (56.3)	9 (37.5)	10 (33.3)	59 (47.6)
Widow	0	0	0	0	1 (4.2)	0	1 (0.8)
Education n (%)							
None	0	1 (4.6)	0	0	0	0	1 (0.8)
Elementary school	1 (5.3)	5 (22.7)	2 (15.4)	3 (18.8)	4 (16.7)	5 (16.7)	20 (16.1)
Middle school	6 (31.6)	8 (36.4)	8 (61.5)	8 (50.0)	9 (37.5)	18 (60)	57 (46)
High school	6 (31.6)	3 (13.6)	3 (23.1)	3 (18.8)	8 (33.3)	7 (23.3)	30 (24.2)
Bachelor's degree	4 (21.2)	5 (22.7)	0	2 (12.5)	2 (8.3)	0	13 (10.5)
Technical major	1 (5.3)	0	0	0	0	0	1 (0.8)
Post graduated	1 (5.3)	0	0	0	1 (4.2)	0	2 (1.6)
Number of children n (%)							
One	1 (5.3)	1 (4.6)	4 (30.8)	0	4 (16.7)	2 (6.7)	12 (9.7)
2 to 3	5 (26.3)	8 (36.4)	3 (23.1)	6 (37.5)	8 (33.3)	11 (36.7)	41 (33.1)
Up to 3	12 (63.2)	12 (54.5)	5 (38.5)	7 (43.8)	11 (45.8)	10 (33.3)	57 (45.9)
No answer	1 (5.2)	1 (4.5)	1 (7.7)	3 (18.8)	1 (4.2)	7 (23.3)	14 (11.3)
Ethnicity n (%)							
No	16 (84.2)	18 (81.8)	6 (46.2)	12 (75.0)	13 (54.2)	19 (63.3)	84 (67.7)
Yes	3 (15.8)	4 (18.2)	7 (53.8)	4 (25.0)	11 (45.8)	11 (36.7)	40 (32.3)

"feel" that they are denying a considerable benefit to their children, there were also cases of women who, despite receiving the recommendation from their physician or nutritionist to give Exclusive BF for up to 6 months and continued for up to 2 years, decided to stop BF when they returned to work because they did not have the time and conditions to extract breast milk or because it was too difficult for them to get their job done and feed their baby from the breast.

> *The doctor tells me to take him off the formula and give him only breast milk, but I tell my husband that I can't takes it away from my baby because otherwise, he won't be satisfied. The doctor told me he is fine, but I should give him formula once a day and my milk twice a day because he is growing very fast. And he is fine, but as I told you, I work in a store, and I get tired, and since my husband works at night, there is no one to help me, I am the only one who gets up at night. I don't rest well, that's why I want to take it away from him* (Mother of a child in infancy, Yucatan, rural health center).

3.3 Barriers from health professionals' perspective

3.3.1 Not enough time, not enough knowledge

A systemic barrier is the time HP has for each patient: 15 min on average. Physicians and most nutritionists think it is enough time. Still, nurses consider that it is not enough to provide them with all the information and, at the same time, be able to clear up their doubts, especially for first-time pregnant women.

Health personnel recognize BF as a very important practice for the child's health. However, when describing what a controlled medical consultation is like, only six of the interviewed physicians mentioned the promotion of BF as part of this care. Nutritionists do not include it because they mostly care for patients referred for a specific situation identified by the doctor. The nurses are shown as the ones who do the most promotion, some during the brief nutritional control procedure and others trying to give talks to groups of pregnant women. Three nurses mentioned specific cases in which they have guided mothers of children in infancy,

recommending grasping techniques and breast massages to promote production.

> *Sometimes if the mother brings her 6 months-old child who already has to start weaning, I have to tell the mother "No, you have to give him this now; it is no longer exclusive breastfeeding." But, if they don't bring him and the mother only continues to breastfeed him, and she doesn't give him (complementary) food, the child will fall into malnutrition. So, if they don't bring the child, well … We don't guide them on these issues*—Nurse, Chiapas, rural.

The medical and nursing staff acknowledged having little knowledge about nutrition and BF. So, when they promote it over follow-up visits during pregnancy, they mention basic and common-sense aspects such as "it is good for healthy growth." No HP mentions the guidance provided regarding breast massage or milk extraction at any stage.

3.3.2 Low users' attendance and no attending

In general, HP reported providing BF information during pregnancy because they knew it was very important. They are unsure if the information is clear enough to women because, although they do not regularly ask them questions or express doubts, they have identified that they pay little or no attention to the recommendations they generally make. So they may not follow the guidance provided.

In relation to women in preconception, HP points out that they are not users who come due to a lack of a culture of preparation for pregnancy; anyway—or because of that—it seems that they do not have a specific strategy to promote BF if any woman attends prenatal controls. The actions related to BF taken by the HP occur mainly during pregnancy, and they hope (or expect) that this will be sufficient to ensure that women do not have barriers to breastfeeding their children. For example, in one state, dolls are used to teach latching techniques to pregnant women, but not during puerperium.

> *Breastfeeding is one of our pillars, one of our strongest actions. We have materials such as the "Jorgitos" (dolls) to teach them latching techniques and the "mama breasts" to teach them how to get milk; in other words, there are some resources to work with pregnant women.* (Nutritionist, Yucatan, urban).

> *During pregnancy-control-consultation, breastfeeding is explained, as well as its importance, the complications that may occur if nothing is done, the use of bottles, and its complications. If she comes and is already breastfeeding, she is oriented on what to do after breastfeeding, on her digestion, on the use of the straw. These little details take time, but women need this orientation* (Physician, Veracruz, Urban Health Center).

According to HP, women in the postpartum period usually do not attend medical consultations unless they have some warning signs. This explains why only 10 interviews and no focus groups with this profile were achieved. Therefore, they generally do not receive any guidance on effective latch-on techniques, breast massaging, or milk extraction at the health center during this period, without mentioning the lack of trained personnel who could provide this information.

In the case of children under 2 years of age, the health unit attendance was higher; however, the main reason for attendance was the vaccine administration and not a control consultation unless the child had a health problem. Some nurses recognized that vaccine administration visits could be used to provide nutritional guidance, including BF. Still, they do not have enough time to vaccinate and comply with the administrative process that it implies.

> *When I apply the vaccines to a child, I would like to do more, check how they are in weight and height, and monitor how their nutrition is going (…), but I am the only nurse here. If at a certain moment, I am vaccinating and at the same time checking one by one, I'll be late. Plus, others are waiting for their vaccines, so sometimes time limits me. Yes, I would like to cover many things, such as guiding mothers, especially first-timers, since they may have many doubts, but the lack of time and nursing staff limits me in everything.* (Nurse, Oaxaca, rural health center).

Regarding the economic costs of commercial milk formulas, the medical position is contradictory, as they recommend using milk formula despite its high cost to the household economy. However, they also recognize that the need for more home resources is a barrier to the following nutritional recommendations.

3.3.3 "The other" promotion

Physicians said they follow up on the type of BF mothers offer their children under 2 years of age, recommending exclusive breastfeeding for up to 6 months and continuing up to 2 years. However, they acknowledge that they recommend giving a formula when women report physical or contextual difficulties to provide it. Nursing and nutrition personnel seem more inclined to promote BF than physicians, showing more sensitivity and knowledge.

> *We have had very, very malnourished children. That's why I always (recommend to moms) breastfeed, latch on well. You hold your baby's head with one hand, and with the fingers of the other hand, you have the nipple, and that's how you're going to feed him. And look at your baby, let your baby look at you, let him see that connection. Give that time to your baby* (Nurse, State of Mexico, rural health center).

The interviews with the HP showed that exclusive BF and continued BF were promoted in the control visits to children under 2 years of age, focusing on the benefits of this food. The medical and nursing staff mentioned that they ask the mothers if they breastfeed their babies and, unless they report any problems in doing so, they are satisfied with the answer "yes," and no further inquiries are made. When difficulties in BF appear, which are mostly "I do not have enough milk and it does not my child satisfied" or "the baby does not like my milk," the nurses make recommendations such as drinking plenty of water and giving

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breast massages or "home remedies" such as drinking *atole*. (A corn flour drink).

Nutrition personnel are the most reluctant to recommend milk formula and do so only when the child or mother is diagnosed with malnutrition. However, because their attention depends on the physician's decision to refer the patient to the nutrition service, the promotion of BF is limited. A case was found in which the breast milk substitute industry gives away unique formulas to mothers through a nutritionist who recognizes that there is a commercial strategy for the company but considers that it is a way to support children with special feeding needs.

> *This formula is for children who do not tolerate regular milk. It is a special milk for children who become constipated and have no infant formula left. This line has one for children under one year old, and this one is for children from one to three years old. We are supported by the Nestlé promoter, who provides us with courses and gives us material and substitutes. We ask her specifically for the children we detect with some pathology, and she brings them to us so that the mother can try it and repurchase it. I know that the company does this to sell more … and yes, it is expensive, about $250 a can* (Nutritionist, Veracruz, urban health center).

Figure 1, shown a synthesis of the barriers to promote breastfeeding in PHC in Mexico.

4 Discussion

In Mexico, there are still barriers to the promotion of breastfeeding for mothers who receive care in public health services. The disappearance of the PROSPERA, the interruption in the continuity of care for pregnant women, the shortage of nutrition professionals within the units, the lack of promotion of breastfeeding from preconception, the recommendation of milk formulas as a source of food, the existence of myths about the quality of breast milk, the lack of material that promotes BF and that is not adapted to the sociocultural contexts of each region, and the lack of reinforcement of breastfeeding during newborn control were the main barriers identified from the perspective of HP and users for the promotion of BF.

The main findings related to the interruption were the disappearance of PROSPERA and the interruption in the continuity of care during the COVID-19 epidemic. This is related to the evaluated years in the study (2020 and 2021); access to and delivery of health services for the population without social security were affected by two reasons. The first was due to mobility restrictions and the saturation of health units due to the SARS-CoV-2 epidemic (COVID-19). This means a reduction in the number of consultations, restricted mobility, and fear of contagion within the health units (31, 34). The second one was the transition that the public health system was going through, repealing the Social Protection System in Health and replacing it with INSABI. In this transition, the HP reported that the decline in control attendance, especially of the "healthy child," began when the health component of the conditional cash transfer program, known as PROSPERA, was canceled in 2019. This program covered 6.5 million households and the disappearance of the co-responsibility of attending control appointments in exchange for receiving economic support (35, 36).

It is evident that women's interest in BF their children, but the limited promotion it gets during pregnancy and the practically non-existent orientation in the postpartum and childhood stages discourage this objective. The idea of low milk production persists in women, and HP reaffirms it even more. The solution that physicians suggest for this and some other difficulties is the use of formula, especially when women feel guilty about not satisfying their children with enough food. These findings show the unfamiliarity and persistent violation of the International Code of Marketing of Breastmilk Substitutes by HP, phenomena previously addressed by other authors in the Mexican context (37, 38).

Counseling on effective latch-on techniques, breast massaging, and milk extraction hardly occurs during the postpartum period because women at this stage usually do not come to the health center. However, it is also considered that there is a well-designed strategy or guidelines to guide the few postpartum women who come for follow-up. Our study agrees with the results published by Hernández Cordero et al. (39), which showed that one of the barriers to breastfeeding identified by the Mexican Health System is the lack of advice from health personnel.

The intervention of more qualified personnel in the nutritional field could counteract this problem; however, nutritionists are the ones who need more presence in the PHCs. On the other hand, nursing staff could also be key in the promotion and orientation of BF since they are more sensitized; most of them do not have sufficient or updated training, and a significant administrative burden keeps them from spending more time on other topics. This reflects the need to strengthen the PHC system with more trained maternal and child nutrition professionals.

Some positive aspects were found within the units that provide care to the population without social security, such as the existence of a Nutrition Directorate in the state of Yucatan. This direction favors the availability of nutrition professionals; however, the overall results show a lack of promotion and counseling of BF. In the case of Chihuahua, some health centers reported that the nutrition professional visits the health center once every 15 days or once a month. This situation also acts as a barrier to the control and monitoring of users (40).

The data collection period was also identified as a limitation for collecting information since we consider that the COVID-19 pandemic was a period of stress, social distancing, and difficulties in accessing health services, mainly by the users. Our study shows the barriers to adequate breastfeeding practices in the selected states of Mexico; however, it is necessary to have more studies in other regions of the country to have a complete vision of the problem.

In conclusion, there are barriers to breastfeeding counseling and promotion in first-level units in six states. Although there are strategies for the promotion of exclusive and continuous breastfeeding at the first level of care, there are still contextual barriers that prevent women from receiving this promotion. This study shows the need to train PSs at the first level of care on issues of breastfeeding and lactation for postpartum mothers, counseling these messages during prenatal visits. Similarly, it is important to

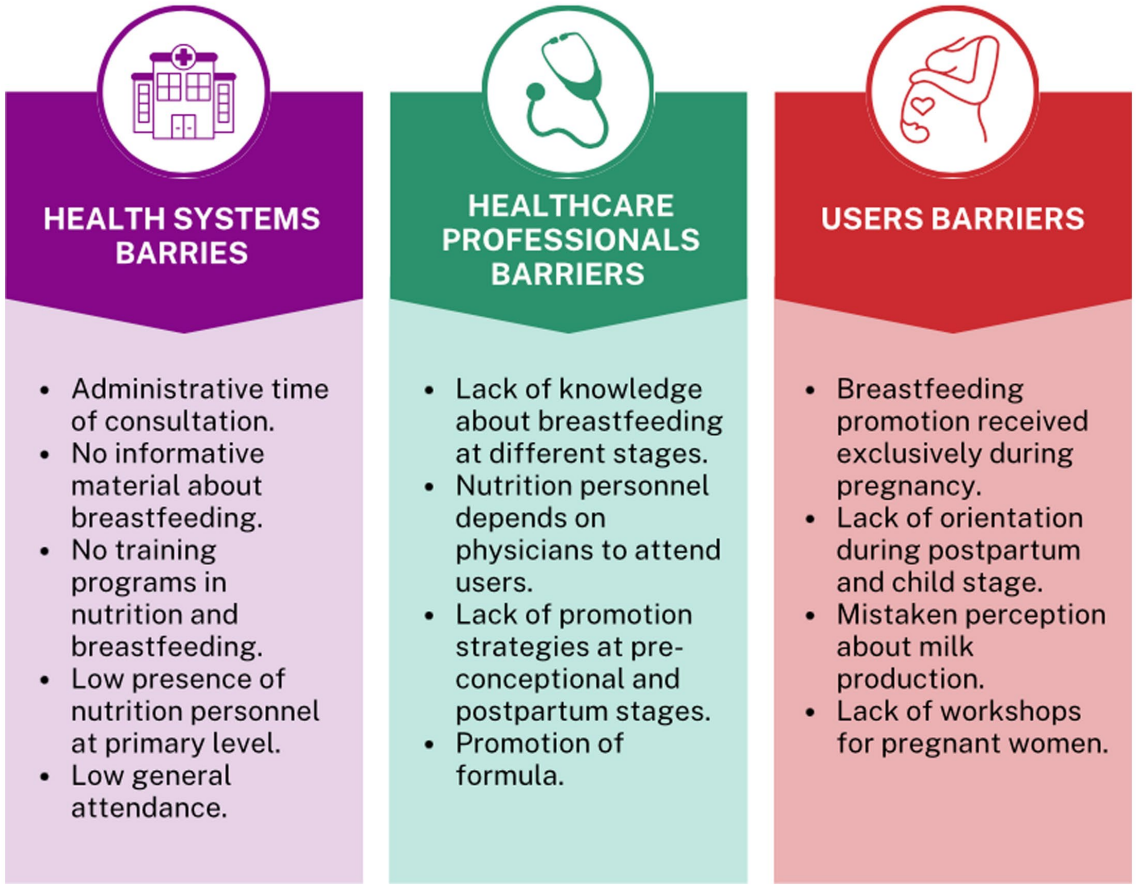

FIGURE 1
Barriers to promote breastfeeding in primary health care in Mexico.

create programs or strategies that allow the control of children's health in PHC to delay the different forms of malnutrition.

Considering the results, we have identified the urgent need to implement actions aimed at improving the quality of nutritional care in PHC on breastfeeding issues. These actions can have a significant impact on optimizing the nutritional status of the maternal-infant population and influencing the prevention of intergenerational transmission of pathological conditions and cardiometabolic risks during the life course.

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 humans were approved by the Ethics Committee of the Universidad Iberoamericana in Mexico City (No. 103/2021) and it was authorized by the Ministry of Health of each state. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

EH-L: Formal analysis, Investigation, Methodology, Supervision, Writing – original draft. CPN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft. SB-M: Supervision, Writing – review & editing. SH-C: Supervision, Writing – review & editing. IO-G: Supervision, Writing – review & editing. MAA: Supervision, Writing – review & editing. MA-M: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft.

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Corrigendum: Barriers to promoting breastfeeding in primary health care in Mexico: a qualitative perspective

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KEYWORDS

breastfeeding, primary health care, child health services, quality of health care, postpartum, infant, prenatal care

A corrigendum on
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In the published article, there was an error in Table 1 as published. The mentions column should not have been included. The corrected Table 1 and its caption appear below.

In the published article, there was an error, the "Ministry of Health in Mexico" was inadvertently duplicated.

A correction has been made to Abstract, methodology, Paragraph 1. This sentence previously stated:

"A qualitative study with a phenomenological approach was carried out in 88 health centers of the Ministry of Health in the states of Chihuahua, Oaxaca, Chiapas, Veracruz, Mexico, and Yucatan. From September to November 2021, we interviewed 88 key health professionals (HPs) (physicians, nurses, nutritionists, and others) from the PHC of the Ministry of Health in Mexico and 80 parents of children under 5 years old. In addition, nine focus groups were conducted with parents and caregivers. The data obtained were triangulated with information from focus groups and semi-structured interviews."

The corrected sentence appears below:

"A qualitative study with a phenomenological approach was carried out in 88 health centers of the Ministry of Health in the states of Chihuahua, Oaxaca, Chiapas, Veracruz, Mexico, and Yucatan. From September to November 2021, we interviewed 88 key health professionals (HPs) (physicians, nurses, nutritionists, and others) from the PHC and 80 parents of children under 5 years old. In addition, nine focus groups were conducted with parents and caregivers. The data obtained were triangulated with information from focus groups and semi-structured interviews."

In the published article, there was an error, "(n=97)" was repeated.

A correction has been made to Materials and methods, 2.1 Population and study unit, Paragraph Number 2. This sentence previously stated:

"For the selection of user participants or their partners, the criteria included: (a) being a woman in preconception, (b) being pregnant, or (c) being a parent of a child under 5 years. Regarding HP, the criterion was initially to have worked in the health center for at least 2 years. However, due to frequent staff rotation, this criterion was eliminated. In both groups, participants needed to be at least 18 years old and provide signed informed consent to participate in the research. At least one interview with an HP (n = 97), an interview with a user (n = 97) in each health center, and 30 focus groups (5 in each state) were expected to be conducted."

The corrected sentence appears below:

"For the selection of user participants or their partners, the criteria included: (a) being a woman in preconception, (b) being pregnant, or (c) being a parent of a child under 5 years. Regarding HP, the criterion was initially to have worked in the health center for at least 2 years. However, due to frequent staff rotation, this criterion was eliminated. In both groups, participants needed to be at least 18 years old and provide signed informed consent to participate in the research. At least one interview with an HP, an interview with a user in each health center, and 30 focus groups (5 in each state) were expected to be conducted."

In the published article, there was an error, a section number was omitted.

A correction has been made to Results, 3.1 Contextual barriers

This section heading previously stated:

"Lack of resources to promote BF (materials and humans)."

The corrected section heading appears below:

"3.1.1 Lack of resources to promote BF (materials and humans)."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

Publisher's note

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TABLE 1 Breastfeeding codebook.

Actor	Category	Description
User	BF knowledge and Information	Overview about the users' knowledge and how they get it
	BF recommendations	Guidance on techniques for effective latching, breast extraction, breast massage users receive at the health center
	BF length	Period that women say they have given exclusive breastfeeding and/or continued breastfeeding
	BF barriers	Difficulties identified by women to follow the BF recommendations during the postpartum period, the infant and the preschool child stage
	Formula	Use or introduction of milk formula in babies reported by women
Health professionals	BF promotion	Strategies or actions to promote exclusive breastfeeding and continued breastfeeding carried out by the HP in the health center
	BF guidance	Breastfeeding information HP give to users during control medical consultation
	BF follow-up	Follow-up given to breastfeeding provided by postpartum mothers and up to 2 years of age of the child
	BF barriers	Difficulties identified by HP for women to follow the BF guidance or recommendations

BF, breastfeeding.

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Where does the time go? Temporal patterns of pumping behaviors in mothers of very preterm infants vary by sociodemographic and clinical factors

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Background: Mothers of very preterm (<32 weeks gestational age [GA]) infants are breast pump dependent and have shorter duration of milk provision than mothers of term infants. The opportunity (i.e., time) cost of pumping and transporting mother's own milk (MOM) from home to the NICU may be a barrier. There is a paucity of data regarding how much time mothers actually spend pumping.

Objective: To investigate the variation in pumping behavior by postpartum week, maternal characteristics, and infant GA.

Methods: Prospectively collected pump log data from mothers enrolled in ReDiMOM (Reducing Disparity in Mother's Own Milk) randomized, controlled trial included pumping date and start time and end time of each pumping session for the first 10 weeks postpartum or until the infant was discharged from the NICU, whichever occurred first. Outcomes included number of daily pumping sessions, number of minutes spent pumping per day, and pumping behaviors during 24-h periods, aggregated to the postpartum week. Medians (interquartile ranges) were used to describe outcomes overall, and by maternal characteristics and infant GA.

Results: Data included 13,994 pump sessions from 75 mothers. Maternal characteristics included 55% Black, 35% Hispanic, and 11% White and 44% <30 years old. The majority (56%) of infants were born at GA 28–31 weeks. Mothers pumped an average of less than 4 times per day, peaking in postpartum week 2. After accounting for mothers who stopped pumping, there was a gradual decrease in daily pumping minutes between postpartum weeks 2 (89 min) and 10 (46 min). Black mothers pumped fewer times daily than non-Black mothers after the first 2 weeks postpartum.

Conclusion: On average mothers pumped less intensively than the minimum recommendation of 8 times and 100 min per day. However, these pumping behaviors represent significant maternal opportunity costs that should be valued by the institution and society at large.

KEYWORDS

preterm infants, milk expression, health disparities, lactation, breast milk, mother's own milk, neonatal intensive care unit

1 Introduction

It is well established that mother's own milk (MOM) feedings from the infant's mother reduce the risk of prematurity related complications during and after the neonatal intensive care unit (NICU) hospitalization, including infections, rehospitalizations, and neurodevelopmental delay in very preterm (VPT, birth gestational age < 32 weeks) and very low birth weight (VLBW, birth weight < 1,500 g) infants (1–18). This risk reduction is attributed to the unique nutritional, immunomodulatory, anti-inflammatory, and epigenetic components of MOM that protect and stimulate the development of many body organs, enzymatic and metabolic pathways, and hormonal responses in the early post-birth period (19–31). Selected specific effects and components include: growth and development of the brain, gut, lungs, programming of growth, tolerance to antigens and induction of selective enzymes via complex complementary mechanisms such as the MOM/infant gut microbiome, oligosaccharides, and MOM-borne lipases, amylases, proteases, adipokines, extracellular vesicles and micro RNAs. These protective functions are especially important for the smallest, least mature infants due to their immunocompromised state. Furthermore, several studies reveal a dose–response relationship between the amount (dose) and duration (exposure period) of human milk (HM) received by the infant and the degree of protection from these acute and chronic illnesses as well as their short- and long-term costs (1, 8–12, 14, 15, 17, 18, 32).

In the US, VPT infants born to non-Hispanic Black (Black) mothers are significantly less likely to receive any MOM at time of NICU discharge when compared to non-Hispanic White (White) and Hispanic mothers (33–36). This disparity in duration of MOM provision directly impacts the ability of Black infants to receive MOM for the recommended duration to garner the many long-term health benefits associated with exclusive MOM feedings for the first 6 months of life and continued MOM feedings through 2 years of age (7, 37). These differences translate into a lifetime of health and economic advantages for MOM-fed versus formula-fed infants and their families.

Mothers of VPT infants encounter many challenges in providing MOM compared to mothers of healthy, term infants. These include preterm delivery, maternal illnesses such as preeclampsia, increased rates of cesarean delivery, stress of the NICU hospitalization, separation from their infant, and reliance on a breast pump to initiate and sustain MOM provision (38). Several studies have demonstrated the importance of pumping behaviors (e.g., daily minutes spent pumping, daily number of pumping sessions, inter-pump intervals) on establishing and sustaining MOM provision during the NICU hospitalization, including initiating milk expression within the first 6 h after birth (39–42), pumping during the early morning (43), and adequate pumping frequency, especially in the first 4–14 days postpartum (35, 39–45). Stimulation of the mammary gland by the infant or breast pump during the transition from secretory differentiation to secretory activation is critical, with programming effects on lactocytes (38, 39, 46). Recommendations for pump-dependent women include pumping 8–12 times daily, especially in the first 14 days postpartum (47, 48). Furthermore, studies suggest a minimum of 5–6 pumping sessions per day during the first 1–3 weeks postpartum is associated with establishing appropriate MOM volume, called coming to volume (CTV, pumping ≥500 mL/day MOM) by 14 days postpartum, or continued MOM provision to NICU discharge (35, 40, 41, 43, 49). A study of 415 mothers of VPT infants from 2008 to 2012 indicated that CTV was the most significant predictor of MOM provision at NICU discharge (odds ratio [OR] 7.46, 95% confidence interval [CI] 3.26 to 17.07, $p < 0.001$) (35). Furthermore, this study showed that differences in pumping frequency during the first 14 days postpartum mediated the Black-White (indirect effect: b = 0.40, 95% CI 0.26 to 0.57) and Black-Hispanic (indirect effect: b = 0.18, 95% CI 0.08 to 0.31) disparity in MOM feeding at NICU discharge with White and Hispanic mothers pumping more frequently than Black mothers. White mothers pumped an average of 5.2 times/day (standard deviation [sd] = 1.2), Hispanic mothers pumped 4.5 times/day (sd = 1.3) and Black mothers pumped 4.0 times/day (sd = 1.2) in the first 14 days (35).

To date, few studies have examined how frequently and when mothers of VPT infants actually pump and how pumping behaviors vary by maternal and infant characteristics (35, 39–41, 43, 49, 50). The aims of this study were to (1) examine the variation in pumping behaviors (i.e., average daily time spent pumping, average daily number of pumping sessions, percent of days with an early morning pumping session [between 1:00 a.m. and 4:59 a.m. (43)], and percent of days with at least 5 pumping sessions) by postpartum week, and (2) provide a descriptive comparison of pumping behaviors by maternal and infant characteristics, including maternal race/ethnicity, age at birth, and primary payment source and infant gestational age (GA).

2 Materials and methods

2.1 Study population

Data for this study were obtained from the ReDiMOM (**Re**ducing **Di**sparity in Receipt of **M**other's **O**wn **M**ilk in Very Low Birth Weight Infants) randomized controlled trial (RCT) at Rush University Medical Center enrolling adult mothers and their inborn VPT infants. Briefly, maternal inclusion criteria for the ReDiMOM RCT included age ≥ 18 years, fluent in English or Spanish, willing and able to share a valid Social Security number and planned to provide at least some MOM feedings. Infant inclusion criteria were < 32 0/7 weeks GA at birth, no significant congenital anomalies or chromosomal defects, and ≤ 144 h (6 days) of age at the time of enrollment. Therefore, mothers who planned to provide MOM were included in the analysis, regardless of the actual proportion of feedings that consisted of MOM. ReDiMOM tests the effectiveness and cost-effectiveness of a three-part intervention bundle designed to offset opportunity (i.e., time) and out-of-pocket costs of MOM provision, including a hospital-grade breast pump for use at home, transportation of pumped MOM from home to the NICU, and payment for time spent pumping (51). This study included pumping records for the first 75 mothers who completed study activities and documented at least one pumping session in the pumping log. Data included in this analysis were limited to pumping records for the first 10 weeks of life (WOL) or the infant's discharge from the NICU, whichever occurred first.

2.2 Measures

Mothers recorded each pumping session either on a paper log or in an electronic REDCap log, including the start and stop date and time of each pumping session. For paper logs, mothers either returned them to study staff or photographed the logs and uploaded the files to

a secure REDCap questionnaire. Study staff manually entered paper log data into an Excel spreadsheet and uploaded the spreadsheet into the study's REDCap database.

Duration of each pumping session was calculated in minutes by subtracting the start date and time from the end date and time. Pumping sessions that were missing the start time, end time or were for 0 min in duration ($n = 216$), greater than 75 min ($n = 138$), less than 0 min ($n = 153$), or included pumping dates prior to the infant's birth date ($n = 10$) were excluded, for a total of 3.6% of pumping records dropped from the analysis. Days without any pumping records that were after the first date of pumping but before discharge were assumed to have 0 min spent pumping and 0 pumping sessions. Overall, 33% of the subjects had pumping data for every day of their infant's NICU stay, after the first date of pumping. An additional 20% of subjects had ≤20% of NICU days with no pumping sessions, and 11% had 21–40% of NICU days with no pumping sessions (Supplementary Figure S1). A sensitivity analysis was conducted by excluding subjects from the calculations after they had discontinued pumping (last recorded pumping date), leaving only those subjects who were still pumping based on the pumping logs in the analysis.

Outcomes included average daily number of pumping sessions (i.e., frequency) by WOL, average daily time spent pumping (i.e., duration) by WOL, and other pumping behaviors, including the percent of days with at least 5 pumping sessions by WOL and percent of days with an early morning pumping session by WOL (43). For each WOL, average daily number of pumping sessions per week was calculated as $\sum_{i=1}^{n} number\ of\ pumping\ sessions_i / n$. Average daily time spent pumping was calculated as $\sum_{i=1}^{n} daily\ \min_i / n$ for each week where i = day and n = number of days in a week. The first week of pumping and the last week before discharge may have had fewer than 7 days of data. For the sensitivity analysis that included pumping records from the first to last date of pumping, the last week of pumping may have had fewer than 7 days of data. To calculate the proportion of days with at least 5 pumping sessions in each WOL, we classified each day as either having at least 5 pumping sessions or not and divided by the number of days in the week. To calculate the proportion of days with an early morning pumping session in each WOL, we classified each day by whether the mother had a pumping session that started between 1:00 a.m. and 4:59 a.m. (43) and divided the number of days in the week with an early morning pumping session by the total number of days in the week. The percent of days with at least 5 pumping sessions and percent of days with an early morning pumping session were calculated only for those subjects who were still pumping at each WOL ("while pumping"), excluding subjects that had discontinued pumping. Results are reported as medians of the average for each outcome.

Independent variables included maternal self-identified race/ethnicity (Black, Hispanic, White), age (<30 years versus ≥30 years), and primary payment source (Medicaid versus private insurance) and infant GA at birth (<28 weeks versus 28–31 weeks). Other variables included to describe the sample were delivery mode (vaginal versus cesarean delivery), 5-min Apgar Score, number of prior deliveries (none, 1, 2, 3 or more), whether the subject provided any MOM to previous children, and maternal goal for MOM provision at NICU discharge (reported at the time of study enrollment).

TABLE 1 Description of the sample, $N = 75$.

Characteristic	
Maternal race/ethnicity, *n* (%)	
Black	41 (54.7)
Hispanic	26 (34.7)
White	8 (10.7)
Maternal age, *n* (%)	
<30 years	33 (44.0)
30 years and older	42 (56.0)
Primary payment source, *n* (%)	
Commercial	34 (45.3)
Medicaid	41 (54.7)
Infant gestational age at birth, *n* (%)	
<28 weeks	33 (44.0)
28–31 weeks	42 (56.0)
Delivery mode, *n* (%)	
Vaginal	17 (22.7)
Cesarean delivery	58 (77.3)
5-Minute Apgar Score, median (IQR)	8 (6, 8)
Prior deliveries *n* (%)	
None	38 (50.7)
1	13 (17.3)
2	16 (21.3)
3 or more	8 (10.7)
Provided MOM to previous child(ren), *n* (%)*	28 (75.7)
Goal to provide exclusive MOM at NICU discharge, *n* (%)**	62 (83.8)

IQR, interquartile range (25th percentile, 75th percentile). IQR, interquartile range (25th percentile to 75th percentile), MOM, mother's own milk, NICU, neonatal intensive care unit. *Denominator is subjects with other children ($n = 37$); **$n = 74$.

2.3 Statistical analysis

Frequency distributions and medians (interquartile range [IQR]) were used to describe the sample and pumping behaviors by WOL. Heatmaps and line graphs were used to graphically display outcomes by WOL. Line graphs included four panels: (A) median of the average number of daily pumping sessions by WOL, (B) median of the average number of daily pumping minutes by WOL, (C) median of the average percent of days each week with at least 5 pumping sessions by subjects who continued to pump MOM by WOL (i.e., while pumping), and (D) median of the average percent of days each week with at least 1 early morning pumping session by subjects who continued to pump MOM by WOL (i.e., while pumping). Line graphs were created for all mothers and stratified by maternal race/ethnicity, maternal payment source, maternal age, and infant GA.

3 Results

3.1 Description of the sample

The sample included 75 mothers and 13,994 recorded pumping sessions, with 55% of the mothers Black, 35% Hispanic and 11% White (Table 1). There was no significant difference in the distribution of mothers by race/ethnicity and ReDiMOM

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TABLE 2 Maternal pumping patterns by week of life.

Week of Life	During NICU stay*			While pumping**		
	N	Average daily number of pumping sessions per mother median (IQR)	Average daily minutes per mother median (IQR)	*N*	Percent of days with at least 5 pumping sessions per mother median (IQR)	Percent of days with early morning pumping session per mother median (IQR)
1	70	3.6 (1.7, 4.8)	69 (36, 108)	70	31.0 (0, 75.0)	16.7 (0, 40.0)
2	74	3.9 (1.7, 5.6)	89 (36, 122)	68	35.7 (0, 85.7)	14.3 (0, 42.9)
3	75	3.7 (0.6, 6.0)	80 (13, 131)	65	42.9 (0, 100.0)	28.6 (0, 57.1)
4	74	3.4 (0.1, 5.7)	71 (3, 128)	60	42.9 (0, 100.0)	14.3 (0, 57.1)
5	74	3.1 (0, 5.6)	62 (0, 119)	54	35.7 (0, 100.0)	14.3 (0, 42.9)
6	72	2.8 (0, 5.4)	57 (0, 106)	49	57.1 (0, 100.0)	28.6 (0, 71.4)
7	67	2.4 (0, 5.5)	54 (0, 108)	43	57.1 (0, 100.0)	14.3 (0, 57.1)
8	59	2.3 (0, 5.0)	56 (0, 103)	36	42.9 (7.1, 100.0)	14.3 (0, 35.7)
9	54	2.0 (0, 5.4)	48 (0, 102)	34	35.7 (0, 100.0)	14.3 (0, 42.9)
10	49	2 (0, 4.8)	46 (0, 98)	29	33.3 (0, 100.0)	25.0 (0, 42.9)

IQR, interquartile range (25th, 75th percentiles). * *N* by week of life varies across the 10 weeks, due to variation in the first day with pumping data reported and infants discharged from the NICU in the first 10 weeks of life. Reported values include subjects that had stopped pumping, with 0 min entered for their data. ** *N* by week of life varies across the 10 weeks, due to variation in the first day with pumping data reported, subjects stopping pumping during the NICU stay, and infants discharged from the NICU in the first 10 weeks of life.

study arm ($p = 0.870$), with 56% of Black, 50% of Hispanic, and 50% of White mothers randomized to the intervention arm. Overall, 44% were < 30 years, 55% were insured by Medicaid, and 56% delivered an infant at 28–31 weeks GA. The average number of pumping sessions/day and pumping minutes/day peaked in WOL 2, with a median of 3.9 pumping sessions/day (IQR 1.7–5.6) and 89 pumping minutes/day (IQR 36–122), decreasing to 2.0 pumping sessions (IQR 0–4.8) and 46 pumping minutes/day (IQR 0–98) in WOL 10 (Table 2). The percentage of days with at least 5 pumping sessions peaked in WOL 6 and 7, with a median of 57.1% (IQR 0–100.0%), partially due to mothers with low pumping frequency discontinuing pumping and being dropped from the analysis. The average percent of days with an early morning pumping session was highest in WOL 3 and 6, with a median of 29%.

3.2 Overall pumping sessions, pumping duration and other pumping behaviors

Figure 1 demonstrates the medians of the average number of daily pumping sessions, average daily pumping durations, and other pumping behaviors by WOL. Panels A and B demonstrate the difference in the number of daily pumping sessions and duration based on utilizing data from the entire sample versus only subjects that continued to pump. A notable but not unexpected difference is evident, with relatively constant frequency and duration noted after week 2 when limiting the data to subjects who continued pumping compared to the steady decline noted when subjects who had discontinued pumping were also included in the data. Panels C and D display pumping patterns for subjects that continued to pump. Using data from subjects who continued to pump, we found a minority of subjects pumped at least 5 times per day in the first 5 weeks post-partum and a very small minority had at least 1 early morning pumping session.

Figure 2 displays a heatmap of the intensity of average daily pumping minutes by WOL by maternal race/ethnicity for all subjects, illustrating both inter-subject (i.e., between mother) and intra-subject (i.e., within mother, over time) variation in minutes/day across time. Figure 2 qualitatively shows greater inter-subject and intra-subject variation in pumping duration for Black mothers compared to Hispanic and White mothers. Supplementary Figure S2 includes heatmaps for the intensity of average number of daily pumping sessions, average daily pumping durations, and pumping patterns by WOL, illustrating greater inter-subject than intra-subject variation in all outcomes (Supplementary Table S1).

3.3 Pumping sessions, pumping duration and other pumping behaviors by maternal and infant characteristics

Figure 3 illustrates the medians of the average number of daily pumping sessions and average daily pumping durations (for the NICU stay), and other pumping behaviors by WOL and by maternal race/ethnicity for the entire sample. The average number of daily pumping sessions and pumping duration show similar patterns (panels A and B). The average number of daily pumping sessions increases between WOL 1 and 6 for White mothers and then remains relatively constant until WOL 10, except for a transient decline at WOL 5 (panel A). For Hispanic mothers, average daily pumping sessions remain relatively constant between WOL 1–6 and then decrease through WOL 10. For Black mothers, average number of daily pumping sessions remains relatively constant between WOL 1–3 and then decreases drastically to WOL 6, after which the median is 0 or discontinued pumping.

Figures 4–6 depict the medians of the average number of daily pumping sessions, average daily pumping durations and pumping patterns by WOL and by payment source, maternal age, and infant GA. The average number of daily pumping sessions remain consistently higher for mothers with commercial insurance compared

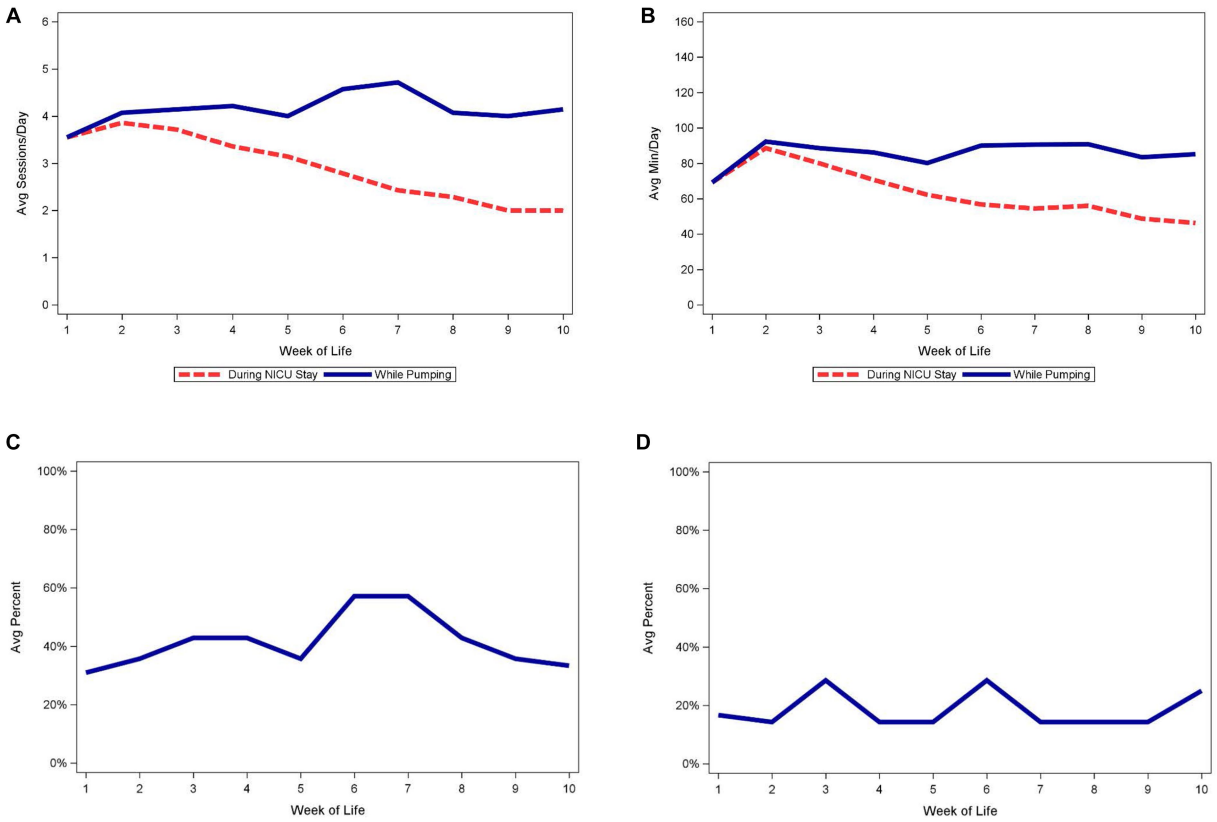

FIGURE 1
Median of average daily pumping sessions and pumping minutes by week of life. **(A)** Median of average daily pumping sessions. **(B)** Median of average daily pumping minutes. **(C)** Median of average percent of days with at least 5 pumping sessions, while pumping. **(D)** Median of average percent of days with at least 1 early morning pumping session, while pumping.

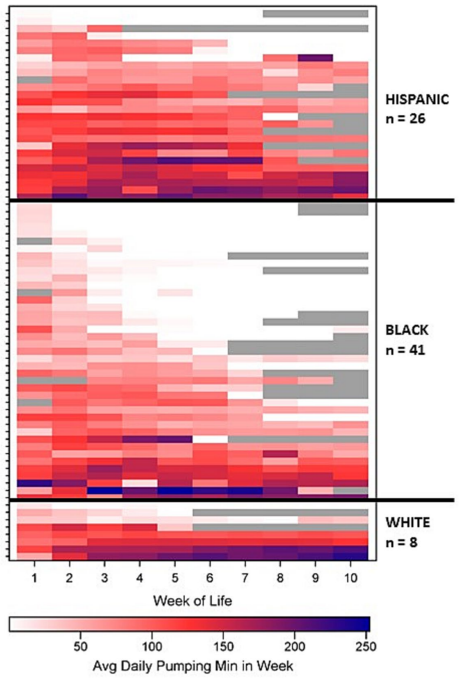

FIGURE 2
Average daily pumping minutes by week of life grouped by maternal race/ethnicity. Gray shading indicates weeks after discharge.

to those with Medicaid (Figure 4A), consistently higher for mothers aged 30 and older compared to mothers under age 30 (Figure 5A), and modestly higher for infants born 28–31 weeks GA compared to those with infants born <28 weeks GA (Figure 6A).

4 Discussion

For mothers of VPT infants, pumping frequency in the early weeks after delivery is positively correlated with later lactation outcomes, including achievement of CTV by postpartum day 14 (38, 43, 45), duration of MOM provision (35, 40, 51), receipt of MOM at NICU discharge and exclusive MOM provision in the NICU (42). Studies examining pumping behavior and lactation outcomes have inconsistently included race/ethnicity in analyses (35, 40, 43) despite disparities in MOM provision (33–36). Although these studies have demonstrated a disparity between White mothers and other mothers in achievement of CTV, or in the receipt of MOM at specific time points, such as postpartum day 14 or NICU discharge, no previous study has described detailed pumping behaviors for mothers of VPT infants, and whether or how these behaviors vary by maternal race/ethnicity and other demographic characteristics.

Our data demonstrate racial and ethnic differences in pumping behaviors, with Black mothers demonstrating shorter and less intensive pumping compared to White and Hispanic mothers. Although our previous research has demonstrated similar rates of MOM provision at

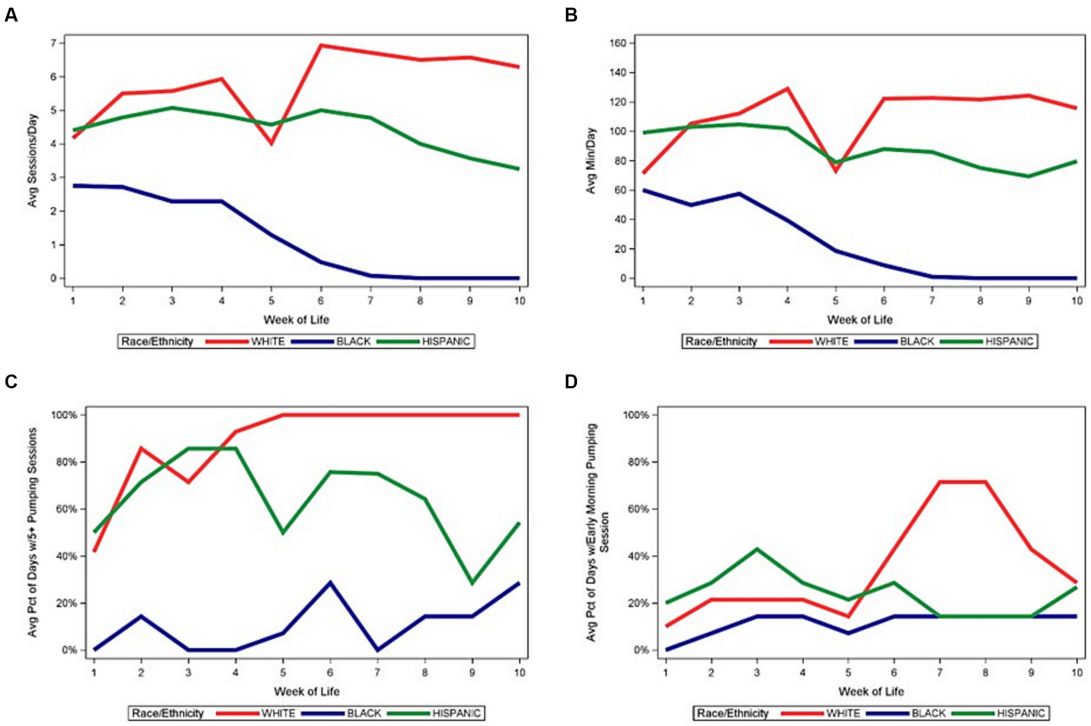

FIGURE 3
Median of pumping patterns by maternal race/ethnicity and week of life during NICU stay. **(A)** Median of average daily pumping sessions. **(B)** Median of average daily pumping minutes. **(C)** Median of average percent of days with at least 5 pumping sessions, while pumping. **(D)** Median of average percent of days with at least 1 early morning pumping session, while pumping.

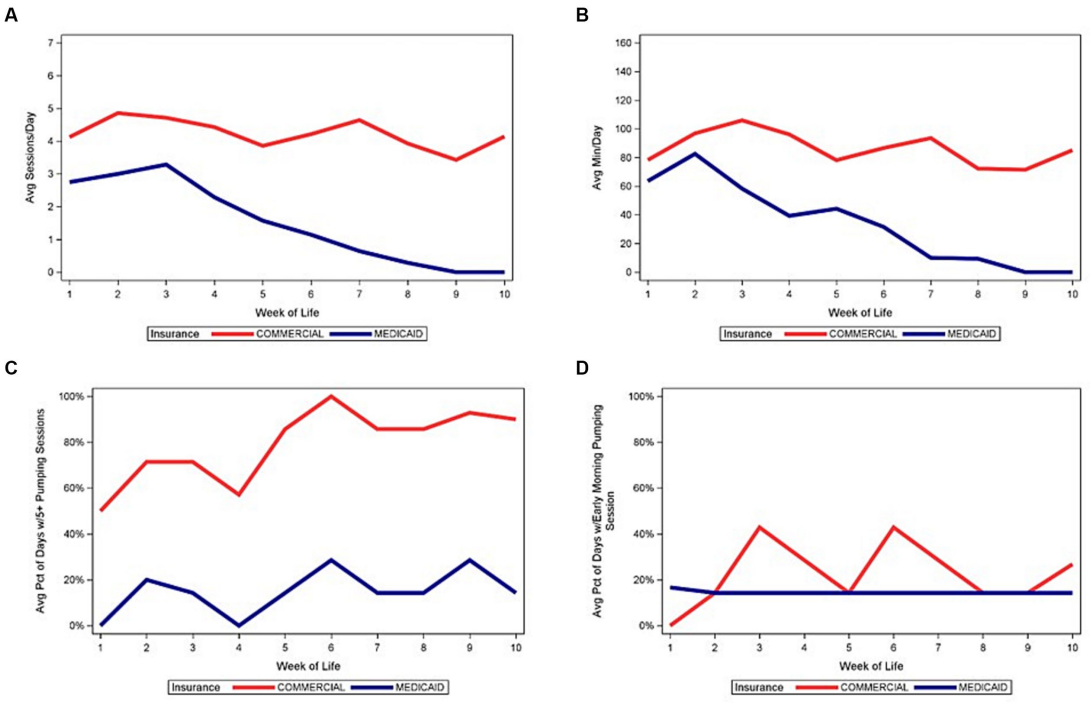

FIGURE 4
Median of pumping patterns by maternal insurance and week of life during NICU stay. **(A)** Median of average daily pumping sessions. **(B)** Median of average daily pumping minutes. **(C)** Median of average percent of days with at least 5 pumping sessions, while pumping. **(D)** Median of average percent of days with at least 1 early morning pumping session, while pumping.

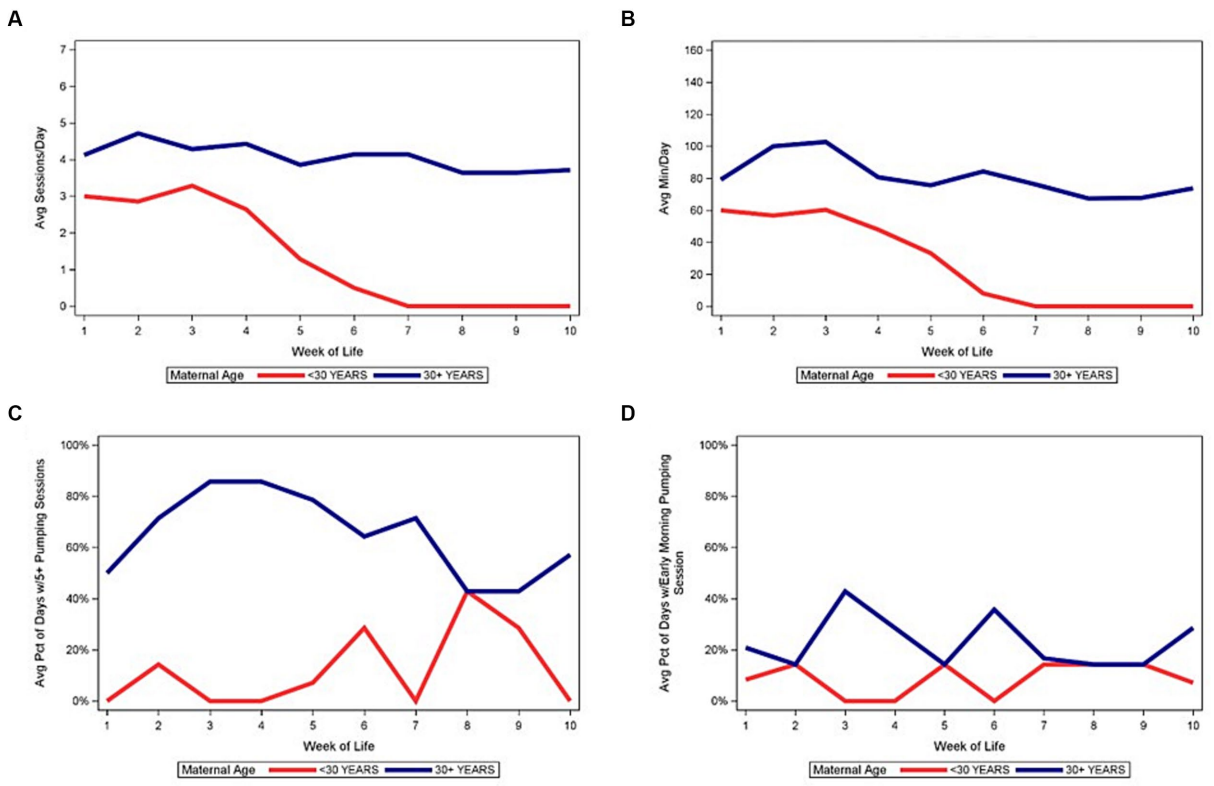

FIGURE 5
Median of pumping patterns by maternal age and week of life during NICU stay. **(A)** Median of average daily pumping sessions. **(B)** Median of average daily pumping minutes. **(C)** Median of average percent of days with at least 5 pumping sessions, while pumping. **(D)** Median of average percent of days with at least 1 early morning pumping session, while pumping.

NICU discharge by White and Hispanic mothers (35), the current data demonstrate that the pumping behavior between White and Hispanic mothers are initially similar but then diverge around 4–6 weeks postpartum. Mothers in this study were enrolled in the ReDiMOM trial, for which randomization was stratified by maternal race/ethnicity and infant GA (52). Therefore, despite the economic intervention available to the intervention arm, the three racial/ethnic groups were similarly distributed between the control and intervention study arms. We also noted differences associated with primary payment source, with greater pumping frequency and duration in commercially insured mothers. During the first 2 WOL, publicly and privately insured mothers pumped a similar number of times each day, but during WOL 2–10, privately insured mothers pumped significantly more frequently.

Intra-individual differences were also evident and widely variable, with the most common pattern consisting of a gradual decline in pumping frequency and duration with prolonged NICU hospitalization. A small minority of mothers were able to maintain a consistent pattern of pumping duration, and they were more likely to be White, aged 30 years or older, or those with commercial insurance. This gradual decline in pumping intensity is commonly seen in mothers of VPT due to challenges associated with prolonged pump-dependency. Researchers have repeatedly demonstrated the vital importance of frequent pumping in the first 1–2 weeks postpartum to program the mammary gland for later milk production, with animal studies demonstrating changes in gene expression and cellular and hormonal changes associated with milk expression frequency (35, 39, 42, 43, 45, 51, 53–56). In the early postpartum period, reductions in pumping frequency can negatively impact milk, with short-term changes in pumping frequency associated with changes in milk biomarkers. This lack of stability in mammary function during this critical early period heightens the importance of understanding pumping behaviors to develop actionable interventions.

Standard lactation recommendations given to mothers of VPT infants include pumping 8–12 times/day in the early postpartum period to mimic a healthy term newborn (47, 48, 57), although the actual minimum pumping frequency requirement is unknown (7). A recent study by Mago-Shah et al. suggested that pumping at least 5 times per day by day 5 and pumping once in the early morning were independently associated with CTV (43), which is in turn associated with continued MOM provision at NICU discharge (35, 58). This is aligned with findings from an observational study of mothers of infants <34 weeks GA by Lai et al. (59) demonstrated that short-term milk production rate decreased with inter-pump intervals ≥5 h and recommended ≥5 daily pumping sessions at regular intervals with a maximum inter-pump interval of 7 h (59). Mago-Shah et al. did not report inter-pump intervals, so it is unclear whether time of day (e.g., early morning) versus inter-pump interval is the primary determinant of subsequent MOM synthesis. Certainly, longer inter-pump intervals contribute to decreased MOM synthesis via the autocrine/paracrine regulatory pathways and the lack of frequent suckling-induced prolactin secretion from the pituitary (60, 61). While we did not routinely have pumping data available on day 5 due to the enrollment time frame through day 6 for ReDiMOM (52), we found that women were unlikely to pump in the early morning after week 1.

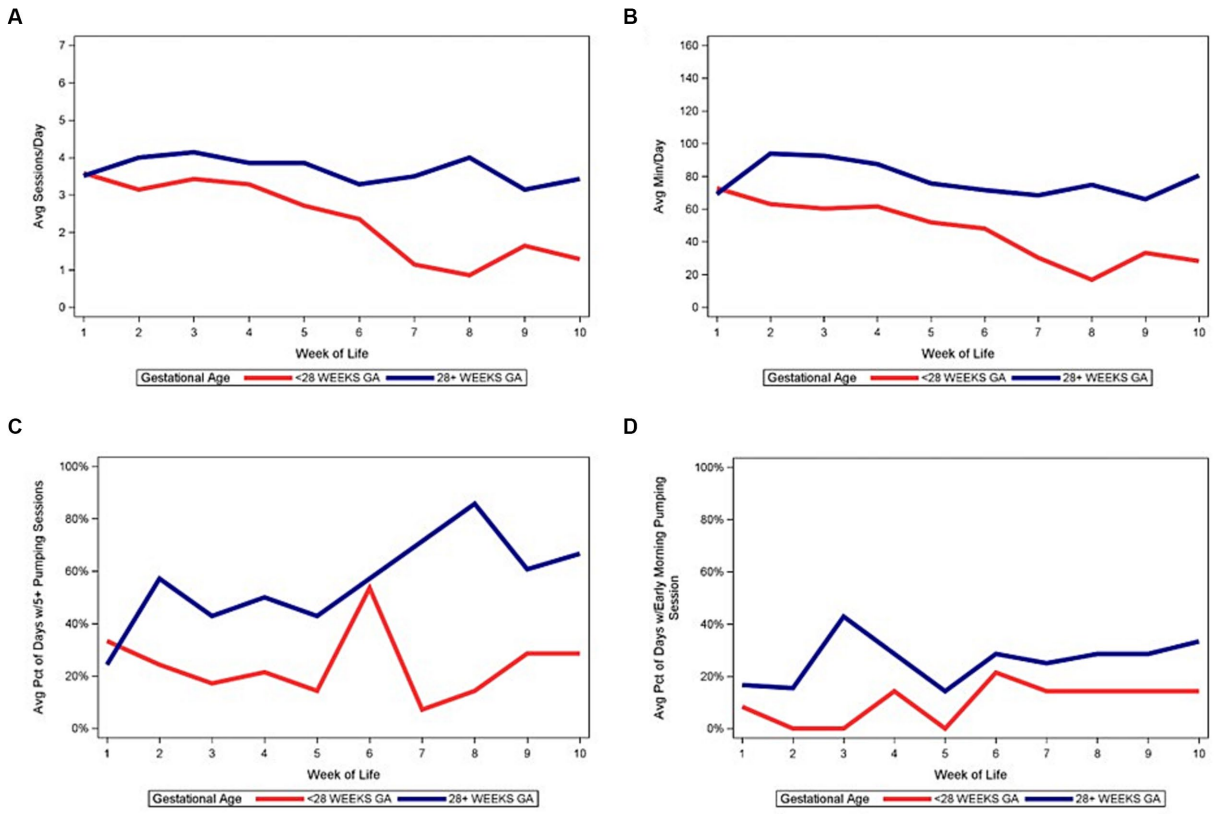

FIGURE 6
Median of pumping patterns by infant gestational age and week of life during NICU stay. **(A)** Median of average daily pumping sessions. **(B)** Median of average daily pumping minutes. **(C)** Median of average percent of days with at least 5 pumping sessions, while pumping. **(D)** Median of average percent of days with at least 1 early morning pumping session, while pumping.

We found dramatically different trajectories when examining daily pumping sessions and daily pumping minutes based on whether mothers who had stopped pumping were included versus excluded from analyzes. Although the number of daily pumping sessions were relatively consistent for mothers while pumping, the inclusion of mothers who stopped pumping illustrated their impact with a downward trajectory in the average number of pumping sessions and daily pumping minutes. Inclusion of mothers who *start* pumping overall versus inclusion of mothers *only while* they are pumping is an important methodological consideration in future research.

Our study has several strengths, including the detailed collection of daily pumping sessions through maternal pumping logs that minimized recall bias; our characterization of pumping patterns by WOL and by maternal race/ethnicity, insurance, age and infant GA, which have been demonstrated to be associated with MOM feeding duration during the NICU hospitalization; and our ability to investigate inter-subject versus intra-subject variation in pumping patterns over time. However, there are limitations that should also be considered. First, we relied on maternal pumping records rather than objective pumping data, however, most studies rely on self-report. ReDiMOM is collecting pumping data using a smart pump that enables electronic capture and storage of pumping behaviors for mothers in the intervention arm, so future analyses can compare the accuracy of maternal self-report with smart pump logs and milk weight data (52). Another limitation is variability in the availability of pumping data for the first week postpartum. Mothers were eligible for enrollment into ReDiMOM from prenatal hospitalization through 144 h (6 days) postpartum, therefore, pumping records may have been missing during the pre-enrollment period. Furthermore, data were not available for the time of first pumping, which could significantly influence lactation outcomes since Parker et al. showed in their randomized controlled trial that pumping initiation within the first 6 h postpartum was associated with greater MOM volume and duration in a similar population (39).

5 Conclusion

The data presented here provide valuable insights into pumping behaviors of a racially, ethnically, and socioeconomically diverse group of mothers of VPT infants. Taken together, the large variation in pumping behaviors by race/ethnicity and socioeconomic status (payment source as proxy) may contribute to disparity in MOM provision for VPT infants, which supports our previous findings that pumping behaviors mediate differences in MOM provision (35). The reasons underlying these disparities in pumping behaviors are unknown and likely multifactorial, with potential reasons including racial/ethnic differences in return-to-work timing, unpaid workload, lactation-supportive work conditions, maternal health conditions, cultural and familial support for MOM provision, and implicit and explicit biases that may impact

mothers in the NICU (33, 35, 47, 62–70). Measuring and acknowledging variation in pumping is a first step (71), which highlights the need to further understand barriers to pumping in order to develop appropriate interventions, education and quality improvement efforts.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors upon completion of the randomized controlled trial.

Ethics statement

The studies involving humans were approved by Rush University Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

AP: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. AT: Data curation, Writing – review & editing. AB: Data curation, Investigation, Writing – review & editing. JJ: Data curation, Investigation, Writing – review & editing. KM: Data curation, Investigation, Writing – review & editing. DM: Data curation, Investigation, Writing – review & editing. PM: Conceptualization, Investigation, Writing – review & editing. TJ: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

PM has received research support and honoraria from Medela.

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.2024.1278818/full#supplementary-material

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Household food insecurity is negatively associated with achievement of prenatal intentions to feed only breast milk in the first six months postpartum

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Background: Household food insecurity (HFI) has been associated with suboptimal breastfeeding practices. Postpartum factors reported by caregivers include stressful life circumstances and maternal diet quality concerns. It is unknown whether prenatal breast milk feeding intentions, a well-established predictor of breastfeeding outcomes, differ by HFI status. We explored associations between HFI and prenatal intentions to feed any and only breast milk in the first 6 months postpartum, and achievement of these intentions.

Methods: We utilized data from self-identified biological mothers with children 6–12 months of age who responded to a retrospective, cross-sectional online infant feeding survey conducted in Nova Scotia, Canada. HFI (yes/no) was assessed using the Household Food Security Survey Module. Prenatal intentions to feed any and only breast milk were assessed based on responses to five options for infant milk feeding plans. Achievement of intentions was assessed by breast milk and formula feeding practices in the first 6 months. Multivariable logistic regressions were conducted, adjusting for maternal socio-demographics.

Results: Among 459 respondents, 28% reported HFI; 88% intended to feed any breast milk and 77% intended to feed only breast milk, with no difference by HFI status. Of those intending to feed any breast milk, 99% succeeded, precluding further analysis. Among mothers who intended to provide only breast milk, only 51% achieved their intention, with lower odds among those with HFI (aOR 0.54, 95% CI 0.29–0.98).

Conclusion: HFI was not associated with intentions for feeding breast milk in the first 6 months postpartum, but mothers with HFI were less likely to achieve their intention to provide only breast milk. Further research is needed to understand the underlying reasons for this and to guide intervention designs to address HFI and help mothers reach their breastfeeding goals.

KEYWORDS

breastfeeding, infant feeding intentions, breastfeeding intentions, food insecurity, breastfeeding disparities, pregnancy, postpartum

Introduction

Household food insecurity (HFI), the insecure or inadequate access to food due to financial constraints, is a major public health concern as it represents broader material deprivation and is a strong determinant of physical and mental health for both children and adults (1–4). The most recent nationally representative Canadian data indicate that 18% of households in the ten provinces experienced food insecurity (5). This includes approximately 6.9 million individuals, including 1.8 million children under 18 years of age (5). Families with children are particularly vulnerable to food insecurity, with one in four Canadian children living in a food insecure household (5–7).

To optimize infant and maternal health and infant development, global infant feeding recommendations include exclusive breastfeeding for the first six months of life (8, 9). The most recent national Canadian data indicate that while 91% of infants initiated breastfeeding, only 35% exclusively breastfed to six months (10). As in other high-income countries, there are social disparities in breastfeeding as this behavior is negatively impacted by the social and structural determinants of health, including HFI (11–13). For instance, secondary data analysis from multiple cycles of the nationally representative, cross-sectional Canadian Community Health Survey found no difference in breastfeeding initiation based on HFI status, but respondents with HFI had lower odds of breastfeeding exclusively to four months (14). Qualitative studies reveal that mothers experiencing HFI may introduce formula or stop breastfeeding early because they are concerned about the quality and/or quantity of their breast milk due to their own inadequate dietary intake (15, 16). The perinatal period is an especially vulnerable time for some families in Canada due to increased expenses and interruptions in earnings; the national parental leave program is indexed to prior employment income up to a maximum of 55%, reducing overall household income, and low-income women may not qualify for parental leave benefits due to eligibility requirements (17, 18). The resultant stress associated with living in food insecure circumstances may negatively impact breastfeeding (16, 17, 19, 20).

Prenatal breastfeeding intentions are a strong and well-established predictor of breastfeeding outcomes (21–23). The practice of infant feeding includes mental processes and planning, but in the context of food insecurity attention has only been paid to actual postpartum feeding behaviors. To further understand the relationship between food insecurity and infant feeding and inform intervention designs, it is important to understand whether HFI status is associated with prenatal breastfeeding intentions and whether mothers experiencing HFI are able to reach their own breastfeeding goals. In this paper, we aimed to explore associations between HFI and 1) prenatal intentions to feed any breast milk in the first 6 months postpartum, 2) prenatal intentions to feed only breast milk in the first 6 months postpartum, and 3) achievement of these intentions.

Abbreviations: CI: Confidence interval; HFI: Household food insecurity; OR: Odds ratio.

Materials and methods

Study setting and participants

The current analysis used data from a retrospective, cross-sectional online infant feeding survey conducted within a larger multi-phased, mixed-methods study which aimed to better understand how food insecurity shapes how infants are fed. In the first phase, interviews were conducted with food insecure caregivers with children under 24 months to investigate how they navigate feeding their infants on a daily basis. Second, based on the interview findings and prior qualitative research, infant food insecurity indicators were created. A larger online survey was created including these infant food insecurity indicators alongside a variety of questions related to infant feeding practices, household food insecurity, and socio-demographic characteristics. Before the survey was launched in the third phase of research, it was pre-tested among 20 caregivers with a child under 24 months of age to ensure the questions were understandable and acceptable.

Any primary caregiver with at least one child between 0–24 months of age who was living in Nova Scotia, Canada was eligible to complete the survey. Primary caregivers were defined as those who were primarily responsible for caring, raising, and feeding the child, and participants selected their caregiving role (e.g., biological mother or father, adoptive mother or father, foster mother or father, grandparent, etc.). To ensure representation of food insecure families in the survey, targeted recruitment was conducted whereby postcards with the survey information were distributed to caregivers through the 25 Family Resource Centers across the province. Family Resource Centers are non-profit, community-based organizations that provide programming and services to families with minimal resources and who are negatively impacted by the social determinants of health. An electronic version of the study postcard was posted in Family Resource Center Facebook groups, when available. For general recruitment, electronic postcards were also posted online through paid Facebook advertisements and in approximately 10 relevant Facebook groups for families of young children in Nova Scotia.

The sample for the current analysis was drawn from respondents who identified their caregiving role as the biological mother. The Household Food Security Survey Module, used to assess HFI status and described in more detail below, has a 12-month recall period; therefore, only survey respondents with infants 6–12 months of age were included in the analytic sample to ensure HFI status reflected the infant feeding period of interest (e.g., the first six months postpartum). Data were excluded from participants with no HFI data, multiple births, or preterm birth.

All surveys were completed between January and April 2022. Participants provided consent online prior to starting the survey.

Data collection and measures

The survey was conducted using the Acadia University Survey System platform and was self-administered; therefore, all data were self-reported by participants.

Participants completed the validated 18-item Household Food Security Survey Module, which assesses the presence or absence of household food insecurity as well as the severity (none [secure],

marginal, moderate, or severe) based on the number of affirmative responses (24). All questions within the Household Food Security Survey Module have a 12-month recall period.

Participants were asked about their prenatal plan for feeding their child in the first six months postpartum (breast milk only, fed directly at the breast; breast milk only, some amount of pumping; mixed feeding of breast milk and formula; formula only; or no infant feeding intention). Participants reporting any of the first three of these choices were classified as intending to feed any breast milk. Participants reporting either of the first two choices were classified as intending to feed only breast milk.

Among participants who intended to feed any amount of breast milk, achievement of this intention was determined by an affirmative response to the question assessing breastfeeding initiation, "Was the child ever breastfed or given breast milk?." Among those who intended to feed only breast milk, achievement of this intention was determined by the participant reporting initiating breastfeeding and not introducing formula before their infant was six months of age.

Among participants classified as intending to feed only breast milk, prenatal intended mode of breast milk delivery (e.g., only at the breast or some amount of pumping) was also assessed since pumping early in the postpartum period has been associated with early cessation of any and exclusive breast milk feeding (25–27). The survey did not collect data on actual mode of breast milk delivery postpartum.

The assessment of feeding intentions and their achievement was only related to milk feeds (e.g., breast milk and formula), consistent with other literature on breast milk feeding intentions (28, 29). Therefore, data on feeding only breast milk cannot be interpreted as following the World Health Organization definition of exclusive breastfeeding, which requires consideration of all types of feeds (9).

Socio-demographic characteristics included as confounders based on availability from the survey and considered to be associated with both HFI and breastfeeding intentions/practices included single parenting (yes, no), highest level of completed education (high school or less, postsecondary), geographic location (urban, rural), parity (primiparous, multiparous), annual household income before tax (low [<$10,000–$39,999], medium [$40,000–$79,999], high [$80,000-≥$150,000]), and age (19–27, 28–36, 37–43 years) (5, 10, 11, 30–32).

Statistical analysis

Frequencies and percentages were calculated for socio-demographic characteristics, breast milk feeding intentions, and achievement of intentions. Chi-square tests were conducted to assess sociodemographic differences by HFI status.

Logistic regression analyses were conducted to investigate the associations between HFI status as the independent variable and three separate outcome variables: (1) intention to feed any breast milk; (2) intention to feed only breast milk; and (3) achievement of intention to feed only breast milk. Almost every participant (99%) with prenatal intention to feed any breastmilk achieved this, so no further analysis was conducted for this outcome. Two regression models were run for each of the three outcome variables: an unadjusted model and a model adjusted for the abovementioned socio-demographic characteristics. Results are presented as odds ratios (OR), 95% confidence intervals (CI), and p values. A p value <0.05 was considered statistically significant.

Small sample sizes within each HFI category (marginal, moderate, or severe) precluded analysis of socio-demographic characteristics, breast milk feeding intentions, and achievement of intentions by severity of HFI.

Frequencies and percentages were calculated, and a chi square test was performed, to assess achievement of intention to feed only breast milk by prenatal intended mode of breast milk delivery (e.g., only at the breast or some amount of pumping). For the outcome variable 'achievement of intention to feed only breast milk', an additional logistic regression model was conducted including prenatal intended mode of breast milk delivery as well as all socio-demographic characteristics.

Statistical multicollinearity among all independent variables was assessed prior to modeling using variance inflation factor (>2.5) and none was identified. Goodness of fit for each multivariable model was assessed using the Hosmer Lemeshow test ($p > 0.05$). IBM SPSS Statistics for Windows, Version 29 (IBM Corp, Armonk, New York, USA) was used to perform all analyses.

Results

Study participants

Overall, 525 survey respondents identified as biological mothers and had infants 6–12 months of age (Figure 1). Of these, 66 were excluded due to missing HFI data (n = 24), multiple births (n = 13), and preterm birth (n = 29). For excluded participants, there was no difference based on HFI status for multiple births, but respondents with HFI were more likely to have preterm births (Supplementary Table S1). In total, 459 participants were included in the study.

The prevalence of HFI was 28% (Table 1). Few participants were single parenting (7%), half were primiparous (51%), and almost half had annual household incomes less than $80,000 (44%). All socio-demographic characteristics differed by HFI status ($p < 0.05$), except for geographic location. For example, a higher proportion of HFI respondents were single parents, had high school education or less, were multiparous, had lower household incomes, and were younger in age.

Prenatal breast milk feeding intentions

Overall, 88% of participants intended to feed any breast milk (Table 2). Among food secure participants, 91% intended to feed any breast milk compared to 80% of those who were food insecure. Seventy-seven percent of all participants intended to feed only breast milk, with 80% of food secure participants intending to do so versus 69% of food insecure participants.

In unadjusted analyses (Table 3), food insecure participants had lower odds of intending to feed any breast milk (OR 0.42, 95% CI 0.24–0.75) and only breast milk (OR 0.55, 95% CI 0.35–0.87) compared to food secure participants. However, HFI status was no longer statistically significant in the adjusted models, in which household income was the only statistically significant predictor of intention to feed any and only breast milk. Compared to participants with medium household incomes, those with low incomes had 54%

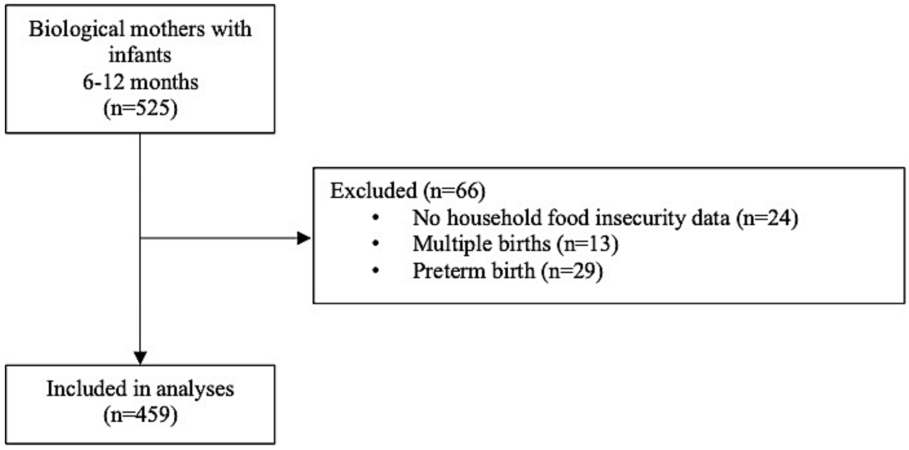

FIGURE 1
Participant flow diagram.

TABLE 1 Maternal characteristics of the study sample.

Characteristic	Total sample *n* (%)	Food secure *n* (%)	Food insecure *n* (%)	*p* value[a]
Household food insecurity status (n = 459)				
Food insecure	127 (27.7)	N/A		
Marginal	45 (9.8)			
Moderate	55 (12.0)			
Severe	27 (5.9)			
Food secure	332 (72.3)			
Education (n = 430)				
High school education or less	63 (14.7)	35 (11.1)	28 (24.1)	**<0.001**
Postsecondary education	367 (85.3)	279 (88.9)	88 (75.9)	
Parity (n = 430)				
Primiparity	218 (50.7)	173 (55.1)	45 (38.8)	**0.003**
Multiparity	212 (49.3)	141 (44.9)	71 (61.2)	
Single parent (n = 428)				
Yes	29 (6.8)	10 (3.2)	19 (16.7)	**<0.001**
No	399 (93.2)	304 (96.8)	95 (83.3)	
Geographic location (n = 412)				
Rural	131 (31.8)	94 (30.6)	37 (35.2)	0.380
Urban	281 (68.2)	213 (69.4)	68 (64.8)	
Household income (n = 425)				
Low (<$10,000 to $39,999)	69 (16.2)	25 (8.0)	44 (38.6)	**<0.001**
Medium ($40,000 to $79,999)	119 (28.0)	75 (24.1)	44 (38.6)	
High ($80,000 to ≥$150,000)	237 (55.8)	211 (67.8)	26 (22.8)	
Age in years (n = 421)				
19–27	84 (20.0)	52 (16.8)	32 (28.6)	**0.026**
28–36	288 (68.4)	221 (71.5)	67 (59.8)	
37–43	49 (11.6)	36 (11.7)	13 (11.6)	

[a]Pearson chi square test.
Denominators differ for each sociodemographic characteristic due to missing data.

TABLE 2 Breast milk feeding intentions in the first six months postpartum by household food insecurity status.

	Total ($N = 459$) n (%)	Food secure ($N = 332$) n (%)	Food insecure ($N = 127$) n (%)	p value[a]
Intention to feed any breast milk				
Yes	403 (87.8)	301 (90.7)	102 (80.3)	**0.002**
No	56 (12.2)	31 (9.3)	25 (19.7)	
Intention to feed only breast milk				
Yes	352 (76.7)	265 (79.8)	87 (68.5)	**0.010**
No	107 (23.3)	67 (20.2)	40 (31.5)	

[a]Pearson chi square test.

lower odds of intending to feed only breast milk (95% CI 0.22–0.97). Participants with high incomes had 3 times higher odds of intending to feed any breast milk (95% CI 1.43–7.48) and almost 2 times higher odds of intending to feed only breast milk (95% CI 1.10–3.53) than those with medium incomes.

The proportion of participants in each of the five prenatal infant feeding intention categories and differences by HFI status are reported in Supplementary Table S2. Among all participants, a higher proportion of food insecure compared to food secure participants had no prenatal infant feeding plan ($p = 0.013$), while a higher proportion of food secure compared to food insecure participants intended to provide breast milk only with some amount of pumped milk ($p = 0.043$). Among the subset of participants who intended to feed only breast milk, there was no difference in the intended mode of breast milk delivery (e.g., at the breast vs. some amount of pumping) by HFI status (Supplementary Table S3).

Achievement of breast milk feeding intentions

Among those who intended to feed any breast milk, 99% achieved their intention by initiating breast milk feeding (Table 4). Among participants who intended to feed only breast milk, 51% achieved this intention in the first six months postpartum; 49% of participants provided both breast milk and formula in the first six months postpartum, and 1% provided only formula (Table 4). Among food secure participants, 52% achieved their intention to feed only breast milk versus 48% of food insecure participants.

There was no association between HFI status and achievement of intention to feed only breast milk in the unadjusted logistic regression model (Table 5). However, in the model adjusted for socio-demographic characteristics, food insecure participants had lower odds of achieving this intention (OR 0.54, 95% CI 0.29–0.98) compared to food secure participants. Parity was also statistically significant; compared to primiparous participants, those who were multiparous had higher odds of achieving their intention to provide only breast milk (OR 2.13, 95% CI 1.31–3.44).

When considering intended mode of breast milk delivery, a higher proportion of participants who achieved their intention to feed only breast milk intended to feed only at the breast versus providing some amount of pumped milk ($p < 0.001$; Supplementary Table S4). When investigating the association between HFI status and achievement of intention to feed only breast milk while adjusting for prenatal intended mode of breast milk delivery as well as all socio-demographic characteristics, the relationship was strengthened (OR 0.46, 95% CI 0.24–0.87; Supplementary Table S5). In addition to parity remaining statistically significant, participants who planned to provide some amount of pumped milk had lower odds of achieving their intention to provide only breast milk (OR 0.25, 95% CI 0.15–0.41) compared to those who planned to only feed at the breast.

Discussion

In this study, we investigated associations between HFI and prenatal intentions to feed any and only breast milk in the first six months postpartum, and achievement of these intentions. We found no difference in breast milk feeding intentions by HFI status. Achievement of intentions was very high for providing any breast milk, but only 51% for providing only breast milk, with lower odds among participants with HFI.

To our knowledge, this is the first study to investigate associations between HFI, prenatal breastfeeding intentions, and achievement of intentions. Breast milk feeding intentions among the group of mothers in this study are consistent with the breastfeeding initiation rate for the Atlantic region of Canada (81%), of which Nova Scotia is a part (10). No national data are collected on breastfeeding intentions; therefore, it is unknown how intentions from this cohort compare to the Atlantic region or to Canada as a whole. Since breastfeeding intentions are modifiable and are associated with later breastfeeding practices, it would be beneficial for national and cohort studies in Canada to collect intentions as part of their infant feeding data (33, 34). This would allow for the investigation of associations between relevant social determinants of health and breast milk feeding intentions to further understand the relationship between prenatal intentions and the social disparities in Canadian breastfeeding practices (10, 11).

In the current study, there was no difference in prenatal intentions based on HFI status. This suggests that postpartum experiences (which include the interplay of individual, interpersonal, community, and societal factors) may contribute to our finding of lower odds of achieving intentions to feed only breast milk, and the lower breastfeeding rates that several others have found among those who are food insecure (12, 14, 19, 35, 36). This merits exploration in future studies, alongside the role that household income may have on prenatal breastfeeding intentions, regardless of HFI status, since income was found to be the only predictor of intentions in adjusted analyses.

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TABLE 3 Logistic regression results: associations between household food insecurity status and breast milk feeding intentions in the first six months postpartum.

	Intention to feed any breastmilk				Intention to feed only breastmilk			
	Unadjusted OR (95% CI)[a]	*p* value	Model 1 OR (95% CI)[b,c,d]	*p* value	Unadjusted OR (95% CI)[a]	*p* value	Model 1 OR (95% CI)[b,c,e]	*p* value
Household food insecurity status								
Food secure	1.00 (ref)	**0.003**	1.00 (ref)	0.492	1.00 (ref)	**0.011**	1.00 (ref)	0.512
Food insecure	0.42 (0.24–0.75)		0.77 (0.37–1.62)		0.55 (0.35–0.87)		0.82 (0.45–1.48)	
Education								
Postsecondary graduation	N/A	N/A	1.00 (ref)	0.537	N/A	N/A	1.00 (ref)	0.165
High school graduation or less	N/A		1.34 (0.53–3.37)		N/A		1.75 (0.80–3.85)	
Parity								
Primiparity	N/A	N/A	1.00 (ref)	0.273	N/A	N/A	1.00 (ref)	0.271
Multiparity	N/A		0.67 (0.33–1.37)		N/A		0.75 (0.45–1.25)	
Single parent								
No	N/A	N/A	1.00 (ref)	0.176	N/A	N/A	1.00 (ref)	0.254
Yes	N/A		2.57 (0.66–10.14)		N/A		1.87 (0.64–5.46)	
Geographic location								
Urban	N/A	N/A	1.00 (ref)	0.600	N/A	N/A	1.00 (ref)	0.782
Rural	N/A		0.83 (0.41–1.67)		N/A		1.08 (0.63–1.85)	
Household income								
Low (<$10,000 to $39,999) vs. Medium ($40,000 to $79,999) [ref]	N/A	N/A	0.46 (0.20–1.13)	**<0.001**	N/A	N/A	0.46 (0.22–0.97)	**0.001**
High ($80,000 to ≥$150,000) vs. Medium ($40,000 to $79,999) [ref]	N/A		3.27 (1.43–7.48)		N/A		1.97 (1.10–3.53)	
Low (<$10,000 to $39,999) vs. High ($80,000 to ≥$150,000)	N/A		0.15 (0.05–0.40)		N/A		0.23 (0.11–0.51)	
Age in years								
28–36	N/A	N/A	1.00 (ref)	0.192	N/A	N/A	1.00 (ref)	0.263
19–27	N/A		0.48 (0.22–1.06)		N/A		0.70 (0.36–1.34)	
37–43	N/A		0.89 (0.30–2.63)		N/A		0.60 (0.29–1.23)	

[a]Sample size for unadjusted models $n = 459$.
[b]Sample size for adjusted models $n = 399$.
[c]Models adjusted for maternal education, parity, single parenting, geographic location, household income, and age.
[d]Hosmer and Lemeshow $p = 0.030$.
[e]Hosmer and Lemeshow $p = 0.659$.

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TABLE 4 Achievement of intention to feed any and only breast milk in the first six months postpartum.

	Intended to feed any breast milk		Intended to feed only breast milk			
	Achieved intention	Did not achieve intention		Achieved intention	Did not achieve intention	
	Initiated breast milk feeding *n* (%)	Did not initiate breast milk feeding *n* (%)		Only fed breast milk *n* (%)	Breast milk and formula *n* (%)	Only formula *n* (%)
Total (N = 403)	398 (98.8)	5 (1.2)	Total (N = 352)	178 (50.6)	171 (48.6)	3 (0.8)
Food Secure (N = 301)	297 (98.7)	4 (1.3)	Food Secure (N = 265)	137 (51.7)	126 (47.5)	2 (0.8)
Food Insecure (N = 102)	101 (99.0)	1 (1.0)	Food Insecure (N = 87)	41 (47.1)	45 (51.7)	1 (1.1)

TABLE 5 Logistic regression results: association between household food insecurity status and achievement of intention to feed only breastmilk in the first six months postpartum.

	Achievement of intention to feed only breastmilk for first 6 months postpartum			
	Unadjusted OR (95% CI)[a]	*p* value	Model 1[b,c,d]	*p* value
Household food insecurity status				
Food secure	1.00 (ref)	0.460	1.00 (ref)	**0.043**
Food insecure	0.83 (0.51–1.35)		0.54 (0.29–0.98)	
Education				
Postsecondary graduation	N/A	N/A	1.00 (ref)	0.811
High school graduation or less	N/A		0.91 (0.43–1.93)	
Parity				
Primiparity	N/A	N/A	1.00 (ref)	**0.002**
Multiparity	N/A		2.13 (1.31–3.44)	
Single parenting				
No	N/A	N/A	1.00 (ref)	0.431
Yes	N/A		0.62 (0.18–2.06)	
Geographic location				
Urban	N/A	N/A	1.00 (ref)	0.259
Rural	N/A		0.75 (0.45–1.24)	
Household income				
Low (<$10,000 to $39,999) vs. Medium ($40,000 to $79,999) [ref]	N/A	N/A	0.65 (0.27–1.60)	0.382
High ($80,000 to ≥$150,000) vs. Medium ($40,000 to $79,999) [ref]	N/A		0.70 (0.40–1.22)	
Low (<$10,000 to $39,999) vs. High ($80,000 to ≥$150,000)	N/A		0.94 (0.38–2.31)	
Age in years				
28–36	N/A	N/A	1.00 (ref)	0.509
19–27	N/A		1.38 (0.72–2.66)	
37–43	N/A		0.81 (0.38–1.74)	

[a]Sample size for unadjusted model $n = 352$.
[b]Sample size for adjusted models $n = 311$.
[c]Model adjusted for maternal education, parity, geographic location, household income, single parenting, and age.
[d]Hosmer Lemeshow test $p = 0.478$.

It is encouraging that almost every participant in this study who intended to feed any breast milk achieved this by initiating breast milk feeding. It is concerning that overall there was a lack of attainment of intention to feed only breast milk, as only half of the participants who wanted to provide only breast milk for the first six months postpartum were able to do so. These findings align with previous evidence that a

high proportion of women across the socio-economic spectrum do not meet their breastfeeding goals and introduce other foods or stop breastfeeding earlier than they want to (28, 32, 37, 38). This underscores the need to strengthen support for all women so that they can achieve their breastfeeding goals, including those who are food insecure (39).

Among mothers with HFI in our study, there was an increased likelihood of not meeting intentions to feed only breast milk. We did not assess reasons for this, but it would be important for future studies to investigate whether the challenges among food insecure women align with those reported in the general population. Although information on breastfeeding intentions or meeting intentions is not available in the Canadian Community Health Survey, our results are consistent with analyses from this nationally-representative dataset which indicate that mothers with HFI were no less likely to start breastfeeding than those without HFI, but they were more likely to stop exclusively breastfeeding earlier (14). Together, these findings reinforce the breastfeeding paradox, that families who can least afford infant formula are the most likely to use it, and highlight that HFI may be an important and underrecognized social determinant of infant feeding (40).

Our finding that HFI was associated with lower odds of achieving intentions to feed only breast milk has potential implications for food insecure families, and for infant food insecurity specifically. Infant food insecurity refers to infant vulnerability with respect to food access, sub-optimal quality, and inadequate quantity due to household financial constraints (16). Non-exclusive breast milk feeding requires infants to receive infant formula, which is an insecure food system for low-income families due to its high cost (15, 16, 20). Qualitative research has found that breastfeeding can be a food security measure for low-income families so that they do not have to worry about purchasing infant formula, but breastfeeding can also be an insecure food system since mothers themselves perceive that the amount and quality of their breast milk is inadequate due to their own poor dietary intake (15, 16, 19, 41–43). To our knowledge, the relationship between maternal or household food insecurity and breast milk volume or composition has not been studied in high-income countries. Research in Nova Scotia found that income assistance and maternity benefits based on minimum wage earnings would not allow families to purchase a basic nutritious diet regardless if their infant was exclusively formula-fed or breastfed, compromising the nutrition of the entire household (17). In the Canadian province of Manitoba, a modest unconditional prenatal income supplement for low-income women was associated with improved breastfeeding initiation, but longer-term breastfeeding outcomes were not assessed and the income supplement did not continue in the postpartum period (44, 45). Thus, there are potential opportunities to reduce breastfeeding disparities and support the health and nutrition of families with young children through improved income supports.

As part of our exploratory analyses, we found that prenatal intended mode of breast milk delivery was an independent predictor of meeting intentions to feed only breast milk. Specifically, intentions to feed some amount of pumped milk compared with feeding only at the breast were associated with lower odds of achieving intentions to provide only breast milk. There is limited literature on pumping intentions, but studies suggest that anticipation of breastfeeding difficulties and concerns about breast milk supply are two main concerns associated with plans to pump and early pump use, which aligns with evidence that pumped/expressed breast milk feeding practices are negatively associated with longer-term breastfeeding outcomes (26, 27, 46–48). As the use of expressed breast milk may reflect lactation difficulties and reduced breastfeeding self-efficacy, this reinforces global guidance that skilled lactation support be available and accessible to all as a standard of care in the early postpartum period (26, 46, 49). We did not collect data on breastfeeding self-efficacy (50) or actual mode of breast milk delivery postpartum, therefore further research is needed to understand associations between pumping intentions, practices, and achievement of breastfeeding goals, and the role of breastfeeding self-efficacy.

Strengths of this study include successful targeted recruitment to ensure representation of food insecure caregivers in the study. The prevalence of HFI in this study (28%) was slightly higher than the national rate for households with children (20%) (5). This enabled investigation of differences in breast milk feeding intentions and achievement of intentions by HFI status. However, our sample was insufficient to support an analysis of breast milk feeding intentions and achievements by severity of HFI. Other strengths include the use of the validated 18-item Household Food Security Survey Model and detailed data collection on the provision of breast milk and formula. The retrospective nature of the survey may have introduced recall bias regarding timing of the introduction of formula, but we used all available data on formula use and the median infant age at survey completions was 9 months. There are additional limitations of this work to consider. First, we did not ask participants at what point in their pregnancy they developed their infant feeding intentions, therefore, HFI status and prenatal intentions may not be concurrent. However, all participants in the analytic sample had infants 6–12 months of age (the median infant age at survey completion was 9 months, as mentioned above) and the Household Food Security Module had a 12-month recall period. As such, a portion of the prenatal period would be captured in most participants' food insecurity responses. Additionally, a recent Canadian study that collected HFI in the pre and postnatal period found that the HFI status of 80% of participants remained the same over both time periods (51). Second, although the survey was pre-tested to ensure questions were understandable and acceptable, the survey was self-administered, therefore, participants could not ask for clarity or more detail on survey questions. Third, as data were self-reported, there is the risk of social desirability bias. Fourth, the survey was only available online and in English, which could have precluded participation for some caregivers. Fifth, the survey was part of exploratory research to better understand how food insecurity shapes how infants are fed. As such, there was a relatively small sample size for multivariable analyses. In addition, the results are based on a convenience sample and are not population-based and not generalizable beyond groups similar to the study sample.

Conclusion

In summary, HFI was not associated with intentions for feeding breast milk in the first 6 months postpartum, but mothers with HFI were less likely to achieve their intention to provide only breast milk. This suggests that mothers experiencing HFI encounter additional challenges that impede exclusive breastfeeding and that differences in achievement of breast milk feeding intentions between food insecure and food secure households may contribute to breastfeeding disparities. Further research is needed to understand the underlying reasons for these differences and to guide intervention designs to address HFI and help mothers reach their breastfeeding goals.

Data availability statement

The datasets presented in this article are not readily available because of participant confidentiality and privacy. Requests to access the datasets should be directed to LF, lesley.frank@acadiau.ca.

Ethics statement

The studies involving humans were approved by Acadia University Research Ethics Board (19-35) and the University Research Ethics Board at Mount Saint Vincent University (2019-067). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. AM: Formal analysis, Writing – review & editing, Methodology. VT: Methodology, Writing – review & editing, Formal analysis. LF: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing, Formal analysis.

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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.2024.1287347/full#supplementary-material

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Associations of maternal inflammatory states with human milk composition in mothers of preterm infants

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Introduction: Overweight/obesity (ow/ob) is increasing in prevalence in pregnant women, and it is associated with other pro-inflammatory states, such as pre-eclampsia, gestational diabetes, and preterm labor. Data are lacking if mothers experiencing inflammatory states who deliver preterm have mother's own milk (MOM) with differing inflammatory markers or pro-inflammatory fatty acid (FA) profiles.

Methods: The aim was to explore associations of maternal pre- and perinatal inflammatory states with levels of inflammatory markers and/or FAs in longitudinal samples of MOM from mothers of preterm infants born <1,250 g. Inflammatory states included pre-pregnancy ow/ob, diabetes, chorioamnionitis (chorio), preterm labor (PTL), premature rupture of membranes (PROM), pre-eclampsia, and cesarian delivery. In MOM, inflammatory markers studied included c-reactive protein (CRP), free choline, IFN-Ɣ, IL-10, IL-1β, IL-1ra, IL-6, IL-8, and TNF-α, and FAs included omega-6:omega-3 ratio, arachidonic acid, docosahexaenoic acid, linoleic acid, monounsaturated FAs, and saturated FAs. The above inflammatory states were assessed individually, and the healthiest mothers (normal BMI, no chorio, and ± no pre-eclampsia) were grouped. Regression analysis tested associations at baseline (day 5) and over time using generalized estimating equations.

Results: A total of 92 infants were included who were delivered to mothers (42% ow/ob) at a median gestational age of 27.7 weeks and birth weight of 850 g. MOM CRP was 116% higher (relative change 2.16) in mothers with ow/ob at baseline than others ($p = 0.01$), and lower (relative change 0.46, 0.33, respectively) in mothers in the two "healthy groups" at baseline (both $p < 0.05$) than others. MOM IL-8 levels were lower with chorio and PTL at baseline. No significant associations were found for other individual or grouped inflammatory states nor for other MOM inflammatory markers nor FA profiles at baseline.

Discussion: In conclusion, MOM CRP levels are positively associated with inflammatory states, such as ow/ob. Reassuringly, there was no association between FA profiles or most other inflammatory markers and maternal inflammatory states. Further studies are needed to determine potential associations or ramifications of MOM CRP in vulnerable preterm infants.

KEYWORDS

human milk, human milk cytokines, preterm infant, maternal obesity, maternal inflammation during pregnancy, human milk composition, pre-eclampsia

Introduction

The prevalence of pre- and peri-pregnancy overweight/obesity (ow/ob) has risen substantially in the last 40 years, and it is associated with co-morbidities affecting both mother and infant, including gestational diabetes, hypertension, pre-eclampsia, and higher rates of premature rupture of membranes (PROM), preterm labor (PTL), and cesarian delivery (1, 2). This increase has significant societal justice and resource utilization ramifications as ow/ob disproportionately affects racialized women and, therefore, their vulnerable infants (2) and is associated with an approximately 30% higher rate of preterm delivery than healthy-weight women (3). This difference may, in part, be explained by higher levels of inflammation (4) as ow/ob is a pro-inflammatory state marked by elevated serum c-reactive protein (CRP) levels (5–8). Higher levels of inflammation in pregnancy may lead to increased insulin resistance and gestational diabetes (7), with associated maternal and neonatal complications. In addition, pre- and peri-pregnancy pro-inflammatory states like ow/ob are often co-morbid with others, including surgical/cesarian delivery, pre-eclampsia, and chorioamnionitis (chorio), which are known to influence fetal metabolic programming and may impact neurodevelopment and increase risks of childhood obesity (7). It is unclear whether there may be a cumulative effect of multiple pro-inflammatory "hits" on the developing fetus (7, 9) and subsequent infant outcomes, such as whether such early-life programming may continue during lactation.

Mother's own milk (MOM) is the gold standard for feeding preterm infants, improving neurodevelopment and lowering rates of co-morbidities, including necrotizing enterocolitis (10). Like many other biomolecules, cytokines from the maternal serum, or potentially the mammary gland, pass into MOM. MOM cytokines and their potential role or effect on the infant are a relatively new and increasing focus of human milk research. Data are lacking on whether mothers experiencing inflammatory states, including ow/ob, who deliver preterm infants have MOM with altered profiles of inflammatory markers compared to mothers without or with fewer inflammatory states. Studies have shown pro-inflammatory fatty acid (FA) profiles, with higher ratios of omega-6 to omega-3 s, in MOM of mothers of term infants who had pre-pregnancy and peri-pregnancy ow/ob than MOM from normal weight mothers, with optimization potentially possible with dietary interventions (11, 12).

The objective of this study was to investigate associations, if any, of maternal inflammatory states with subsequent levels of inflammatory markers and FAs in MOM from mothers of preterm infants. Inflammatory states studied included pre-pregnancy ow/ob, diabetes, chorio, PTL, PROM, pre-eclampsia, and caesarian delivery. Inflammatory markers included cytokines, CRP, and free choline, which have been reported to inversely correlate with CRP (13, 14). FAs studied included omega-6:omega-3 ratio, docosahexaenoic acid (DHA), arachidonic acid (ARA), linoleic acid, monounsaturated FAs, and saturated FAs. This was a preplanned analysis of a larger exploratory study (15) describing longitudinal cytokine and other inflammatory marker profiles in MOM from mothers of preterm infants.

Abbreviations: BMI, body mass index; chorio, chorioamnionitis; FA, fatty acid; MOM, mother's own milk; ow/ob, overweight/obesity; PTL, preterm labor; PROM, premature rupture of membranes; CRP, c-reactive protein.

Materials and methods

Study site and participants

This study is an exploratory secondary analysis of data and MOM samples from a prospective, triple-blind, randomized controlled trial (16) (OptiMoM; NCT02137473) of 127 very low birth weight (VLBW) infants and their mothers. Infants were eligible if birth weight was <1,250 g and were feeding MOM and/or consented to supplemental donor milk. Exclusion criteria included receipt of formula or fortifier before enrollment, no feeds within 14 days of birth, and congenital anomalies affecting growth. Enrollment in the original study occurred at the Hospital for Sick Children and Mount Sinai Hospital, two-level III NICUs, in Toronto, Canada, in 2014–2015, an ethnically diverse city where half of the population are visible minorities and/or immigrants (17). Mothers provided signed informed consent for themselves and their infants. Mothers previously enrolled in the OptiMoM study who still had frozen MOM sample(s) available that were collected at the end of each postpartum week (±2 days) from each of postpartum weeks 1–4, 7–8, or 10–11 were eligible for this secondary study. Mothers of singletons and multiples were included, with the first infant of multiples enrolled in the original study utilized for infant-level data. As the exploratory analysis required data from the electronic medical record (EMR) that had not been originally collected (maternal pre-eclampsia, PTL, PROM, diabetes, and infant diet at NICU discharge), eligible mothers were re-consented via phone to allow access to the EMR to obtain these data and to use frozen MOM samples for testing outside the original consent of "nutritional components." Mothers whose infants died during the original study were not reapproached, nor was contact attempted for dyads known to have been lost to follow-up. In these few cases, only data that were available from the original study were utilized (mother/infant medical/sociodemographic data, MOM FAs). MOM samples were only retested for components that fell within the nutritional realm (free choline). In all eligible mothers, frozen MOM samples that were subject to secondary analysis were matched (ideally another aliquot of the same sample with the same time/date, or if not available, then within 2 days) to MOM samples that had been previously analyzed for FAs. Longitudinally collected MOM samples were analyzed for cytokines and inflammatory markers (CRP, free choline, IFN-Ɣ, IL-10, IL-1β, IL-1ra, IL-6, IL-8, and TNF-α) and FA composition (omega-6:omega-3 ratio, percentage of ARA, DHA, linoleic acid, monounsaturated FAs, and saturated FAs) at baseline and over time. The above inflammatory states were assessed separately, and mothers, with and without such states, were grouped for comparisons *a priori* to better understand the effect of multiple inflammatory morbidities (or lack thereof). To group "healthier" mothers, we first excluded mothers with ow/ob as this diagnosis is well known to be associated with long-term inflammation (4–8). With a relatively small "n" and high prevalence of ow/ob in the population, we then stepwise added the two short-term inflammatory diagnoses in our database that are more definitively associated with inflammation (chorio and pre-eclampsia as PTL, for example, may or may not be associated with inflammation depending on the etiology, such as infection vs. incompetent cervix). Mothers with inflammatory states were then compared to healthy group 1 (normal BMI, no chorio) and healthy group 2 (normal BMI, no chorio, and no pre-eclampsia). Both the

original trial and this exploratory analysis were approved by both hospitals' Research Ethics Boards.

Assessment of milk inflammatory markers

All milk analyses aside from FA testing were performed by the Analytical Facility for Bioactive Molecules, The Hospital for Sick Children, Toronto, Canada. Milk samples were thawed and centrifuged to remove the top fat layer. The skimmed product was separated into aliquots and used in duplicate for all analyses. A custom cytokine multiplex bead panel assay was performed for IL-1βeta, IL-1ra, IL-6, IL-8, IL-10, IFN-gamma, and TNF-alpha (HCYTOMAG-60 K-07 human cytokine magnetic kit; Millepore Sigma). Detection limits were 1.09, 0.95, 1.90, 1.69, 0.81, 0.66, and 1.00 pg./mL, respectively. CRP was quantified with a magnetic bead multiplex assay (HNDG2MAG-36 K-01.Neurodegenerative MAG Panel 2; Millipore Sigma) using assay buffer; samples were run at 1:100 dilution with a detection limit of 0.003 pg./mL. Sample data for cytokine and CRP were processed with Milliplex Analyte version 5.1.0.0. Free choline was quantified using a choline colorimetric enzyme assay kit (MAK056; Sigma-Aldrich) with a detection limit of 0.01 nmol/μL. Sample results in nmol/μL were converted to ng/μL by multiplying by the molecular weight of choline (104.2 ng/nmol).

FA analyses on aforementioned paired unskimmed milk samples had previously been performed via gas chromatography and described (18), with each subtype reported as the percentage of total FAs. The omega-6:omega-3 ratio was calculated from respective percentages of omega-6 and omega-3 FAs.

Statistical analysis

Baseline clinical characteristics were summarized using descriptive statistics. Continuous variables were summarized as means and standard deviations or medians and interquartile ranges (IQRs), and dichotomous and polytomous variables were summarized as frequencies.

To visualize the potentially nonlinear time profile of serial MOM inflammatory markers (log-transformed) and FA profiles, in keeping with our previous studies, we modeled the time effect using natural cubic splines and generalized estimating equations (GEEs) with an identity link function and independent working correlation matrix. The corresponding pointwise 95% confidence intervals (CIs) were estimated using a robust sandwich estimator (15). Cytokines, CRP, and free choline were log-transformed; FAs were reported as percentages in the original data; FA values were multiplied by 100 to compensate for the large differences in units.

We assessed and quantified the association of maternal inflammatory states with MOM inflammatory markers and FA profiles using GEEs with identity link functions and an independent work correlation matrix. Due to the lack of MOM sample availability before day 4 and low numbers of samples in weeks 10–11 (less than 10% of subjects contributed samples in this time period), we analyzed all MOM samples from day 5 (henceforth, referred to as baseline) to day 54. Sample collection time (days) was considered a covariate and modeled using natural cubic splines. Specifically, we investigated the interaction between time and maternal inflammatory states and assessed if any maternal inflammatory states altered the time profiles of the milk inflammatory markers or FA profiles. The corresponding pointwise 95% CIs and p-values were estimated using a robust sandwich estimator.

Finally, we quantified the associations between FAs and inflammatory markers in the cohort overall as well as "healthy group 1" and "not healthy" (aka not in "healthy group 1"). Each association was adjusted for a time trend and was estimated using GEE with identity link functions and an independent working correlation matrix.

It is noteworthy that considerable proportions of serial observations were left censored (i.e., below the detection limit of the kits; see Supplementary Table S1, for missingness at each time point). We used a censored likelihood imputation method to account for these partial observations (19). Specifically, we imputed the left-censored observations 15 times each and estimated the final model using Rubin's rule. Concurrent values of FAs were used as predictors in the imputation model. All statistical analyses assumed a statistical significance of 5% and were conducted using R v3.5.3.

Results

Of the 127 VLBW infant–mother dyads in the OptiMoM study, 92 infant–mother dyads had MOM samples that met inclusion criteria (Supplementary Figure S1). Reflecting Toronto's diversity, over two-thirds of the OptiMoM study cohort consisted of infant–mother dyads who were visible minorities, with the majority East or South Asian (16). A total of 226 MOM samples were analyzed and grouped into six time points: weeks 1, 2, 3, 4, 7–8, and 10–11. Inflammatory marker levels over time have been previously reported for the cohort in general (15). Subject characteristics are shown in Table 1—maternal co-morbidities and pro-inflammatory states were frequent, with a mean of 2.3 and a maximum of 5 inflammatory diagnoses per mother; every mother carried at least one inflammatory diagnosis. Ow/ob was very common; when this diagnosis was excluded, mothers had a mean of 2.0 additional diagnoses. Mother–infant dyads with remaining samples at each time point are illustrated in Figure 1. In this large exploratory analysis of cytokines, we found few associations, so given the large amount of data analyzed, we report and show figures only those that were statistically significant with potential clinical relevance (for full results, see Supplementary Table S2).

In the two "healthy" groups, "healthy group 1" (normal BMI and no chorioamnionitis) consisted of $n = 33$ mothers. "Healthy group 2" (normal BMI, no chorio, and no pre-eclampsia) consisted of $n = 23$ mothers. MOM CRP concentrations were 116% higher (odds ratio, interquartile range [OR, IQR] 2.16 [1.21, 3.88]; Table 2) in ow/ob mothers at baseline than others and remained higher throughout the study (Figure 2). CRP concentrations were lower in mothers in the two "healthy groups" at the day 5 baseline than in the rest of the cohort, as seen in Table 2. In all groups, CRP decreased over time (Figure 2); similar rates of change were seen between ow/ob and healthy groups ($p = 0.38$). CRP rates of change over time only differed in "healthy group 2" compared to the rest of the cohort (Figure 2), where CRP concentrations decreased continuously over time instead of plateauing after an initial decrease in the rest of the cohort. No significant associations with other maternal inflammatory states were noted with CRP concentrations at baseline, nor differences in rates of change over time.

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TABLE 1 Subject characteristics.

N=92	Median [IQR] or n (%)		
	Entire Cohort	Non-overweight/ obese ($n = 53$)	Overweight/obese ($n = 39$)
Infant gestational age at birth (weeks)	27.7 [25.6, 29.6]	27.4 [26.3, 29.9]	27.7 [25.4, 29.1]
Infant birth weight (grams)	850 [735, 1035]	910 [740, 1070]	800 [710, 930]
Male infant	37 (45%)	20 (41%)	17 (52%)
Exclusive MOM feeds during NICU admission	46 (49%)	26 (49%)	20 (53%)
Maternal age (years)	33 [31, 37]	33 [31, 37]	33 [29, 38]
Pre-pregnancy BMI (kg/m^2) category			
Normal/underweight (<25)	53 (58%)	53 (100%)	–
Overweight (25 – <30)	12 (13%)	–	12 (30.8%)
Obese (≥30)	27 (29%)	–	27 (69.2%)
Diabetes	4 (5%)	2 (4%)	2 (6%)
Cesarian delivery	57 (61%)	32 (60%)	24 (63%)
Chorioamnionitis	31 (38%)	20 (43%)	11 (31%)
Pre-eclampsia	20 (24%)	11 (22%)	9 (27%)
Preterm labor	41 (49%)	26 (53%)	14 (42%)
Premature rupture of membranes	26 (32%)	18 (37%)	8 (25%)

MOM: mother's own milk; NICU: neonatal intensive care unit; BMI: body mass index.
Note: some characteristics don't add up to N=92 due to missing data.

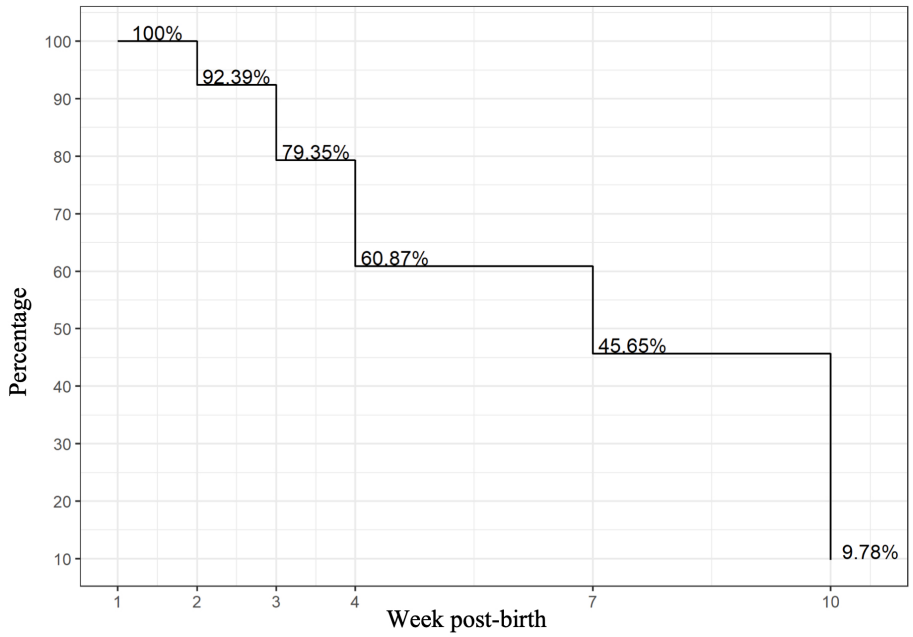

FIGURE 1
Percentage of infant-mother dyads with milk samples available for analysis by week.

MOM IL-8 levels, an inflammatory cytokine, were lower in mothers with chorio (relative change [95% CI] 69.4% [8.06, 89.9%], $p = 0.04$) and PTL (relative change [95% CI] 63.3% [2.08, 86.2%], $p = 0.05$) at baseline than mothers without these diagnoses; trajectories over time did not differ. No other cytokines had any significant associations with maternal inflammatory states. Although there were no baseline differences, free choline demonstrated a significantly different time trend/shape in mothers who had cesarian sections ($p = 0.01$) compared to vaginal deliveries. As seen in Figure 3, free-choline levels decrease for the first month and then increase in mothers with cesarian sections; an opposite trajectory is seen in mothers who deliver vaginally. Differences in free choline trajectories

were also seen in the "healthy group 2" ($p = 0.05$) compared to the rest of the cohort, and they had a borderline different trajectory in those with PTL ($p = 0.06$) compared to others (Figure 3). No significant associations were found for other individual or grouped inflammatory states for other MOM inflammatory markers at baseline (day 5), nor did trajectories differ over time when compared to the cohort as a whole.

In exploring FAs in relation to maternal inflammatory states, we found few associations; again, only statistically significant results are shown given copious data (for full results, see Supplementary Table S2). "Healthy group 1" had lower levels of saturated FAs at baseline (change −4.24, [95% CI −7.71, −0.773], $p = 0.02$), and mothers with chorio had higher baseline levels of saturated FA (change 4.06 [0.149, 7.98], $p = 0.04$) when compared to others. "Healthy group 2" had different trajectories for DHA content compared to the cohort, with healthy mothers having higher MOM DHA levels over time than those not in "healthy group 2" (Figure 4A). Obese/overweight mothers had different trajectories for omega-6:omega-3 ratio than normal weight mothers (Figure 4B), as well as a trend toward lower monounsaturated FAs (MUFA) levels at baseline (change −3.38 [−6.91, 0.152], $p = 0.06$). Differences in omega-6:omega-3 trajectory with chorio vs. no chorio were also observed (Figure 4C). MUFA trajectory differences were seen with PTL and PROM (Figures 4D,E, respectively) compared to the rest of the cohort.

Finally, we explored associations between MOM inflammatory markers and FAs (Table 3). Given a plethora of data, only statistically significant associations are shown, focusing on markers reported in earlier sections (such as CRP and IL-8; for full results, see Supplementary Table S3).

TABLE 2 Regression results for c-reactive protein.

N=92; $n = 226$ MOM samples	Relative Change [95% CI] at day 5 compared to entire cohort	p value
Overweight/obese $n = 39$ (42%)	2.16 [1.21, 3.88]	0.01
Healthy Group 1: normal BMI, no chorio $n = 33$ (36%)	0.46 [0.23, 0.91]	0.03
Healthy Group 2: normal BMI, no chorio, no pre-eclampsia $n = 23$ (25%)	0.33 [0.16, 0.66]	<0.01

MOM: mother's own milk; BMI: body mass index; chorio: chorioamnionitis.

Discussion

This study provides evidence that the pro-inflammatory state associated with pre- and peri-pregnancy ow/ob, as well as other inflammatory states, such as pre-eclampsia, are associated with MOM with temporarily higher markers of inflammation. Overweight and obesity are known to be pro-inflammatory, as well as associated with many co-morbidities, including gestational diabetes, hypertension, and pre-eclampsia, which in and of themselves are pro-inflammatory (4).

Impact of inflammation on MOM

Although MOM is the gold standard for feeding preterm infants, before this study, data were lacking as to whether the inflammatory markers from maternal inflammatory states were passed to infants through preterm MOM, such as has been reported in term infants whose ow/ob mothers produced MOM with a pro-inflammatory FA profile (11, 12). This is potentially important because, in the vulnerable preterm population, increased inflammation, in general, is associated with conditions such as increased bronchopulmonary dysplasia and poorer long-term outcomes (4). Although there were some minor differences at baseline and in rates of change over time that we detailed, overall MOM from "inflamed" mothers was quite similar to those with fewer inflammatory diagnoses, at least from a cytokine and FA standpoint. Differences in the baseline for IL-8 interestingly showed lower levels in mothers experiencing inflammatory states as well as a positive correlation with DHA, contrary to previous work in a

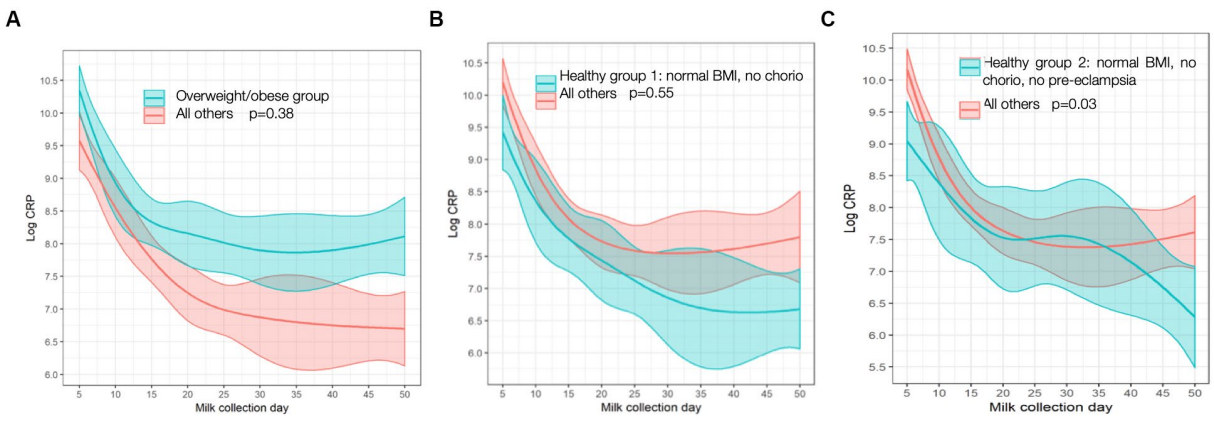

FIGURE 2
C-reactive protein (CRP) trajectory in: **(A)** Overweight/obese vs. all others; **(B)** "Healthy group I" vs. all others; **(C)** "Healthy group 2" vs. all others. p values are for differences in trajectory over time. BMI, body mass index; chorio, chorioamionitis.

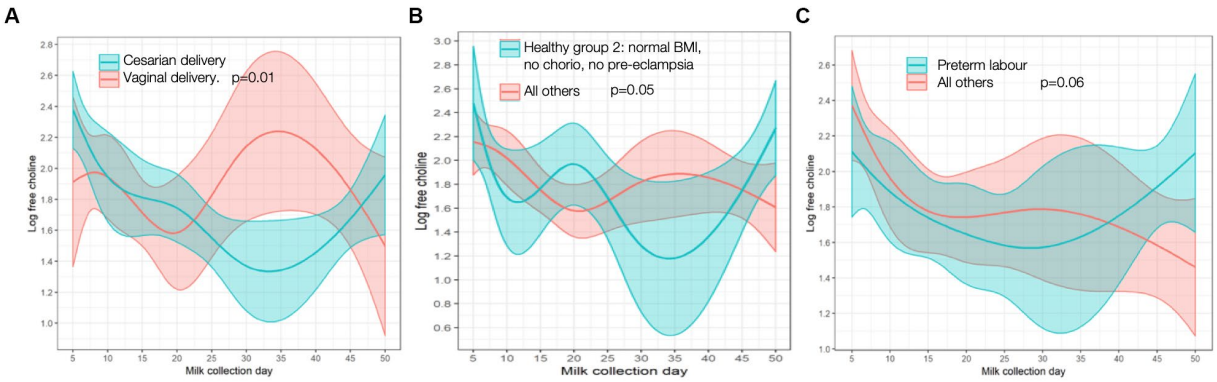

FIGURE 3
Free choline trajectory in **(A)** cesarian vs. vaginal delivery; **(B)** "Healthy group 2" vs. all others; **(C)** preterm labor vs. all others. *P*-values are for differences in trajectory over time. BMI, body mass index; chorio, chorioamionitis.

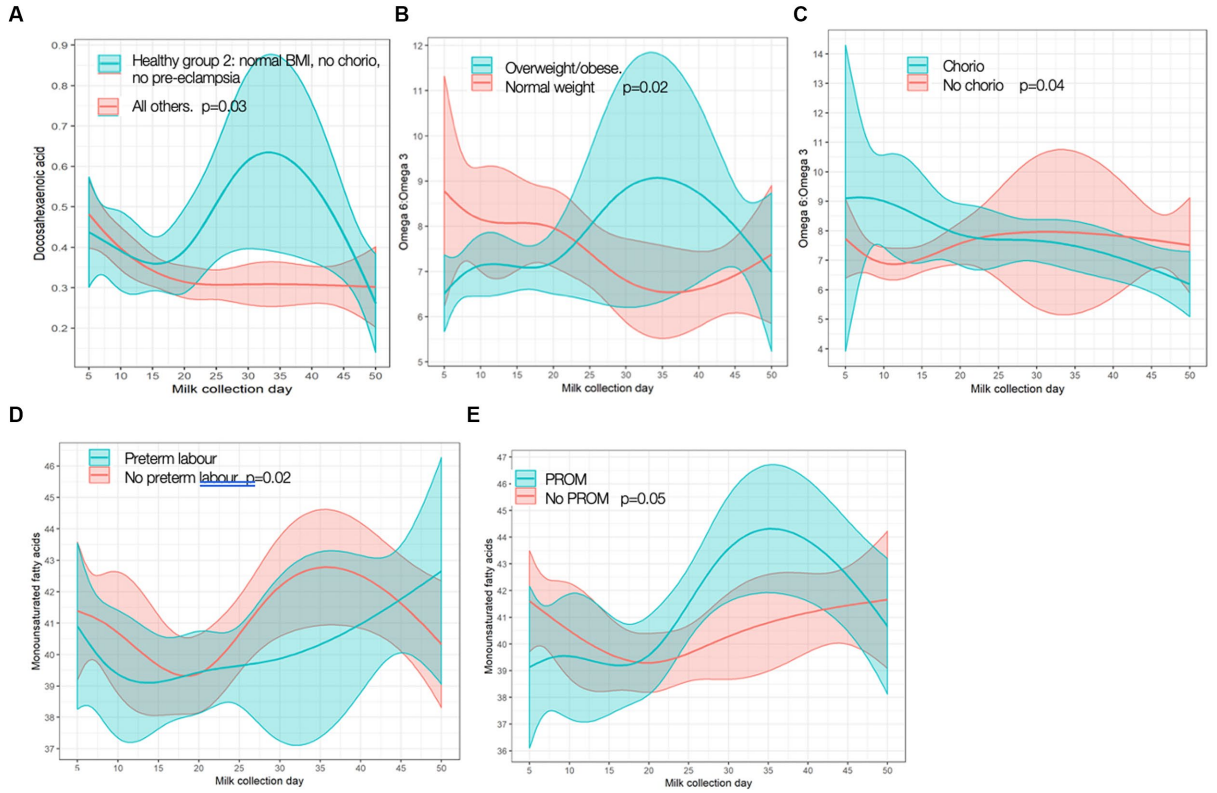

FIGURE 4
Significant differences in fatty acid trajectories. **(A)** Docosahexamoic acid (DHA) in Healthy Group 2 vs. all others. **(B)** Omega-f; pmggg-3 ratio over time in overweight/obese vs. normal weight. **(C)** Omega-qpmggg-3 ratio over time in chorioamnionitis (chorio) vs. no chorio. **(D)** Monounsaturated fatty acid (MUFA) over time in preterm labor (PTL) vs. no PTL. **(E)** MUFA over time in premature rupture of membranes (PROM) vs. no PROM p values are for differences in frajectory over time.

pre-eclamptic term population by Erbağcı et al. (20). This provides some early evidence that MOM from mothers who gave birth while experiencing inflammatory states is relatively similar to that of healthier mothers, especially after the first few weeks postpartum, providing reassurance to mothers who worry they might make "inferior" MOM.

C-reactive protein

Higher levels of CRP demonstrated in MOM samples from mothers experiencing inflammatory states than less-inflamed, normal-weight mothers suggests that the inflammation affecting the fetal environment could continue to impact the preterm infant after

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TABLE 3 Associations between mother's own milk inflammatory markers and fatty acids.

Predictor variable	Response variable	Coefficient [95% CI]	*p*
Log CRP	DHA	Overall: −0.023 [−0.046, 0.001]	0.056
		Healthy: −0.031 [−0.06, −0.002]	0.03
		Non-healthy: −0.005 [−0.037, 0.026]	0.74
	Omega-6	Overall: 0.247 [−0.173, 0.667]	0.25
		Healthy: 0.752 [0.053, 1.45]	0.04
		Non-healthy: 0.012 [−0.506, 0.529]	0.96
Log IL-8	DHA	Overall: 0.024 [0.002, 0.045]	0.04
		Healthy: 0.017 [−0.028, 0.061]	0.46
		Non-healthy: 0.025 [0.003, 0.047]	0.03
	Omega-6	Overall: −0.26 [−0.76, 0.24]	0.31
		Healthy: −0.16 [−0.006, 0.566]	0.67
		Non-healthy: −0.353 [−1.003, 0.296]	0.29
Log IL-6	DHA	Overall: 0.003 [−0.006, 0.012]	0.49
		Healthy: −0.002 [−0.016, 0.012]	0.76
		Non-healthy: 0.008 [−0.003, 0.018]	0.17
	Omega-3	Overall: 0.043 [0.002, 0.083]	0.04
		Healthy: 0.071 [0.01, 0.133]	0.02
		Non-healthy: 0.028 [−0.022, 0.077]	0.27

CRP: C-reactive protein; Healthy = "Healthy Group 1" (no chorioamnionitis, normal body mass index); IL: interleukin.

birth through MOM. The significant difference in CRP was demonstrated at baseline (day 5), and CRP trajectory did not differ between most groups. Therefore, although all CRP levels decreased over time, obese/overweight mothers continued to make milk with higher CRP, similar to Whitaker et al. who showed MOM CRP to be positively associated with maternal BMI at 1 month postpartum (21). In addition, we found a negative association between CRP and DHA levels in healthy mothers but not in the mothers experiencing inflammatory states. Perhaps, there are cumulative impacts of inflammation from birth (i.e., PTL and cesarian section) on top of the pro-inflammatory pregnancy states (ow/ob, pre-eclampsia, diabetes, etc.), which lead to higher levels of CRP in the MOM of more inflamed mothers at baseline and although this effect dissipates somewhat over time postpartum, at-risk mothers never reach the levels of their non-inflamed counterparts. In theory, these differences in MOM could result in "intergenerational transmission of disease risk" (21). Reassuringly, however, unlike in previous studies of term infants, there were few significant differences between any other inflammatory markers in MOM between inflamed and non-inflamed groups of mothers, including IL-6, which is a precursor to CRP. This lack of difference in MOM IL-6 by maternal BMI has previously been reported in mothers of term infants (21). It is still unclear whether the short-term period of raised levels of CRP in MOM could have a clinically significant impact on preterm infant health. CRP itself is an indirect measure, not a cause, of inflammation, and there is no evidence of which we are aware that it would be absorbed by the infant, but it could, in theory, bind to infant gastrointestinal tissues with unclear effect. Interestingly, MOM choline, which previously has been reported to be inversely correlated to MOM CRP, did not show similar associations (13) in our cohort. Overall, the lack of disparities in more direct measures of inflammation, such as cytokines, is reassuring. It is likely that the robust benefits of MOM far outweigh any potential risks, especially given that formula is well known to lead to a higher risk of NEC and donor milk lacks many of the benefits of MOM (10).

Fatty acid profiles

There were some differences in FA levels between groups. Healthier normal-weight mothers had more favorable FA profiles, including lower levels of saturated fat and higher MUFA levels at baseline and a DHA trajectory that increased in the first weeks postpartum. These findings are similar to a previous small study of mothers of preterm infants by Robinson et al., although 40% of mothers in that study were taking DHA supplements (22). We did not have data on DHA supplementation or nutritional intake in our cohort. In addition, grouping overweight and obese mothers together, as BMI and potential inflammation is a spectrum, could affect our results, as previous analyses from a larger number of mothers from this cohort reported that it was specifically the obese mothers who had lower MOM DHA levels (18). Studies have shown that maternal dietary intake has a significant impact on FA composition in MOM, particularly dietary fat and FA composition (23, 24). There could be potential to optimize MOM in at-risk populations by increasing dietary DHA, for example, which could potentially counteract some of the FA differences seen in the "inflamed" MOM and perhaps improve infant outcomes (25–27).

Clinical significance

The significance of these results is multi-fold. Globally, there continues to be an increase in pre-pregnancy and peri-pregnancy ow/ob. This study, although small, adds to the very limited body of work on inflammatory markers in preterm MOM and suggests the need for further research on the potential impacts of ow/ob on MOM and its subsequent effects on the next generation, especially in vulnerable preterm populations. DHA and choline, for example, are likely important in fetal and infant brain and eye development (27); therefore, suboptimal MOM levels could have long-term ramifications. Work in term populations suggests MOM cytokines like IL-6 and TNFα can correlate with infant adiposity (28). As preterm delivery and pregnancy complications/inflammatory states are more common in racialized groups, research is an important area of advocacy, particularly to mitigate inequities in minority populations (1, 2).

Study limitations

This study's limitations include the relatively small sample size, particularly at later time points when fewer leftover MOM samples were available for analysis, the lack of maternal dietary records or information on nutritional supplementation (such as DHA), and the lack of infant outcome data. There is a need for future studies to

prospectively evaluate the profile of inflammatory markers and FAs in MOM of preterm infants born to mothers with and without inflammatory states, controlling for dietary intake (especially the FA profile of the diet). Future studies should include short-term outcome data, such as incidence of necrotizing enterocolitis, bronchopulmonary dysplasia, and sepsis, and long-term outcome data, including growth and neurodevelopment. Because of the exploratory nature of this study and relatively small sample size, there was a large number of associations examined and no correction for multiple comparisons, which increases the risk of Type 1 error. Future research testing is needed to test the hypotheses raised by this exploratory analysis. In addition, while healthy groupings examined in this study are clinically relevant to this population, it is possible that other differences between the groups not measured or adjusted for in the present work may be driving some relationships.

Conclusion

In summary, in the early postpartum days (day 5), we report for the first time that inflammatory states in mothers are associated with higher CRP in MOM. As rates of change did not differ over time, ow/ob status was associated with MOM with higher CRP levels throughout the study. Reassuringly, there were few other associations between most other cytokines/inflammatory markers or FA profiles and maternal inflammatory states; however, we found some suggestions of a more ideal FA profile in mothers with fewer inflammatory diagnoses. Prospective studies are needed to further investigate the inflammatory profile of MOM from mothers experiencing inflammatory states, including ow/ob who deliver preterm infants, potential long-term impacts to vulnerable preterm infants, and how or if MOM can be optimized.

Data availability statement

The datasets presented in this article are not readily available because this analysis involved secondary use of data from a previous study (for which primary and senior authors were not PIs). Requests to access the datasets should be directed to rebecca.hoban@seattlechildrens.org.

Ethics statement

The studies involving humans were approved by Mount Sinai Health System (Toronto, Canada) and The Hospital for Sick Children (Toronto, Canada). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

EL-C: Data curation, Writing – original draft, Writing – review & editing. DO'C: Data curation, Funding acquisition, Resources, Writing – review & editing. SU: Data curation, Funding acquisition, Resources, Writing – review & editing, Conceptualization. KH: Data curation, Investigation, Validation, Writing – review & editing. ES: Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. HN: Data curation, Investigation, Writing – review & editing. RH: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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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.2023.1290690/full#supplementary-material

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COVID-19 booster enhances IgG mediated viral neutralization by human milk *in vitro*

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Background: Facilitated by the inability to vaccinate, and an immature immune system, COVID-19 remains a leading cause of death among children. Vaccinated lactating mothers produce specific SARS-CoV-2 antibodies in their milk, capable of neutralizing the virus *in vitro*. Our objective for this study is to assess the effect of COVID-19 booster dose on SARS-CoV-2 antibody concentration and viral neutralization in milk, plasma, and infant stool.

Methods: Thirty-nine mothers and 25 infants were enrolled from December 2020 to May 2022. Milk, maternal plasma, and infants' stool were collected at various time-points up to 12 months following mRNA COVID-19 vaccination. A subgroup of 14 mothers received a booster dose. SARS-CoV-2 antibody levels and their neutralization capacities were assessed.

Results: Booster vaccination led to significantly higher IgG levels within human milk and breastfed infants' stool. *In vitro* neutralization of VSV-gfp-SARS-CoV-2-S-gp, a laboratory safe SARS-CoV-2 like pseudovirus, improved following the booster, with a 90% increase in plasma neutralization and a 60% increase in milk neutralization. We found that post-booster neutralization by human milk was highly correlated to SARS-CoV-2 IgG level. In support of our correlation result, Protein G column depletion of IgG in milk yielded a significant reduction in viral neutralization ($p = 0.04$).

Discussion: The substantial increase in neutralizing IgG levels in milk and breastfed infants' stool post-booster, coupled with the decrease in milk neutralization capabilities upon IgG depletion, underscores the efficacy of booster doses in augmenting the immune response against SARS-CoV-2 in human milk.

KEYWORDS

human milk, COVID-19, booster, antibodies, neutralization, IgG, stool

Introduction

Maternal vaccination during pregnancy and breastfeeding plays a crucial role in ensuring the health and protection of mothers and infants. Current guidelines from the Centers for Disease Control and Prevention recommend the whooping cough vaccine (Tdap), Influenza, Respiratory syncytial virus (RSV) and COVID-19 vaccinations for pregnant and/or lactating women (1). Extensive research has demonstrated the efficacy of maternal vaccination in safeguarding breastfeeding infants (2–5).

The initial two dose mRNA vaccination series has been shown to significantly enhance immunogenicity and elicit protection against COVID-19 infection in adults (6, 7) and children as young as 6 months old (8, 9). Halasa et al. (10) found that maternal vaccination during pregnancy was associated with lowered risk of COVID-19 hospitalizations in infants under 6 months. Though, our group and others show a waning of SARS-CoV-2 antibodies 6 months post vaccination completion (11–13), studies have now shown that the mRNA booster dose significantly reduces the incidence and severity of COVID-19 infections compared to unvaccinated or placebo-treated controls among the general population (14, 15). One study found that infants of mothers receiving a third mRNA dose during pregnancy had shorter hospital stays and decreased rates of hospitalizations compared to infants of unvaccinated and unboosted mothers (16).

The predominant antibody isotype in human milk is IgA, followed by IgG and IgM. IgA, particularly sIgA, plays an important role in pathogen neutralization in mucosa with broad binding activity (17). Although the placental transfer of IgG from pregnant mothers to the infants' systemic circulation is well established (18, 19), little is known about the human milk IgG function as it traffics to the infants' intestinal tract.

In our previous work, we have established the presence of SARS-CoV-2 IgA and IgG antibodies in human milk and breastfeeding infant stool following maternal mRNA COVID-19 vaccination during lactation (20, 21). Notably, we and others observed a significant increase in these antibodies after the initial two-dose series (22–26), with peak levels occurring 7 to 10 days after the second dose and a subsequent decline at 6 months post-vaccination (11). In this study, we aimed to analyze the SARS-CoV-2 antibody titers and *in-vitro* neutralization capability in human milk, maternal plasma, and infants' stool at 12 months post-initial vaccination series to investigate the potential booster effect.

Methods

Participants recruitment and study design

This prospective observational study was conducted at the University of Florida with institutional review board approval. The inclusion criteria comprised breastfeeding women aged 18 years and older who had either pre- or post-COVID-19 vaccination status and provided informed consent. Thirty-nine breastfeeding mothers and 25 infants were recruited at different timepoints between December 2020 and May 2022, either before or after receiving COVID-19 vaccination from Pfizer/BioNTech, Moderna, or Johnson & Johnson. Of those, 5 mother's and 1 infant's samples were not included in the analysis (3 mothers only participated at 1 time-point; and two participants received the J&J vaccine). Given significant differences in effectiveness and antibody response with J&J compared to mRNA vaccines, those two mother-infant dyads were excluded. Participants completed a questionnaire collecting maternal/infant demographics, medical and family history, and vaccination side effects upon agreeing to participate.

Maternal plasma, milk and infant stool samples were collected up to 7-time points relative to COVID-19 vaccination completion: pre-vaccination, 15–30 days after the first vaccine dose and then at 7–30 days, 60–75 days, 90–105 days, 6 and 12 months following 2-dose vaccination series completion (Supplementary Figure 1). Not all participants contributed samples at every listed collection time point. In our prior publications, we presented the results up to 6 months post-maternal vaccination. In this paper, data from 34 mothers and 24 infants was included with a focus on a subgroup analysis of longitudinal samples collected at 6- and 12-months post-vaccination series. We examined 9 paired milk samples and 13 paired maternal plasma samples to study the booster effect. These samples were collected at 6 and 12 months, with the booster shot administered in between.

Sample collection and processing

Maternal blood samples were collected via venipuncture or finger prick in ethylenediaminetetraacetic acid-coated (EDTA) tubes at designated time points and centrifugated at 2000 × g for 10 min to separate plasma from cellular matter. Milk samples (10 mL−30 mL) were collected and stored at −20°C within 4 hours of collection. The samples were aliquoted and underwent centrifugation (500 × g for 15 min) to separate the aqueous layer, which was further centrifugated (3000 × g for 15 min) to obtain the final aqueous layer stored at −20°C. Stool samples were collected in diapers, refrigerated overnight or submitted on the same day, and stored at −80°C. The stool samples were diluted in sterile DPBS, vortexed, and centrifugated (1500 × g for 20 min) to obtain the supernatant. This supernatant was then placed in a clean tube, vortexed and centrifugated (10,000 × g for 10 min) to obtain the final supernatant, which was stored undiluted at −20°C.

SARS-CoV-2 antibody measurement

Measurement of SARS-CoV-2-Specific IgA and IgG concentrations was performed using previously validated ELISA kits (20). Dilutions were made for plasma and milk samples, while infant stool samples were run undiluted. Negative controls and duplicate samples were included.

In vitro neutralization

The neutralization capability of milk and plasma samples was assessed using SARS-CoV-2 spike glycoprotein-expressing vesicular stomatitis virus (VSV-gfp-SARS-CoV-2-S-gp) and infection competent BHK cells expressing the human ACE2

receptor in a fashion similar to those utilized in Stafford et al. with modification (11). In short, serially diluted milk (1:5, 1:20, 1:80, 1:320, 1:1280) or plasma (1:20, 1:100, 1:500, 1:2500, 1:12500) samples were incubated with VSV-gfp-SARS-CoV-2-S-gp for 1 h and after incubation this sample/virus mixture was added to BHK-ACE2 cells and incubated for 48 h. Following the 48 h, cell proliferation was measured using the MTT assay. The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability. Metabolically active cells reduce tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT] to purple formazan crystals (27, 28). Metabolically active cells have NAD(P)H-dependent oxidoreductase activity that assists in reducing MTT to formazan. The formazan crystals are then dissolved in DMSO and OD is read at 570 nm. OD values were normalized to a scale of 0%−100% infectivity using control values. Half maximal effective concentration (EC50), or the sample concentration in which 50% of cells are viable, values were calculated for each sample and used for quantitative analysis. Samples were run in duplicate.

Plaque reduction assay

Plaque reduction assays, as described by Baer and Kehn-Hall with modifications were used to confirm our MTT neutralization results (29). Neutralization assays were initially set up as MTT assays, however after 48 h of infection, cells were fixed in 10% formalin for 1 h, followed by crystal violet staining for 15 min. Plates were then washed, imaged, and optical density was measured. EC50 values were calculated as stated above for quantitative analysis. Samples were run in duplicate.

IgG depletion

IgG depletion was performed using NAb Spin Protein G columns (ThermoFisher) using manufacturer's instructions. Milk samples were diluted 1:2 in sterile DPBS and passed through Protein G columns to deplete samples of IgG. IgG-specific depletion was confirmed using SARS-CoV-2 IgG and IgA ELISA plates. Neutralization assays were then performed as described above in paired diluted milk samples and Protein G-depleted milk samples.

Statistical analysis

Descriptive statistics characterized the demographics and clinical features of the study sample. Ordinary one-way ANOVA, Mann–Whitney *U*-tests, and paired *T*-tests were employed for comparisons, and Spearman correlation analysis was conducted to explore relationships between variables. Statistical test results were reported as p-values, and software such as SPSS and GraphPad Prism 9 were used for analyses and figure creation. An alpha threshold of $< 5\%$ (or $p < 0.05$) was used for declaring statistical significance. Geometric mean values with geometric mean SD were shown in the figures.

TABLE 1 Study participants characteristics[a].

	N (%) or mean ± standards deviation
Maternal characteristics ($n = 34$)	
Age (years)	34 ± 3.6
Race	
White	33 (97)
Asian	1 (3)
Ethnicity	
Non-Hispanic	23 (67)
Hispanic	6(18)
Not disclosed	3(9)
Body mass index (Kg/m^2)	24.5 ± 3.9
History of allergies[c]	9 (26)
History of asthma[c]	4 (12)
History of inadequate immune response to vaccine[c]	2 (6)
Family history of cancer[c]	17 (50)
Family history of autoimmune disorder[c]	3 (9)
Antibiotic use in 6 months before enrollment[c]	10 (29)
Time postpartum at enrollment (months)	5.3 ± 5
Vaccine brand (first 2 doses)[c]	
Moderna	13 (45)
Pfizer	20 (55)
Booster brand (third dose)	
Moderna	8 (57)
Pfizer	6 (43)
Infant characteristics ($n = 24$)	
Infant gender	
Female	10 (42)
Male	14 (58)
Infant age at the time of first stool collection (months)	4.8 ± 5.4
Infant age at 12 months stool sample collection (months)	18 ± 7

[a]Categorical data are given as the number of participants and, in parentheses, the percentage of the total. Continuous data are provided as means ± standard deviations.
[c]Missing data from 1 subject.

Results

To assess how SARS-CoV-2 vaccination influences antibody composition, 39 lactating women and 25 infants were enrolled in the study. We analyzed the data from 34 mothers and 24 infants, with a focus on a subgroup analysis of longitudinal samples collected at 6- and 12-months post-vaccination series. We examined 9 paired milk samples and 13 paired maternal plasma samples to study the booster effect. These samples were collected at 6 and 12 months, with the booster shot administered

in between. Pre-vaccination samples consisted of 25 milk and 16 plasma samples; 7–30 days post second dose samples included 24 milk and 23 plasma samples; 6-month samples included 15 milk and 18 plasma samples; 12 months included 11 milk and 15 plasma samples. Due to the small number of participants, samples from 60–75 days and 90–105 days timepoints were excluded from the analysis. 40 infant's stool samples were collected up to 12 months post-vaccination. The study population consisted primarily of White non-Hispanic women in their mid-30s and their infants with a median infant age of 10 months at enrollment (Table 1).

Predominant SARS-CoV-2 IgG response in milk, plasma and breastfed infants' stool after mRNA COVID-19 booster

SARS-CoV-2 IgG and IgA concentrations were measured in maternal milk and plasma at 6 and 12 months post-initial vaccination series, with the booster administered in between. After the mRNA booster, there was a significant increase in SARS-CoV-2 IgG levels, evident in both human milk and plasma compared to pre-booster samples ($p < 0.0001$ in both) (Figure 1). Before the booster vaccination, SARS-CoV-2 IgG and IgA levels peaked between 7–30 days after the 2-dose vaccine series, with a predominant IgA response in milk. Although SARS-CoV-2 IgA did not show a significant increase after the booster, it did remain consistently above pre-vaccination levels, in which 9/11 (82%) milk and 12/15 (80%) plasma samples were above the positive cutoff. Throughout the 12-month period, we observed a less dynamic response in IgA levels in both human milk and plasma. The booster primarily triggered a rise in IgG levels, indicating a shift in the immune response toward a stronger IgG-mediated protection against SARS-CoV-2 (Figure 1).

Furthermore, a notable rise in SARS-CoV-2 IgG levels was evident in infant stool samples following the mother's booster dose, in comparison to pre-COVID negative controls. While we did not observe significant variations in antibody levels across specific time-points, the significance emerged when comparing the negative control group with all post-maternal vaccination samples, particularly those after the booster ($p = 0.002$) (Supplementary Figure 2).

SARS-CoV-2 antibodies concentration positively correlate between milk and plasma

Using Spearman correlations, we found that SARS-CoV-2 antibodies in milk and plasma were positively correlated pre- and post-booster dose, where higher concentrations of milk IgG correlated with higher concentrations of plasma IgG ($p = 0.007$, $R = 0.61$ for IgA and $p < 0.0001$, $R = 0.83$ for IgG) (Supplementary Table 1).

Furthermore, we observed higher concentrations of SARS-CoV-2 IgG in milk and plasma in individuals who received the booster more recently, suggesting a time-dependent effect.

Milk and plasma neutralization capabilities increase after the mRNA booster

In this study we wanted to robustly assess the potential of antibodies, present within the milk and plasma of booster vaccinated individuals, to neutralize SARS-CoV-2 viral activity *in vitro*. As such, we utilized two analysis methods to assess antibody-mediated inhibition of viral activity, using a laboratory-safe SARS-CoV-2-like pseudovirus. Whereas the MTT assay measured the ability of the pseudovirus to inhibit cellular metabolic activity, the plaque reduction assay was utilized to measure pseudovirus-mediated cell death (determined by changes in levels of intact, adherent cells).

In vitro viral neutralization significantly increased in plasma by 90% after mRNA booster dose compared to pre-booster ($p = 0.007$), with a 60% increase in milk post booster dose ($p = 0.08$) (Figure 2A). These trends were confirmed using plaque reduction assays (Figure 2B). We found that plasma neutralization and plasma IgG concentration were significantly correlated ($p = 0.0005$, $R = -0.64$) pre and post mRNA booster dose (Supplementary Table 2). Milk neutralization and milk IgG and IgA concentrations were also correlated ($p = 0.02$ and 0.03, $R = -0.56$ and -0.52, respectively). These significant, negative correlations show the higher the SARS-CoV-2 IgG, the lower the EC50 value and higher neutralization.

Our results, from both the plaque reduction assay and MTT, show that cell survival and cellular activity is protected and preserved in cells treated with boosted plasma or milk during *in vitro* VSV-gfp-SARS-CoV-2-S-gp infection.

Milk IgG depletion significantly decrease milk *in-vitro* neutralization capabilities

After observing the exponential increase in milk IgG concentration post-booster, along with its correlation with viral neutralization (Figures 3A, B), we aimed to investigate the role of IgG in *in vitro* viral neutralization. To accomplish this, we depleted IgG from milk samples using Protein G flow columns, which specifically bind human IgG. Subsequently, we measured viral neutralization using MTT assays. The results revealed a significant reduction in viral neutralization after the removal of IgG from the milk samples ($p = 0.04$) (Figure 3C).

To further validate our findings, we utilized SARS-CoV-2 IgG and IgA ELISAs, which confirmed that IgG concentrations were notably decreased through Protein G depletion, while IgA concentrations remained unaffected (Supplementary Figure 3).

Discussion

A recent publication shows that COVID-19 remains a leading cause of death among children aged 0–19 from 2021 to 2022, with COVID-19 in infants under 1 year old, ranking as the seventh leading cause of death (30). A combination of emergent strains of SARS-CoV-2, an immature infant immune system, and a requirement for 6 months of age before vaccination, has prompted

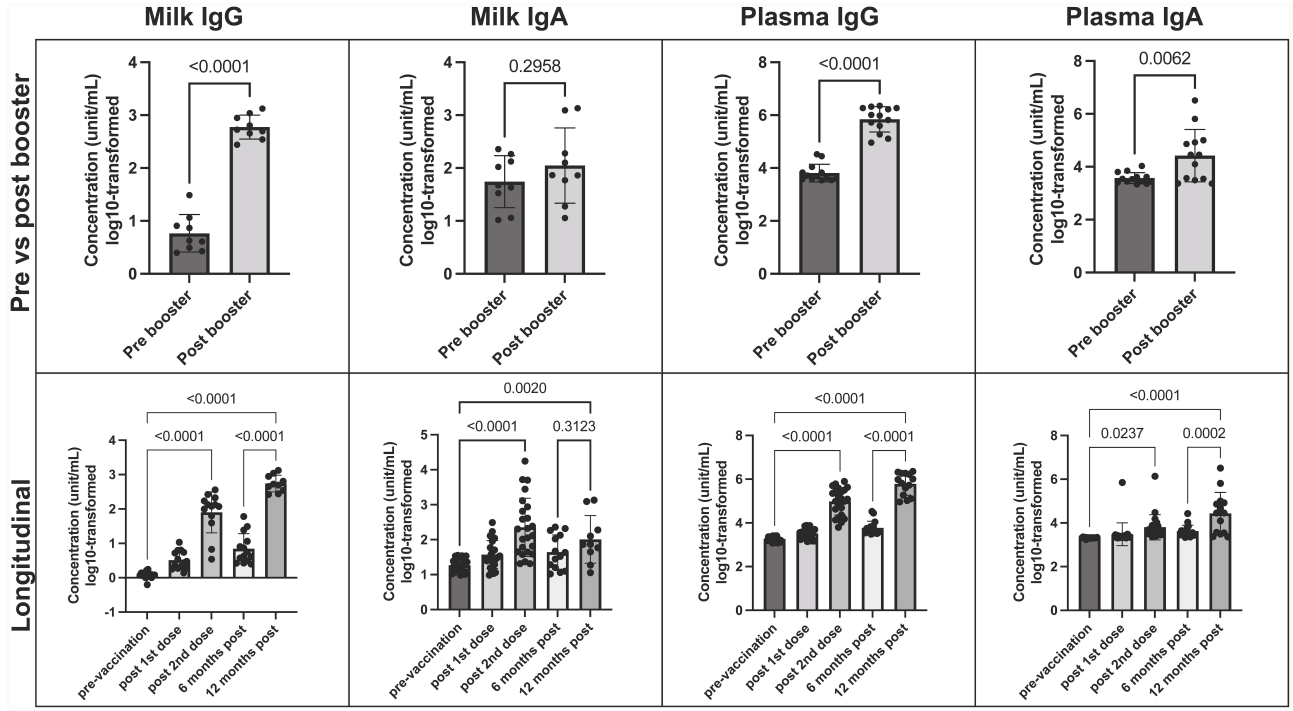

FIGURE 1
COVID-19 Booster promotes elevated SARS-CoV-2 specific IgG in human milk. Concentration of SARS-CoV-2 IgA and IgG in milk and plasma post booster dose **(top)**, and over 12-month vaccination course following initial COVID-19 vaccination **(bottom)**. Y axis = log10-transformed unit/mL; Graphs shown as geometric mean + SD. Pre vs. post booster statistical analysis = paired *T*-test; Longitudinal statistical analysis = Ordinary one-way ANOVA.

the CDC to recommend COVID-19 vaccination for pregnant and lactating mothers to combat infant risk during this critical life stage. Indeed, Halasa et al. (10) indicated that maternal vaccination with 2 doses of mRNA vaccine was associated with reduced risks of COVID-19 hospitalization, including critical illness among infants younger than 6 months of age. We and others have previously shown a significant increase in human milk derived SARS-CoV-2 IgA and IgG levels after initial mRNA COVID-19 vaccination series (20, 22–26). This increase was subsequently followed by a decline, noted 6 months post-vaccination (11, 31, 32). Notably, in this paper we show a significant increase in SARS-CoV-2 specific IgG within human milk post-booster immunization. Similar observations have been described by other researchers studying the humoral response to COVID-19 vaccines (32–35). IgG in milk was highly correlated to the increase in IgG present in the plasma post-booster. Notably, the ability of the milk to neutralize viral activity post-booster was highly IgG dependent, which drastically contrasted with pre-booster milk derived neutralization which was IgG independent (presumably IgA dependent).

Maternal immunity, primarily IgG, is transferred to the fetus during pregnancy via the placenta. Antenatal vaccination and/or infection infers pathogen-specific IgG transfer to the fetus, that wane over time (36). A recent study showed that the durability and quantity of placental derived IgG varied within infants as COVID-19 vaccination resulted in significantly higher transplacental persistence compared to infection after 6 months, with only 8% of infants born to infected mothers possessing antibodies at that time. The transplacental transfer of COVID-19 antibodies was also significantly lower at 6 months if the vaccination/infection occurred during the 3rd trimester compared to the second (37). Our study suggests that the consumption of human milk, from individuals receiving the COVID-19 booster, by infants may provide protection during the period of waning transplacental antibodies and the development of the infant's own immune system.

Although a critical role of human milk derived IgA, in the protection of postnatal infants has been well established, virus-specific IgG concentrations in milk have also been shown to be correlated with decreased risk for infant infection. Mazur et al. found that RSV Prefusion-F protein (pre-F) specific IgGs in human milk act as a clinical correlate for infant protection against respiratory syncytial virus illness. Particularly, pre-F IgG are lower in mothers' milk whose children became ill with RSV, compared to mothers of infant that did not develop RSV (38). Similarly, Fouda et al. found that milk-derived anti-HIV IgG has been shown to be correlated with lowered risk of mother-to-child transmission of HIV. Anti-HIV IgG in milk were positively correlated to plasma IgG, and IgG purified from both sample types had similar neutralizing capacity (39). These IgG in milk were shown to be correlated with neutralization and induction of antibody-dependent cellular cytotoxicity (36, 39).

Although there are lower levels of IgG compared to IgA in the gastrointestinal tract, recent studies have uncovered that among milk components, it is IgG, rather than IgA, that plays a crucial

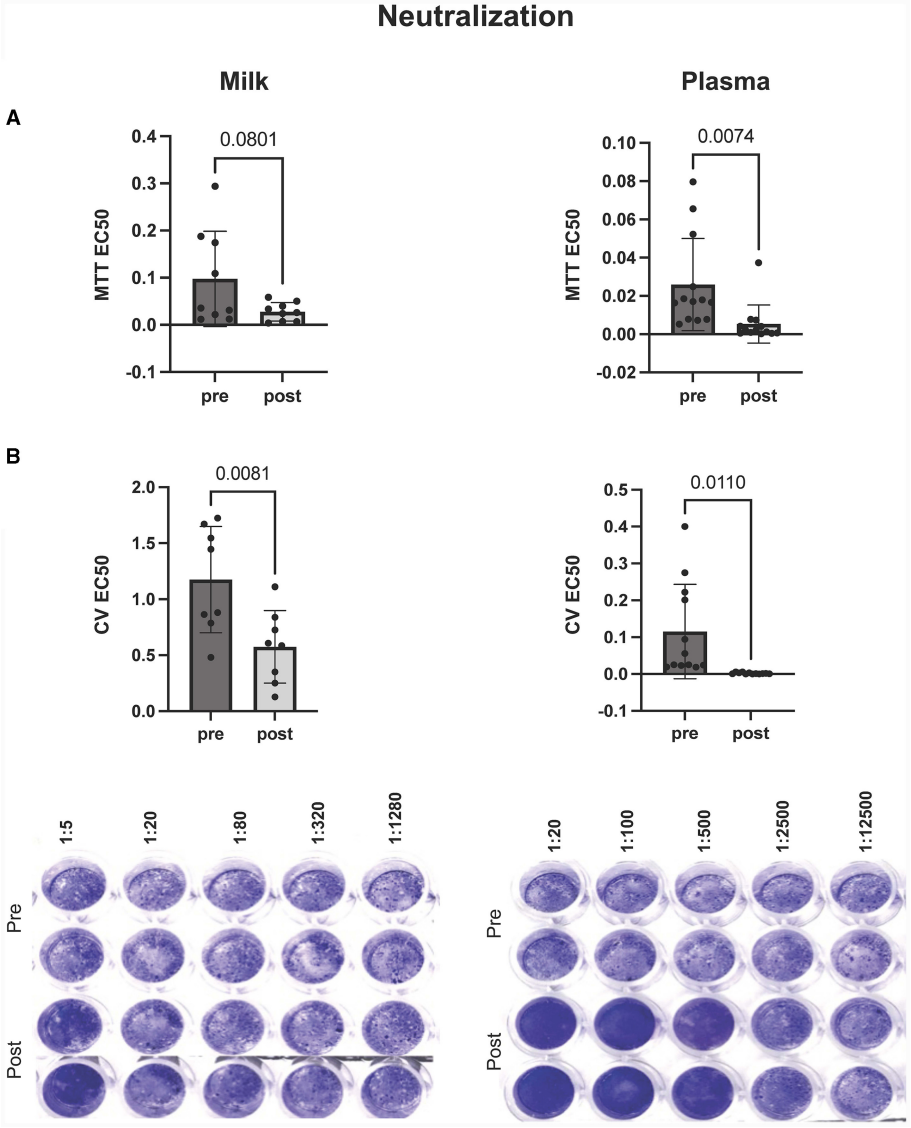

FIGURE 2
Inhibition of virus mediated cell death by milk (left) and plasma (right) pre and post mRNA COVID-19 booster dose. Neutralization of virus was assessed by **(A)** MTT and **(B)** plaque reduction assays. Y-axis = EC50 (half maximal effective concentration, sample concentration at which 50% of cells are protected from infection). Graph shown as geometric mean + SD; Statistical analysis = paired, *T*-test.

role in reducing pathogen loads and minimizing intestinal damage in breastfed pups following maternal infection or immunization in rodent models (40, 41). Caballero-Flores et al. (40) proved that maternal IgG coats, promotes phagocytosis and reduces pathogen attachment to intestinal mucosa in rodents pups (40). Another possible mechanism is that IgG binds to a broader microbial index (42). Indeed, Wang et al. (43) demonstrated that mRNA COVID-19 vaccination and/or SARS-CoV-2 infection significantly broadened the cross-reactivity of IgG in milk toward other human coronaviruses. In contrast to mice, evidence in humans has shown the passive diffusion of antibodies from the gut to the lamina propria is prevented by tight junction closure in infants born full-term (40 weeks) (44). Notably, however, it has been demonstrated, that the human neonatal Fc receptor (FcRn) was sufficient to promote transcytosis of IgG from the intestinal lumen to the lamina propria in a bidirectional manner using transgenic mice (45). Additional evidence suggesting a role of FcRn in modulating IgG mediated immunity at the lamina propria/intestinal lumen interface is the demonstration that FcRn receptors, present on the apical membrane of intestinal epithelial cells, can bind to IgG molecules found in the amniotic fluid following ingestion and facilitate endocytic transfer to the fetus (46, 47). Although it is possible that IgG may passively transfer from lumen to the lamina propria in extremely preterm infants (who may have a permeable gut), it is tempting to speculate that FcRn receptors may serve to actively transport maternal IgG derived from milk feeding into the systemic circulation of the infant.

We found a significant increase in SARS-CoV-2 IgG in the stool of infants receiving human milk post-vaccination. Interestingly, we previously found a statistically significant increase in SARS-CoV-2 specific IgG in infant stool up to 6 months post-maternal

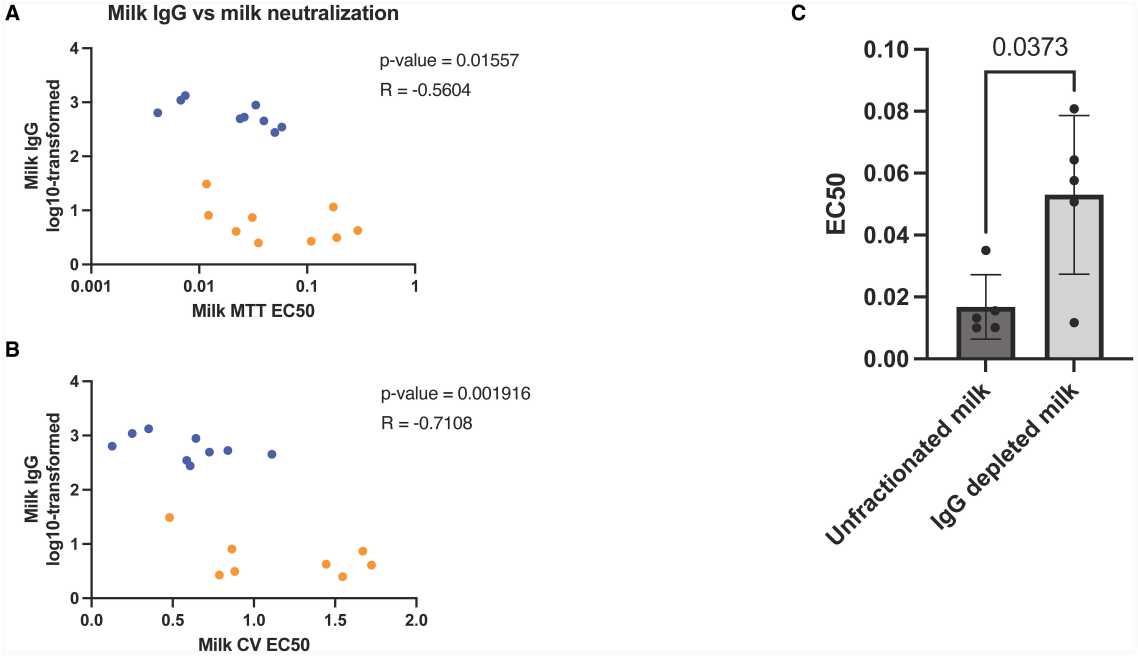

FIGURE 3
Human milk mediated viral neutralization is closely correlated to COVID-19 specific IgG post-booster. Spearman correlations of log10-transformed milk IgG vs milk neutralization assessed by MTT **(A)** and plaque reduction assay **(B)**. Orange dots represent pre booster dose values, blue dots represent post booster dose values. Graph showing neutralization capacity of milk before and after IgG depletion **(C).** Y-axis = EC50 (half maximal effective concentration, sample concentration at which 50% of cells are protected from infection). Graph shown as geometric mean + SD; Statistical analysis = paired, *T*-test.

vaccination, even when the predominant human milk antibody isotype was IgA (11). Notably, ACE2 is highly expressed on the luminal surface of intestinal epithelial cells and SARS-CoV-2 is able to infect mature enterocytes within the intestine through expression of ACE2 (48, 49). Given the existing data, it is tempting to speculate that maternally derived SARS-CoV-2 specific IgG antibodies within the infant lumen may serve to inhibit viral binding to ACE2 receptors. Although future studies are necessary to fully elucidate the specific mechanisms whereby luminal IgG confers infant protection, these works highlight the protective role of maternal milk-derived IgG. Our current studies add to the existing body of literature, further underscoring the importance of human milk-derived IgA and IgG in promoting infant health.

Limitations

This study highlights the role of human milk IgG and the transfer to breastfeeding infants after maternal mRNA vaccination. There are limitations to some of the results. First, there was a small sample size and limited diversity in this cohort of mothers. Although participants contributed data at various time points, no subjects contributed on all occasions. Furthermore, whether infants or mothers might have had a previously undiagnosed COVID infection, and how this may have interfered with results, cannot be known. We did not test the IgA secretory component, rather we assumed that IgA found in milk is majority secretory IgA. We did not explore other immune factors in milk (i.e., cell mediated immunity, cytokines, lactoferrin) that might be increased after maternal vaccination and contribute to milk neutralization capabilities. The mean age of our infant subjects at the time of enrollment was 5 months, and all were more than 6 months old by the 12 months stool collection time-point. Based on age, we assume that all of the enrolled infants had a mixed diet of solid food and human milk by 12 months collection time-point; this in combination with small sample size might have interfered with the stool antibodies concentration and neutralization capacity results, since exclusively breastfeeding infants may have higher antibodies levels.

Although we detect sIgA in stool, we cannot say for sure they are coming from milk rather than own infant intestinal mucosal production. However, several previously published works report that breastfeeding is positively associated with infant fecal sIgA concentration in the first 4 months of life, most notably when compared to sIgA in formula-fed fecal samples (50, 51).

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 author.

Ethics statement

The studies involving humans were approved by the University of Florida Institutional Review Board 202003255. The studies were conducted in accordance with the local legislation and institutional

requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

VVa: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. LS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. JN: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. LP: Conceptualization, Methodology, Writing – review & editing. VVi: Investigation, Writing – review & editing. TC: Investigation, Writing – review & editing. OD'A: Investigation, Writing – review & editing. SD: Investigation, Writing – review & editing. AH: Investigation, Writing – review & editing. JF: Investigation, Writing – review & editing. MA: Investigation, Writing – review & editing. AV: Investigation, Writing – review & editing. NC: Conceptualization, Methodology, Writing – review & editing. IK: Methodology, Writing – review & editing. JY: Methodology, Writing – review & editing. JL: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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.2024.1289413/full#supplementary-material

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Mammary epithelium permeability during established lactation: associations with cytokine levels in human milk

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Objective: The cytokine profile of human milk may be a key indicator of mammary gland health and has been linked to infant nutrition, growth, and immune system development. The current study examines the extent to which mammary epithelium permeability (MEP) is associated with cytokine profiles during established lactation within a sample of US mothers.

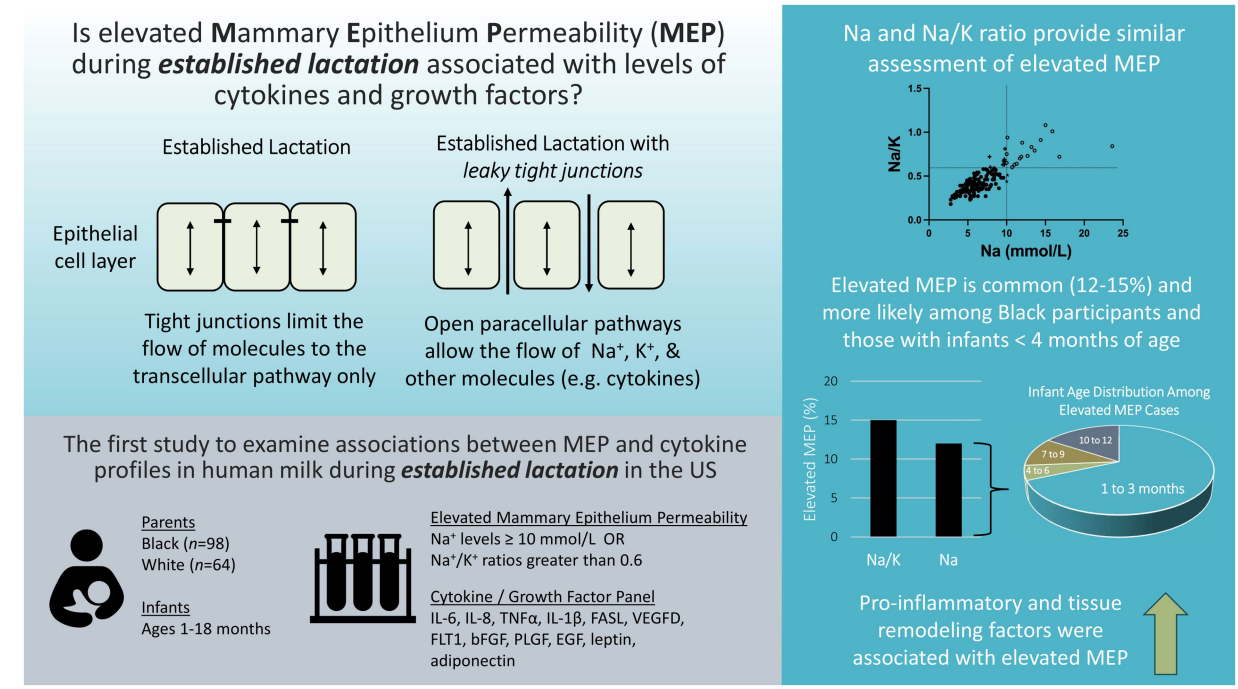
Methods: Participants were drawn from a previous study of human milk cytokines. The present analysis includes 162 participants (98 Black, 64 White) with infants ranging from 1 to 18 months of age. Levels of cytokines were determined previously. Here we measure milk sodium (Na) and potassium (K) levels with ion-selective probes. Two approaches were used to define *elevated MEP*: Na levels ≥10 mmol/L and Na/K ratios greater than 0.6. Associations between maternal–infant characteristics, elevated MEP, and twelve analytes (IL-6, IL-8, TNFα, IL-1β, FASL, VEGFD, FLT1, bFGF, PLGF, EGF, leptin, adiponectin) were examined using bivariate associations, principal components analysis, and multivariable logistic regression models.

Results: Elevated MEP was observed in 12 and 15% of milk samples as defined by Na and Na/K cutoffs, respectively. The odds of experiencing elevated MEP (defined by Na ≥ 10 mmol/L) were higher among Black participants and declined with older infant age. All cytokines, except leptin, were positively correlated with either Na or the Na/K ratio. A pro-inflammatory factor (IL-6, IL-8, TNFα, IL-1β, EGF) and a tissue remodeling factor (FASL, VEGFD, FLT1, bFGF, PLGF, adiponectin) each contributed uniquely to raising the odds of elevated MEP as defined by either Na or the Na/K ratio.

Conclusion: This exploratory analysis of MEP and cytokine levels during established lactation indicates that elevated MEP may be more common in US populations than previously appreciated and that individuals identifying as Black may have increased odds of experiencing elevated MEP based on current definitions. Research aimed at understanding the role of MEP in mammary gland health or infant growth and development should be prioritized.

KEYWORDS

human milk, sodium, Na/K ratio, cytokine, growth factor, inflammation, mammary epithelium permeability, subclinical mastitis



GRAPHICAL ABSTRACT

1 Introduction

Human milk is a complex biological fluid containing a multitude of cellular and molecular components integral to the health or disease state of the mother and infant. In particular, inflammatory markers in human milk appear to be associated with infant nutrition (1, 2), growth (3, 4), and immune system development (5, 6), and may be useful indicators of current (7, 8) and future mammary gland health (9, 10).

Human milk contains a variety of cytokines that act locally in the mammary gland and/or influence the growth and development of infant tissues (11). Cytokines represent a large class of secreted bioactive molecules that modulate cell-to-cell communication to impact cellular growth, viability, and differentiation, as well as immune and inflammatory responses (5). In human milk, cytokines are produced by leukocytes that have migrated into the mammary tissue from systemic circulation as well as by tissue-resident cells, such as mammary epithelium, fibroblasts, and adipocytes (5, 12). Specific cytokines are upregulated during mammary gland differentiation during pregnancy, in response to milk stasis, and during involution (13, 14). This upregulation is distinct from the rise in pro-inflammatory cytokines observed during an infection, such as mastitis. In the setting of infection, pro-inflammatory cytokines modulate the immune response of the mammary gland (7, 8).

Adipokines, such as leptin and adiponectin, are a subset of cytokines produced by adipose tissue that serve as endocrine signaling molecules with roles in regulating metabolism and body composition (11, 12). Both also play an important role in modulating mammary gland development and tissue remodeling during the lactation cycle (15). Growth factors are a class of cytokines linked to tissue growth and remodeling. They may retain bioactivity after ingestion and are important for the development of the infant intestinal barrier (11). Within the mammary gland, growth factors are important for angiogenesis, maintaining lactation, and regulating involution (16, 17).

Measurement of mammary epithelium permeability (MEP) may provide important information for the interpretation of cytokine concentrations in human milk. Prior to secretory activation (i.e., onset of mature milk), paracellular pathways between mammary epithelial cells are open allowing communication between the maternal bloodstream and the mammary gland (18). Following birth, rapid tight junction formation within the mammary epithelium facilitates paracellular pathway closure, a shift important for the establishment and maintenance of milk synthesis and secretion (19, 20). The paracellular pathway is particularly important for the movement of ions across the mammary epithelium. For example, sodium (Na) levels are high in colostrum, but decrease rapidly during the first 5 days postpartum in response to paracellular pathway closure. It has been proposed that once Na levels decrease below 10 mmol/L, milk maturity has been achieved (18, 21). In contrast, potassium (K) accumulates in milk as paracellular pathways close (18, 22). Both Na alone and as a ratio with potassium (Na/K) have been used to assess MEP. Na/K ratios of less than 0.6 have been used to indicate tight junction closure and milk maturity (18).

As long as the paracellular pathways remain closed during established lactation, milk secretion is maintained and levels of Na and the Na/K ratio remain low (19). However, both milk accumulation and inflammation have been linked to the re-opening of tight junctions and rising Na or Na/K ratios (19, 23). Our lab recently demonstrated that shifts in the Na/K ratio were closely linked to anti-SARS-CoV-2 antibody levels in human milk within a single individual over time, confirming an association between permeability and

immune factors (24). Knowledge of MEP could provide important context for the interpretation of cytokine levels in human milk.

We previously examined levels of human milk cytokines in a cohort of Black and White participants during established lactation to determine associations with obesity, race, and risk factors for breast cancer (24). Of note, certain pro-inflammatory cytokines (IL-1β, FASL), growth factors (bFGF, EGF), and adipokines (leptin, adiponectin) were elevated among participants with a BMI >30. Levels of 1L-1β and leptin were found to be higher in Black participants.

The goals of the current study were (1) to determine the extent to which MEP as indicated by Na and Na/K ratios is associated with cytokine profiles in human milk during established lactation and (2) to determine if race or BMI are associated with elevated MEP. We hypothesized that higher levels of human milk pro-inflammatory cytokines would be associated with elevated permeability and that similar to observed patterns for the cytokines listed above, elevated MEP would be more common among participants with Black race or a BMI >30.

2 Methods

2.1 Study population

This is a secondary analysis of selected data from a study examining racial differences in cytokines in human milk in relation to breast cancer etiology (24). For the present study, we selected participants for whom archived whole milk samples were available. All participants had signed a consent form for a study approved by the Institutional Review Board of the University of Massachusetts Amherst (#749). Briefly, for the original study, all participants completed questionnaires on demographics and health history. Milk samples were collected between 2007 and 2013 from lactating females aged 18 years and older living in the continental United States (US). Participants had collected milk in the morning upon waking by expressing the full contents of each breast into separate glass or BPA-free plastic containers via hand expression or use of their own pump. Archived aliquots of whole milk used in this project had been stored at −20°C. Descriptive statistics regarding collection and storage and presented in Supplementary Table S1.

Of the 292 participants in the original study, frozen aliquots of whole milk were available from 167 participants (24). Of these, 5 mother-infant dyads were identified as being more than 3 SD above the mean for infant age (>798 days or 2.18 years of age). Breastfeeding practices employed by these participants were not typical of the remainder of the sample and were therefore excluded. Our final sample consisted of 162 participants (98 Black, 64 White) with infants ranging in age from 1 to 18 months.

2.2 Measurement of sodium and potassium ions

Levels of Na and K were measured using ion-selective electrode probes (Medica EasyLyte Na/K Analyzer) in 2022, providing new data for the current analysis. Briefly, 1 mL aliquots of whole milk were thawed, centrifuged at 3220 *g* for 3 min at 4°C and the Na and K concentrations (mmol/L) were determined in the clarified whey fraction. A total of 18 samples were run in duplicate with mean coefficients of variation (CV) of 7.8% for Na and 4.4% for K. A ratio was calculated between Na and K for each participant. Using Mann–Whitney *U* tests, neither Na nor the Na/K ratio were associated with prescription medication use, over-the-counter pain medication use, or shipping methods, $ps < 0.10$. Spearman rank correlations with years frozen at −20°C, and number of freeze–thaw cycle were also non-significant, $ps < 0.10$.

2.3 Measurement of cytokines and growth factors

Assays for pro-inflammatory cytokines, growth factors, and adipokines were performed in the original study (24). Briefly, multiplex and single-analyte electrochemical-luminescent sandwich assays from MesoScale Discovery (MSD, Gaithersburg, MD) were used to measure 15 analytes, of which 12 were selected for the present analysis and are shown in Table 1. IFN, TIE-2, and VEGFC

TABLE 1 Lower limits of detection (LLOD) for human milk analytes from Murphy et al. (24).

Analytes	Analyte symbol	LLOD (pg/mL)
Interleukin-6	IL-6	0.14
Interleukin-8	IL-8	0.12
Tumor necrosis factor α	TNFα	0.09
Interleukin-1β	IL-1β	0.07
Fas ligand	FASL	0.415
Vascular endothelial factor D	VEGFD	3.26
Fms-related tyrosine kinase 1	FLT1	1.53
Placental growth factor	PLGF	0.42
Basic fibroblast growth factor	bFGF	0.15
Epidermal growth factor	EGF	0.075
Leptin	Leptin	93.96
Adiponectin	Adiponectin	0.08

TABLE 2 Descriptive statistics for Na, K, and the Na/K ratio ($N = 162$) and criteria for defining elevated MEP (cutoffs bolded) (18).

	Median	IQR	Elevated MEP criteria
Sodium (mmol/L)	6.2	5.0–8.0	**≥10 mmol/L** 90th percentile $N = 19$
Potassium (mmol/L)	14.6	12.7–17.4	N/A
Sodium-Potassium Ratio (Na/K)	0.41	0.34–0.52	**≥0.60** 85th percentile $N = 25$

were excluded due to low detectability (<35%). Thirty-eight samples, eight standards, and two of three controls were tested in duplicate on each plate according to the manufacturer's protocols. The lower limit of detection (LLOD) for each included analyte was determined empirically, as previously reported in Murphy et al. (25), and is also reported in Table 1. Coefficients of variation (CVs) and intraclass correlation coefficients (ICCs) are also available in Murphy et al. (24).

Mean concentrations of human milk analytes were calculated for all 162 samples with duplicate values above the LLOD. The percent of mean values below the LLOD was calculated for each analyte. Values below the LLOD were imputed with the LLOD/2. Per Keizer et al. (26), single imputation with LLOD/2 has equivalent performance to maximum likelihood imputation when less than 10% of the sample is missing. For the 162 participants included in the current study, the majority of analytes (8 of 12) had less than 10% of samples below the LLOD. However, IL-6, IL-1β, TNFα, and bFGF ranged from 13 to 20% of values below the LLOD.

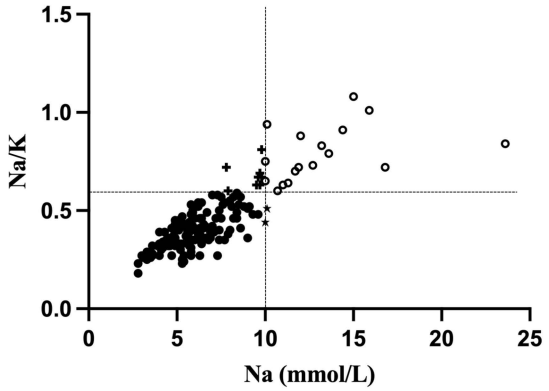


FIGURE 1
Scatterplot of sodium (Na; mmol/L) and the sodium-potassium (Na/K) ratio. Elevated MEP cases identified only by a Na level ≥ 10 mmol/L are indicated with a star (n = 2). Elevated MEP cases identified only by a Na/K ratio ≥ 0.6 are indicated with a plus sign (n = 8). Elevated MEP cases identified by both criteria are located in the right upper quadrant and are shown as open circles.

2.4 Statistical analysis

Data were cleaned and coded. All analyses were performed in SPSS 28.0. Descriptive statistics were examined. Spearman rank correlations and Mann–Whitney U tests were performed to explore bivariate associations. Two definitions of *elevated MEP* were examined in our dataset: Na ≥ 10 mmol/L (18, 21), and Na/K ratio ≥ 0.6 (18). Multivariable logistic regression models were fitted to predict increased MEP from maternal–infant factors. Principal component analysis with varimax rotation was used to identify affinities between pro-inflammatory cytokines, growth factors, and adipokines after normalizing (natural log) and centering analytes. Based on sample size, 0.45 was designated as the threshold for factor loadings (27). Factor scores were computed based on these results. Multivariable logistic regression models were fitted to examine factor scores as predictors of elevated MEP.

3 Results

3.1 Descriptive statistics for Na and the Na/K ratio

Descriptive statistics for Na, K, and Na/K ratios are presented in Table 2. Median concentrations of Na and K were 6.2 mmol/L (range 2.8–23.6) and 14.6 mmol/L (range 10.1–28.1) respectively. The median Na/K ratio in this sample was 0.41 with a range of 0.18–1.08. Na and the Na/K ratio were strongly correlated, $r(160) = 0.79$, $p < 0.001$. Two sets of criteria for identifying elevated MEP were examined. Using the criteria defined in the literature of 10 mmol/L for Na (18, 21), the cutoff for elevated MEP was at the 90th percentile and a total of 19 cases of elevated MEP were identified. Using the criteria of 0.6 for the Na/K ratio (18), the cutoff for elevated MEP was at the 85th percentile and 25 cases were identified. A total of 17 cases were identified by both criteria. Two cases were identified by Na ≥ 10 mmol/L alone, while 8 cases were identified solely by Na/K ≥ 0.6 (Figure 1).

3.2 Associations of MEP with mother-infant characteristics

Demographic and selected health characteristics of the 162 participants for the whole sample and by participant race are presented in Table 3. There was a weak negative association between maternal age and Na, $r(160) = -0.19$, $p < 0.05$, and the Na/K ratio, $r(160) = -0.18$, $p < 0.05$. Human milk Na levels also declined with older infant age, $r(160) = -0.51$, $p < 0.001$, but this association was not observed for the

TABLE 3 Median (IQR) or *N* (%) for maternal–infant characteristics for the entire sample (*N* = 162) and by participant race.

	N	Whole sample	*N*	Black	*N*	White	*p*-value
Maternal Age (Years)	162	31.0 (27.8–35.0)	98	31.0 (27.8–34.0)	64	32.0 (27.3–36.0)	0.21
Parity (Multipara)	162	88 (54%)	98	52 (53%)	64	36 (56%)	0.69
BMI (kg/m^2)	162	25.9 (22.1–30.0)	98	26.3 (22.4–30.4)	64	24.6 (21.7–29.6)	0.11
Menses Since Birth (yes)	159	61 (38%)	97	43 (44%)	62	18 (29%)	0.06
Hours Since Pumping	123	2.0 (1.0–4.0)	87	2.0 (1.0–4.0)	36	3.0 (1.0–4.9)	0.15
Infant Age (weeks)	162	21.5 (11.8–38.7)	98	25.7 (13.1–44.4)	64	17.6 (10.6–30.0)	0.07

TABLE 4 Multivariable logistic regression model predicting elevated mammary epithelium permeability (MEP) as indicated by Na ≥ 10 mm/L (*n* = 162).

	OR (95% CI)
Infant Age (weeks)	**0.96 (0.93–0.99)**
Race (Black)[a]	**3.35 (1.03–10.82)**

Model 1: [a]Reference: White.
Bolded values indicate a significant finding.

TABLE 5 Descriptive statistics for human milk analytes and Spearman rank correlations with Na and the Na/K ratio.

	N	Median (IQR)	Na	Na/K ratio
IL-6	162	1.53 (0.45–3.74)	0.35***	0.36***
IL-8	157	249.93 (132.46–629.40)	0.13	0.31***
TNFα	162	0.95 (0.46–2.51)	0.33***	0.28***
IL-1β	162	0.70 (0.27–1.81)	0.15	0.28***
FASL	162	43.23 (34.17–57.15)	0.28***	0.18*
VEGFD	161	275.92 (209.91–409.83)	0.25**	0.11
FLT1	161	1671.69 (1363.21–2423.49)	0.24**	0.12
PLGF	161	72.30 (41.77–127.57)	0.24**	0.26***
bFGF	162	0.98 (0.57–1.76)	0.41***	0.33***
EGF	162	4294.31 (3010.36–6052.75)	0.20*	0.22**
Leptin	162	1056.22 (490.75–2190.75)	0.11	0.07
Adiponectin	162	20.80 (15.70–28.46)	0.28***	0.17*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Na/K ratio. The majority of cases of elevated MEP occured in infants less than 4 months of age: 68% of cases identified by Na ≥ 10 mmol/L and 52% by Na/K ≥ 0.6.

Maternal and infant characteristics were evaluated in multivariable logistic regression models predicting elevated MEP. Infant age and maternal race were significant predictors in the model predicting elevated MEP as indicated by Na ≥ 10 mmol/L (Table 4). For every additional week of infant age, the odds of elevated MEP as defined by Na declined by 4%. Black participants had a higher likelihood of experiencing elevated MEP by 3.3 times. BMI over 30 was not associated with elevated MEP as indicated by Na ≥ 10 mmol/L. No maternal or infant characteristics, including race or BMI, were significant in the model predicting elevated MEP as defined by the Na/K ratio ≥ 0.6. Medication use, shipping method, nor storage conditions were significant predictors of elevated MEP as indicated by Na or Na/K.

3.3 Human milk cytokines and associations with MEP

Descriptive statistics for the human milk cytokines are presented in Table 5. Both Na and the Na/K ratio had several significant positive associations with the selected cytokines. Nine analytes each were positively correlated with Na or the Na/K ratio, although the specific analytes varied by MEP indicator (see Table 5). Only leptin was not associated with either Na or the Na/K ratio. The overall pattern suggested that Na was more strongly associated with growth factors, while the Na/K ratio was more strongly associated with pro-inflammatory cytokines.

Principal components analysis, performed to consolidate the analytes, identified three unique factors (see Table 6). IL-6, IL-8, TNFα, IL-1β, and EGF loaded onto a factor representing the pro-inflammatory signature. The second factor was composed primarily of analytes involved in tissue growth and remodeling

TABLE 6 Analyte factor loadings for principal component analysis with varimax rotation.

	Factor 1 Pro-inflammatory	Factor 2 Tissue remodeling	Factor 3 Leptin
IL-6	**0.840**	0.188	0.201
IL-8	**0.849**	0.202	−0.078
TNFα	**0.829**	0.095	0.225
IL-1β	**0.843**	0.031	−0.017
FASL	0.212	**0.570**	0.315
VEGFD	−0.051	**0.871**	−0.070
FLT1	0.091	**0.816**	0.123
PLGF	0.216	**0.728**	−0.045
bFGF	0.393	**0.452**	0.388
EGF	**0.460**	0.423	−0.024
Leptin	0.054	−0.007	**0.892**
Adiponectin	0.395	**0.480**	−0.277

Factor loadings over the threshold of 0.45 are bolded.

TABLE 7 Multivariable logistic regression models and 95% confidence intervals predicting increased mammary epithelium permeability (MEP) from pro-inflammatory and tissue remodeling factor scores.

	OR (95% CI)
Elevated MEP Na ≥ 10 mmol/L (n = 156)	
Infant Age (weeks)	0.97 (0.93–1.00)
Race (Black)[a]	3.90 (0.98–15.54)
Pro-inflammatory Factor	**2.40 (1.39–4.17)**
Tissue Remodeling Factor	**2.55 (1.42–4.57)**
Elevated MEP Na/K ≥ 0.6 (n = 156)	
Pro-inflammatory Factor	**2.45 (1.51–4.00)**
Tissue Remodeling Factor	**2.02 (1.25–3.26)**

Model 1: [a]Reference: White.
Bolded values indicate significant associations.

and included FASL, VEGFD, FLT1, PLGF, bFGF, and adiponectin. A single analyte, leptin, loaded onto the third factor. Pro-inflammatory and tissue remodeling factor scores were calculated based on this analysis.

Pro-inflammatory factor scores were higher in primipara as compared to multipara, Mann–Whitney $U = 2{,}391$, $p < 0.05$. The pro-inflammatory factor was more strongly correlated with Na/K, $r(154) = 0.31$, $p < 0.001$, than with Na alone, $r(154) = 0.22$, $p < 0.01$. In contrast, the tissue remodeling factor was more strongly correlated with Na, $r(154) = 0.32$, $p < 0.001$, than with the Na/K ratio, $r(154) = 0.19$, $p < 0.05$.

Multivariable logistic regression models were examined to evaluate pro-inflammatory and tissue remodeling factor scores as predictors of elevated MEP (Table 7). Controlling for infant age and maternal race, higher pro-inflammatory and tissue remodeling factor scores each uniquely raised the odds of elevated MEP as indicated by Na ≥ 10 mmol/L. A similar pattern was observed for the Na/K ratio where pro-inflammatory and tissue remodeling factors were uniquely associated with elevated MEP during established lactation.

4 Discussion

To our knowledge, this is the first study to examine associations between MEP and cytokine profiles in human milk during *established lactation* among parents living in the US. Of note, the percentage of participants with elevated MEP was higher in this cohort than was observed in a European cohort during established lactation using the same criteria (Na/K > 0.6) (28). Elevated MEP during established lactation occurred in 5% or less of participants in a European cohort as compared to the observed 15% in the current study. However, worldwide prevelance of elevated MEP varies significantly (28, 29). More study will be needed to identify appropriate thresholds for different sub-populations.

Historically, MEP has been studied primarily in relation to secretory activation, subclinical mastitis (SCM), mastitis, and involution. Secretory activation occurs in the first days postpartum and is associated with a precipitous decline in MEP (18, 21), while SCM and mastitis may occur at any time during lactation and are associated with significant increases in MEP (2, 28, 30–32). Therefore, MEP has been linked to developmental processes, in addition to

infectious processes such as subclinical mastitis or mastitis. Our aim is to understand the physiological process of MEP which can arise as the result of multiple lactation-related states.

The milk samples assessed in the present study were all from participants nursing infants between 1 and 18 months of age, and to our knowledge, no participant was experiencing symptoms of mastitis at the time of milk collection. While we did not specifically inquire about plans for weaning, it is possible that some participants may have been in the midst of this process. MEP, as indicated by elevated Na or Na/K, is known to increase during involution (33). Of note, the odds of elevated MEP (defined as Na ≥ 10 mmol/L) declined with advancing infant age overall, possibly representing normal changes in mammary function over the course of the lactation cycle. This is in line with previous research showing a decline in subclinical mastitis with older infant age (28, 30). However, few studies have examined these trends into the second year postpartum.

As predicted, Black participants in our cohort were more likely to experience elevated MEP (defined as Na ≥ 10 mmol/L), despite a non-significant older infant age in this sub-group. Since this was the first study to assess MEP in a sizable ($n = 98$) sample of Black females in the US, it is unknown whether the 3.3 times greater odds of elevated MEP among Black participants was due to socioeconomic conditions impacting the frequency of breastfeeding or pumping, represents normal variation in healthy breast tissue, or was a sign of inflammation in the mammary gland. Elevated MEP among Black participants is consistent with our previous report demonstrating higher levels of some pro-inflammatory cytokines in the milk of Black women in this cohort (24).

Levels of all cytokines examined were positively associated with either Na or the Na/K ratio, with the exception of leptin. A body of research has previously identified links between cytokines and permeability. Many of these studies have suggested that inflammation may drive increased permeability (2, 4, 31, 32). Indeed, during mastitis open paracellular pathways may be adaptive, allowing cytokine-producing leukocytes access to the alveolar lumen (34). However, permeability may also increase in response to the rising alveolar pressure associated with milk accumulation. Eventually, this can lead to tissue remodeling as seen during involution. Both pro-inflammatory cytokines and tissue growth factors play a role in this process (14, 19).

In the current study, pro-inflammatory and tissue remodeling factors each uniquely raised the odds of experiencing elevated MEP as indicated by either Na ≥ 10 mmol/L or the Na/K ratio ≥ 0.6. However, when considering continuous measures of Na or the Na/K ratio, a pattern emerged where the pro-inflammatory factor was more strongly associated with the Na/K ratio, while the tissue remodeling factor was more tightly linked with Na. To our knowledge, this is the first study to examine growth factors in relation to MEP. While additional research is needed to confirm this finding, unique patterns of Na and Na/K with specific cytokines could be used to identify the physiologic processes underlying elevated MEP.

Identifying the etiology underlying MEP has important implications for both parent and infant. Elevated MEP has been linked to delayed onset of lactation (18), low milk supply (35), reduced milk nutrient content (28), and inadequate infant growth (4). Infant growth faltering has also been identified in relation to elevated cytokines in human milk (3). Growth factors in human milk may also affect the development of the infant intestinal barrier (11). Identifying persistently increased permeability could also have important implications for identifying breast cancer risk, given the established role of cytokines in tumorigenesis (9, 24, 36). Taken together, both MEP and cytokines could have important implications for both parent and infant health.

An important direction for future research is to determine how to most appropriately measure MEP. In the present study, analyses based on Na levels and Na/K ratios provide slightly different results. Using cut-off values from the literature (18), Na was the more conservative indicator of elevated MEP in this study, identifying 19 cases as compared to the 25 cases identified using the Na/K ratio. Of note, a subset of 17 cases was identified by both indicators, while 2 cases were identified by Na alone, and 8 cases by the Na/K ratio alone. As noted above, continuous measures of Na were also more closely associated with tissue growth and remodeling cytokines, while the Na/K ratio seemed to be more tightly linked with pro-inflammatory cytokines. Additional research is needed to determine how best to interpret human milk Na levels and Na/K ratios during established lactation.

There were several strengths to the current study including a cohort with a significant number of Black female participants, representation of parents with up to 18 months of established lactation, and a panel of 12 cytokines. However, there is also an important limitation. This secondary analysis used milk and questionnaire data from a study aimed at understanding factors associated with breast cancer risk. Therefore, the study design and questionnaires were not optimally structured to assess factors known to be associated with MEP. In addition, more research is needed to determine if extended freezer storage at −20°C might affect the measurement of Na by ion selective electrode.

5 Conclusion

Results presented here highlight the importance of measuring mammary epithelium permeability in studies of normal developmental and inflammatory processes in the human mammary gland, as well as in studies of the effects of human milk on infant health. Surprisingly, the human mammary gland is the only organ for which we lack routine clinical tests for normal function (37, 38). Rich information may be obtained through the measurement of MEP indicators during established lactation. Research aimed at understanding the importance of MEP for mammary gland health or infant growth and development should be prioritized.

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 humans were approved by University of Massachusetts Amherst Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KK: Conceptualization, Formal analysis, Writing – original draft. SS: Conceptualization, Methodology, Writing – review & editing. EB: Data curation, Investigation, Methodology, Validation, Writing – review & editing. BP: Writing – review & editing. DA: Writing – review & editing. KA: Conceptualization, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing.

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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.2024.1258905/full#supplementary-material

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Association between maternal stress and premature milk cortisol, milk IgA, and infant health: a cohort study

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Background: Maternal stress is pervasive in the neonatal intensive care unit (NICU). Maternal stress is associated with changes in human milk (HM) immunomodulatory agents, which may impact neonatal health. We sought to determine the association between maternal stress, HM immunoglobulin A (IgA) and cortisol, and to assess how these milk components correlate with infant immune and neurodevelopmental outcomes. We then compared how these associations persist over time.

Methods: The study design involved a cohort study of exclusively breastfeeding mothers and their singleton moderately preterm (28–34 weeks) infants admitted to the NICU. We collected maternal serum, maternal saliva, and first-morning whole milk samples, and administered maternal stress questionnaires at 1 and 5 weeks postpartum. We analyzed the samples for HM IgA (using a customized immunoassay in skim milk) and for HM and salivary cortisol (using a chemiluminescent immunoassay). Infant illness was assessed using the Score for Neonatal Acute Physiology II (SNAP II) and SNAP II with Perinatal Extension (SNAPPE II), and infant neurodevelopment were assessed using the Test of Infant Motor Performance. We analyzed changes in HM IgA and cortisol over time using paired *t*-tests. Furthermore, we performed correlation and regression analyses after adjusting for gestational age (GA), corrected GA, and infant days of life.

Results: In our study, we enrolled 26 dyads, with a mean maternal age of 28.1 years, consisting of 69% white, 19% Black, and 8% Hispanic. Cortisol: Salivary and HM cortisol were closely associated in week 1 but not in week 5. Though mean salivary cortisol remained stable over time [2.41 ng/mL (SD 2.43) to 2.32 (SD 1.77), $p = 0.17$], mean HM cortisol increased [1.96 ng/mL (SD 1.93) to 5.93 ng/mL (SD 3.83), $p < 0.001$]. Stress measures were inversely associated with HM cortisol at week 1 but not at week 5. IgA: HM IgA decreased over time (mean = −0.14 mg/mL, SD 0.53, $p < 0.0001$). High maternal stress, as measured by the Parental Stressor Scale: neonatal intensive care unit (PSS:NICU), was positively associated with HM IgA at week 5 ($r = 0.79$, $P \leq 0.001$). Higher IgA was associated with a lower (better) SNAP II score at week 1 ($r = -0.74$, $p = 0.05$). No associations were found between maternal stress, salivary cortisol, HM cortisol, or HM IgA and neurodevelopment at discharge (as assessed using the TIMP score). Furthermore, these relationships did not differ by infant sex.

Conclusion: Maternal stress showed associations with HM cortisol and HM IgA. In turn, HM IgA was associated with lower measures of infant illness.

KEYWORDS

human milk, breastmilk, cortisol, stress, neurodevelopment, infant

Introduction

The provision of mother's milk for sick neonates promotes infant feeding tolerance, growth, and neurodevelopment, while serving as a preventive measure against life-threatening diseases such as necrotizing enterocolitis (1). However, providing mother's milk also adds to the family's burden of stress during a tumultuous time (2, 3). Mothers in the neonatal intensive care unit (NICU) report high levels of stress, with more than 40% experiencing clinical depression by the time of NICU discharge (4). While the human milk (HM) quantity and the onset of lactogenesis 2 have been shown to be negatively impacted by maternal stress (5, 6), the composition of HM may also be affected by maternal stress.

Preterm HM has been shown to have higher levels of immune modulatory agents, such as secretory IgA (7), which is possibly protective against infection and inflammation. IgA amounts in HM are highly variable and vary between individuals, by health conditions, and over time. Nevertheless, these levels may be related to maternal stress, and the nature of these relationships remain poorly understood, especially in medically fragile premature infants and high-stress environments such as neonatal intensive care units (NICUs). Stress-responsive biological markers, such as cortisol, also vary in this way, though studies have demonstrated synchrony between maternal and breastfed infant salivary cortisol up to 12 months postpartum (8, 9).

Other research has linked maternal serum cortisol and secretory IgA with maternal mood and neonatal autonomic stability (10). These findings emphasize the potential to improve infant and maternal well-being by improving lactation support in the NICU and maximizing the benefits of HM composition. Some studies suggest that maternal stress reduction interventions may increase serum and HM secretory IgA levels (11), reduce subjective stress and salivary cortisol, and increase breastmilk production in NICU mothers (12).

Therefore, determining the impact of maternal stress on human milk (HM) and immunomodulatory agents may prove important for maximizing maternal and neonatal health. Specifically, we sought to determine: (1) whether reported measures of maternal stress were associated with immunologic markers in maternal circulation and HM and (2) whether HM composition was associated with infant health and neurodevelopment during NICU hospitalization.

Materials and methods

Study design

We conducted an observational cohort study at a Level 4 NICU that serves as a regional perinatal center. Our goal was to determine the trajectory of maternal stress (captured via surveys and biometric assays), its transmission to infants via HM (captured via composition assays), and relationships with infant outcomes (infant illness scores and neurodevelopmental testing; Figure 1).

We recruited dyads of mothers and moderately preterm infants (born at 28–32$^{6}/_{7}$ weeks gestation with an appropriate-for-gestational age birth weight). We approached mothers and lactating parents of infants, hospitalized in the NICU, who were less than 7-days old. The inclusion criteria included the intention to exclusively breastfeed, the mother's routine pumping of breastmilk at least six times per day (indicating support for ongoing supply), and the absence of clinical indications for the supplementation of formula or donor milk at the time of enrollment. We excluded infants who were out of their mother's legal custody, who had a medical contraindication to breastfeeding, and who received more than 50% of base feeds as donor human milk or infant formula at enrollment or discharge.

Maternal/infant dyads were seen for two study visits: at enrollment (week 1 postpartum) and 4 weeks post enrollment (week 5 postpartum).

Sample collection

Maternal saliva samples were self-collected via spit collection at the time of the first-morning milk expression (5 a.m.–8 a.m.) on the day of the study visit. Mothers were given a salivary collection kit along with the instructions. Research assistants educated mothers on when and how to collect the sample. Saliva collection kits were brought back to the lab, where they were immediately spun at 10,000 g, and the supernatant was transferred to −80°C until analysis.

Human milk samples were self-collected by each mother before the day of the study visit. Mothers were provided a sterile collection kit and were instructed to perform a full-breast collection using their own electric breast pump (mothers expressed their breast completely until milk stopped flowing) at the time of their first daily expression (between 4 a.m. and 8 a.m.). Samples obtained outside this window were excluded from analysis. Upon expression, milk was swirled to homogenize, and a 5 mL of aliquot was removed and transferred to the lab. In the lab, 1 mL of the whole milk was spun at 10,000 g for 10 min at 4°C to separate fat from skim. A micropipette was used to pull skim from fat, using cold pipette tips to prevent fat from melting. The aliquot of skim milk, fat, and the remaining 4 mL of whole milk was then frozen at −80°C until analysis.

Biological measures

Salivary cortisol

Cortisol was analyzed in duplicate as instructed using a commercially available chemiluminescent immunoassay (IBL

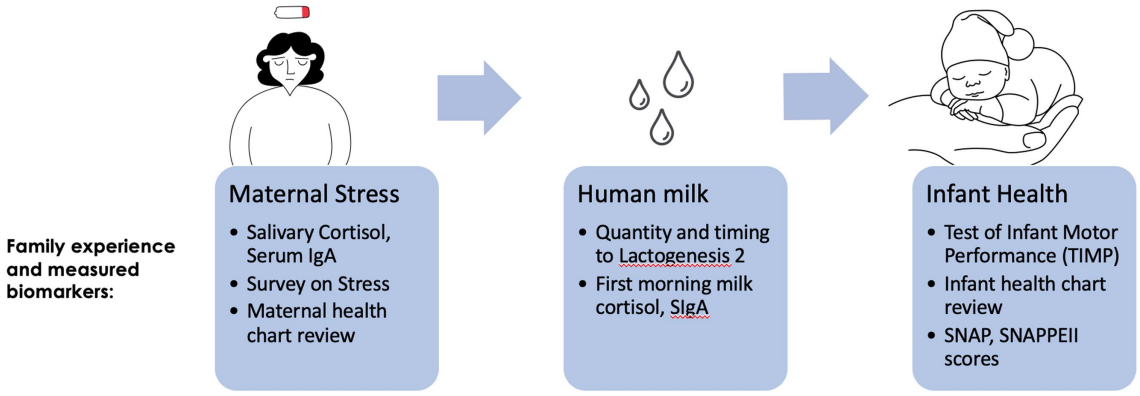

FIGURE 1
Flow diagram of the pilot study with measured biomarkers.

Immuno-Biological Laboratories, Hamburg, Germany, catalog: RE62111).

HM cortisol

Milk cortisol was measured in whole milk following the method published by Hahn-Holbrook et al. (13) using an adapted salivary chemiluminescent immunoassay (IBL Immuno-Biological Laboratories, Hamburg, Germany, catalog: RE62111). In short, 200 µL of whole milk was thawed. A measure of 100 µL of sample was spiked with 2.5 ng of cortisol. Both spiked and un-spiked aliquots were then extracted by adding 500 µL of chilled dichloromethane to milk, vortexing and incubating on ice for 10 min. Samples were then centrifuged for 5 min at 1,500 g, and the top aqueous phase was removed and discarded. A measure of 100uL of the lower phase was transferred to a new tube and evaporated to dryness in a chemical fume hood. A measure of 50 µL of distilled water was used to resuspend the tube contents after sitting for 10 min at room temperature. Resuspended samples (both spiked and un-spiked) were analyzed in duplicate following the kit instructions, loading 20 µL of extraction per well. Relative luminescence units were measured within 10 min of assay completion. A four-parameter logistic curve was generated, and the results were corrected for extraction efficiency by adjusting according to the percent recovery of the spiked sample.

HM IgA

HM IgA was measured via custom immunoassay as previously described (14). In short, 96-well MaxiSorp plates were coated with 100 µL of anti-human IgA (Bethyl Lab A80–102A) at 1:100 dilution in 0.05 M carbonate–bicarbonate overnight at 4°C. The following day, the well contents were removed and the plate was blocked by adding 200 µL of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature. The blocking solution was removed, and the plate was washed for five cycles with a 300-µL wash of PBS-T solution containing 0.05% Tween-20 using a BioTek microplate washer. Then, the samples and controls were added to the plate for 2 h at room temperature. Skim milk was diluted at a concentration of 1:5,000 in 1% BSA in PBS before running, and the controls were prepared as a serial dilution of control serum (Bethyl Lab RS10–110) in 1% BSA in PBS. Following the incubation process, the plate was washed as described above, and then 100 µL of secondary antibody (Bethyl Lab A80–102P) was added at a concentration of 1:100,000 in 1% BSA in PBS for 1 h at room temperature. After this incubation, the plate was washed as described above, and then 100 µL of prepared TMB solution [BD Biosciences (Franklin Lakes, NJ) OptEIA TMB substrate reagent set (BD 555214)] was added to each well, followed by a 15-min incubation at room temperature in the dark. To stop the reaction, 50 µL of 0.18 sulfuric acid was added to each well, and the plate absorbance was read at 450 nm. A 5-parameter logistic curve was generated, and sample concentrations were calculated.

HM sodium and potassium

HM Na and K concentrations were measured using ion selective electrodes (Sodium: B-722; potassium: B-731; Horiba, Japan) as previously validated (15). In short, the electrodes were calibrated, and 300 µL of milk was added to the sensor for measurement. The samples were analyzed in duplicate. If duplicates were more than a 10% disparity, a triplicate measurement was performed. The sodium-to-potassium ratio (Na:K ratio) was calculated as a biomarker of lactogenesis 2 (16, 17).

Interview/observational measures

Maternal demographics were collected at the time of enrollment. These included measures of maternal psychosocial and sociodemographic status (including income, education, family household composition, marital status, occupation, race, ethnicity, medical history, illness history, and medication use). Additionally, measures known to be associated with perinatal morbidity (e.g., hypertension, diabetes, and preterm labor) and breastfeeding difficulty (e.g., type of delivery, ICU stay, and infant separation) were collected by surveys and chart reviews (2, 3, 18–20). The demographic measures (listed above) were derived from the Centers for Disease Control and Prevention's Pregnancy Risk Assessment Monitoring System and were adapted for use in Monroe County (21).

Maternal stress was measured at enrollment and at 4 weeks of age. Questionnaire measures of maternal stress included standard assessments of stress that had been previously used and validated in prior research (22, 23). These assessments comprised the Edinburgh

Postnatal Depression Scale (EPDS), the Adverse Childhood Events Scale (ACES), the State–Trait Anxiety Inventory (STAI) (24–28), and the Patient-Reported Outcomes Measurement Information System (PROMIS) measures for sleep (29), and Cognitive Abilities Short Form 4a (30). We also included measures of social support, prenatal smoking, substance use, and health behaviors, such as physical activity (31). NICU-specific maternal stress indicators included the Parent Stress Scale: Neonatal Intensive Care Unit (PSS: NICU) (32) and the Postpartum Bonding Questionnaire (33, 34).

For breastfeeding measures, we collected information regarding time to first expression, time to first colostrum expressed, time to lactogenesis 2 (as per maternal report, defined by an increase in milk production > 15 mL/expression), exclusivity, weekly milk production, and time of first feed at the breast (35). Exclusivity was defined as the receipt of mother's own only, with or without fortification.

Infant neurodevelopment was measured at 4 weeks of age or at discharge, whichever was first. The Test of Infant Motor Performance (TIMP) scale measures the cognitive and motor performance of premature infants (36, 37). This scale has been validated by the scale developers for use in research and was administered by a trained occupational therapist. Although initially validated for infants of 34 weeks gestation or more, this scale has been used for younger populations as well, including the changes in scores with maturation over time, which is the goal of our pilot study (22, 38, 39). Infant health was measured at enrollment and at 4 weeks of age. The Score for Neonatal Acute Physiology II (SNAP II) and the Score for Neonatal Acute Physiology with Perinatal Extension (SNAPPE II) are validated tools for determining the severity of illness and the mortality risk in newborns of all birthweights (40). The SNAP II evaluates the mortality risk from the severity of illness, including blood pressure, PO2/FiO2, lowest temperature, serum pH, multiple seizures, and urinary output. The SNAPPE II extends this evaluation to the perinatal period by including factors such as newborn weight, appearance, pulse, grimace, activity, and respiration (APGAR) score, and being small for gestational age to determine the mortality risk. Both scores are determined based on values obtained in the first 12 h of life.

Statistical analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Means, standard deviations, and ranges were used to summarize the continuous measures, while frequencies and percentages were used for categorical measures. Within patients, changes in HM and salivary cortisol as well as HM IgA over time were assessed using paired *t*-tests or Wilcoxon signed-rank tests, as appropriate. At each time point, Pearson's correlation and Spearman correlation analyses were performed to evaluate associations between maternal stress and human milk cortisol and IgA, as appropriate. Due to the high skewness of cortisol in biological samples (35), HM and salivary cortisol were log-transformed in the analyses. Partial correlation coefficients were estimated after adjusting for gestational age (GA), corrected GA, and infant day of life (DOL). For binary stress variables, t-tests or Wilcoxon rank sum tests were used to evaluate the relationship between HM, salivary cortisol, and IgA. A *value of p* of <0.05 was considered statistically significant. No adjustment for multiple tests was made.

TABLE 1 Subject characteristics.

Variable	*N* = 26
Maternal age[1]	28.1 years (range 18.5–38.7, SD 5.7)
Race[2]	
White	69% (18)
Black	19% (5)
Ethnicity	
Hispanic	8% (3)
Insurance type[2]	
Medicaid/Medicare	30.8% (8)
Private	65.4% (17)
Uninsured	3.8% (1)
Marital status[2]	53.85% married (14)
Education[2]	
High school or less	30.7% (8)
Some college or more	69.23% (18)
Infant gestational age	Range 28.5–33.5 weeks; SD = 1.8
Infant SNAP II score[1]	6.5 (range 0–21, SD 5.66)
Infant SNAPPE II score[1]	8 (range 0–21, SD 6.15)
Time to first expression[1]	1.96 days (range − 0.31–8.15, SD 2.06)[3]
Time to L2 (by self-report)[1]	3.29 days (0–33 days, SD 6.49)
Time to first feed at the breast[1]	18.5 days (2–62, SD 16.7)

SD, standard deviation; L2, lactogenesis 2; SNAP II, Score for Neonatal Acute Physiology; SNAPPE II, Score for Neonatal Acute Physiology with Perinatal Extension-II. [1]Reported as mean (range, standard deviation). [2]Reported as % (*n*). [3]Negative "time to first expression" represents antenatal hand expression.

The sample size calculation was based on the ability to detect differences in maternal cortisol levels between stressed and non-stressed mothers (based on PSS) in the NICU. A sample of 25 achieves 80% power to detect an effect size of 1.2, assuming balanced group sizes at a two-sided significance level of 5%.

Results

Cohort characteristics are presented in Table 1: 26 mothers (with a mean maternal age of 28.1 years, consisting of 69% white, 19% Black, and 8% Hispanic) and their infants (gestational age range: 28.5–33.5; SD = 1.8) were enrolled between May 2019 and March 2020. Time to lactogenesis 2 by parental report was collected. That lactogenesis 2 had occurred by the time of sample collection was verified by a Na:K ratio < 0.8 (35). Cortisol and IgA levels are described in Table 2. Mothers reported high levels of stress, postpartum mood disorders, anxiety, and sleep disorders, which were stable or worsened over time (Table 3).

Timing of lactogenesis 2

No demographic variables, markers of maternal stress, or HM analytes correlated with time to lactogenesis 2 (as reported by the patient or by the Na:K ratio).

TABLE 2 Human milk and salivary cortisol (raw and log-transformed) and human milk IgA[1].

	1 week ($N = 24$)	5 weeks ($N = 17$)	Mean difference ($N = 17$)	P-value[2]
Cortisol				
Human milk cortisol	1.93 ng/mL (range 0.12–6.52, SD 1.97)	6.02 ng/mL (range 1.1–15.3, SD 3.9)	Mean 4.05 (range −0.6–14.74)	$p < 0.001$*
Human milk cortisol, log-transformed	0.10 (range −2.09–1.88, SD 1.19)	1.57 (range 0.09–2.73, SD 0.697)		0.0001*
Maternal salivary cortisol	2.25 ng/mL (range 0.04–9.24, SD 2.35)	2.43 ng/mL (range 0.48–6.86, SD 1.76)	Mean −0.13 (range −5.37–3.76)	0.8
Maternal salivary cortisol, log-transformed	0.117 (range −3.22–2.22, SD 1.52)	0.56 (range −0.798–1.925)		0.17
IgA				
Breastmilk IgA	1.13 mg/mL (range 0.42–1.91, SD 0.47)	0.89 mg/mL (range 0.25–1.86, SD 0.45)	−0.145 mg/mL (range −0.86–0.89, SD 0.53)	<0.0001*

*Statistically significant. [1]HM and salivary cortisol was measured using chemiluminescent immunoassay, and IgA was measured using customized immunoassay in skim milk.
[2]For comparison over time.

Cortisol

Neither maternal age, parity, infant gestational age nor time to lactogenesis 2 was associated with HM cortisol concentrations.

Maternal salivary cortisol remained stable over time (Table 2; 2.25 ± 2.35 ng/mL–2.43 ± 1.77 ng/mL, $p = 0.17$). HM cortisol increased from 1.93 ± 1.97 ng/mL at week 1 to 6.03 ± 3.93 ng/mL at weeks 5 ($p < 0.0001$). Human milk and salivary cortisol were closely associated in week 1 but not in week 5 (Table 4; Figure 2). Measures of maternal stress were associated with HM cortisol at week 1 but not at week 5 (Table 4). No associations were found between EPDS, sleep, ACES, and PSS:NICU other categories and either HM or salivary cortisol at both time points. PROMIS clarity of thought at week 1 was positively associated with maternal salivary cortisol. The change in anxiety (STAI) between weeks 1 and 5 was positively associated with the increase in HM cortisol, but this association did not hold for salivary cortisol (Table 4).

No associations were found between maternal salivary or HM cortisol and infant illness scores at any time point (SNAP II and SNAPPE II; data not shown).

IgA

Human milk IgA decreased over time from 1.13 ± 0.47 mg/mL at 1 week to 0.89 ± 0.45 mg/mL at 5 weeks ($p < 0.0001$; Table 2; Figure 3). Neither maternal age, parity, infant gestational age nor time to lactogenesis 2 was associated with HM IgA concentrations. High stress on the PSS:NICU scale at week 5 was associated with HM IgA (Table 4; Figure 4). The change in stress (via PSS:NICU score, overall stress with hospitalization) was positively associated with the change in HM IgA (Table 4; Figure 4).

HM IgA at week 1was negatively associated with infant SNAP II score at week 1 ($r = -0.75$, $p = 0.05$); no associations were found between HM IgA and SNAPPE II. No other associations with maternal stress measures were found.

Neurodevelopment

No associations were found between maternal stress, salivary cortisol, HM cortisol, or HM IgA and neurodevelopment at discharge (TIMP; data not shown). These relationships did not differ by infant sex.

Discussion

In our study of mothers of NICU infants over the first 5 weeks of hospitalization, maternal salivary cortisol remained stable, while HM cortisol increased over time. However, with the exception of the PROMIS clarity of thought scale, maternal anxiety and stress were negatively associated with HM *but not* with salivary cortisol. These associations were found during the early postpartum period (week 1) but were not evident 1 month later (week 5). Conversely, HM IgA decreased over time. At week 5, maternal stress on the PSS:NICU scale was positively associated with HM IgA, suggesting that mothers with higher stress had higher levels of IgA in their milk. HM IgA was in turn negatively associated with the infant illness score (SNAP II) at week 1, indicating that higher IgA was associated with lower SNAP II scores (a healthier infant).

HM cortisol trends and relationship between salivary and milk cortisol

This positive correlation between maternal salivary and HM cortisol has been reported in other studies involving term and preterm infants (41–43). However, Romijn et al. detected this association in term infants at 1 month postpartum, unlike our analysis, in which the association dissipated over time. Other studies have found that HM cortisol concentrations are highest in the colostral phase and decrease in mature milk after term birth (44, 45). This finding contrasts with the studies of preterm milk over time, which show higher cortisol levels compared to term milk (46, 47), with levels increasing over time (47). These conflicting reports may represent differences in cortisol secretion in term vs. preterm milk and have differing impacts on the recipient infant. The concentrations of HM cortisol detected in our study (collected from a full-breast expression) were comparable to those reported in other preterm milk studies and lower than those reported in studies that collected hindmilk samples (42, 47, 48).

TABLE 3 Maternal stress measures over time (week 1 to week 5).

Stress measure	Week 1, $N = 25$	Week 5 $N = 21$	P-value[1]
PSS:NICU[2] "How stressful has the experience of having your baby hospitalized been for you?"	4.47 (very stressful, range 2–6, SD 1.2)	4.87 (3–6, SD 1.14)	0.36
PSS:NICU mean all questions combined	3.37 (1.7–5.9, SD 1.15)	3.31 (1.8–5.6, SD 1.13)	0.81
EPDS[3]	12.3 (3–24, SD 5.34)	12.18 (3–27, SD 6.82)	0.23
EPDS[3] score > 13, depression	46% of participants ($n = 12$ of 26)	36% of participants ($n = 8$ of 22)	0.69
Sleep[4], moderate to severe sleep difficulty	68% (17)	52.4% (11)	0.18
Sleep[4], T-score	62.3 (36.6–78.5, SD 12.98)	60.5 (36.6–80.3, SD 9.9)	0.07
Maternal anxiety (STAI[5])	36 (20–64, SD 10.9)	39 (23–73, SD 16.01)	0.21
Maternal high anxiety (STAI[5] score > 53)	8% of participants ($n = 2$)	23% of participants ($n = 5$)	0.0006*

Results reported as mean (range, standard deviation). SD = standard deviation. *Statistically significant. [1]For comparison over time. [2]Parental Stressor Scale: Neonatal Intensive Care Unit. [3]Edinburgh Postnatal Depression Scale. [4]Adult PROMIS—Sleep Disturbance—Short Form, Patient-Reported Outcomes Measurement Information System. Raw scores are converted to T-scores based on patient response rate. T-scores are used for interpretation. A score of 55 or below indicates no to mild sleep disturbance, a score over 60 indicates moderate sleep disturbance, while a score of 70 and over indicates severe sleep disturbance. [5]State–Trait Anxiety Inventory.

TABLE 4 Associations between maternal stress and human milk cortisol and IgA over time (week 1 to week 5).

Associations with HM cortisol (log-transformed)		
Independent variable	Week 1	Week 5
Maternal Salivary Cortisol (log-transformed)	r = 0.75	r = 0.49
	$p < 0.001$*	$p = 0.06$
PSS:NICU[1] sub-score, stress associated with parental role	r = − 0.44	r = 0.22
	$p = 0.047$*	$p = 0.44$
PROMIS[3]: clarity of thought	r = 0.54	r = −0.06
	$p = 0.01$*	$p = 0.83$
Maternal anxiety (STAI[2])	r = −0.40	r = 0.18
	$p = 0.007$*	$p = 0.52$
Change in HM Cortisol vs. Change in Maternal Anxiety (STAI[2])	r = 0.58	
	$p = 0.03$*	
Associations with HM IgA		
Independent variable	**Week 1**	**Week 5**
PSS: NICU[1] score, overall stress with hospitalization	r = 0.35	r = 0.79
	$p = 0.18$	$p \leq 0.001$*
Change in PSS:NICU[1] score, overall stress with hospitalization vs. Change in HM IgA	r = 0.70	
	$p = 0.02$	

HM, human milk. *Statistically significant. **Higher score = worse outcomes associated with higher mortality rates. [1]Parental Stressor Scale: Neonatal Intensive Care Unit. [2]State–Trait Anxiety Inventory. [3]Patient-Reported Outcomes Measurement Information System. [4]Score for Neonatal Acute Physiology.

HM IgA levels and trends

IgA levels in our study were similar to those observed in other studies, which have been reported at 1.6–2 mg/mL in transitional and mature milk (49). Though IgA levels are typically higher in preterm milk, this difference is more pronounced in colostrum, which we did not collect (7). Human milk IgA decreased from weeks 1 to 5; however, associations between maternal stress and IgA were mostly stable over time. This decrease in HM IgA over time has been reported in other studies (50, 51). There is significant variation in HM IgA concentration among individuals. The milk IgA content further varies by age, parity, mode of delivery, BMI, gestational age, and over time (52–54). IgA is greater in HM from the mothers of infants <1,000 g and may be higher in male infants (55). However, we detected no difference in HM composition based on infant sex in this study. Some research has found that stress and maternal mood influence HM secretory IgA (56). There are significant data to suggest that HM IgA provides passive protection to infants against specific infectious diseases (57–59), and also shapes the developing microbiome (49, 60, 61).

HM cortisol and IgA and stress

We detected a negative association between maternal stress (PSS:NICU) and anxiety and HM cortisol in the early postpartum period. However, as anxiety increased over the course of

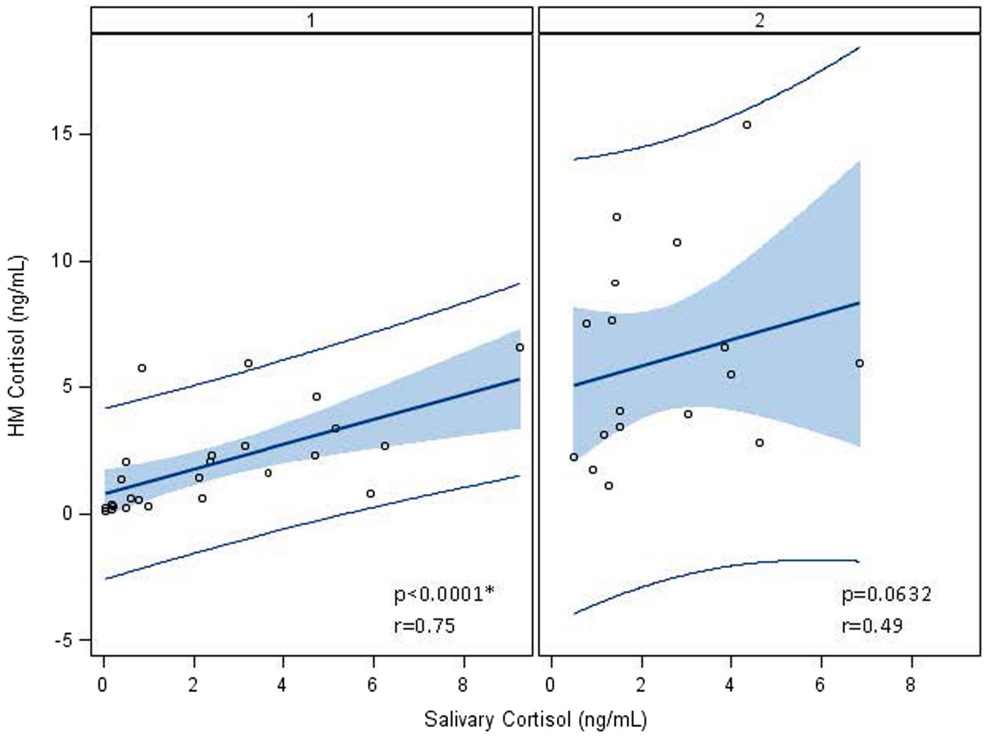

FIGURE 2
Association between log-transformed human milk and salivary cortisol over time, from visit 1 (week 1) to visit 2 (week 5). At visit 1 (week 1 postpartum), human milk (HM) cortisol was associated with maternal salivary cortisol. At visit 2 (week 5 postpartum), this association no longer held, and cortisol measures were log-transformed to account for the skewness of cortisol in biological samples. *Statistically significant.

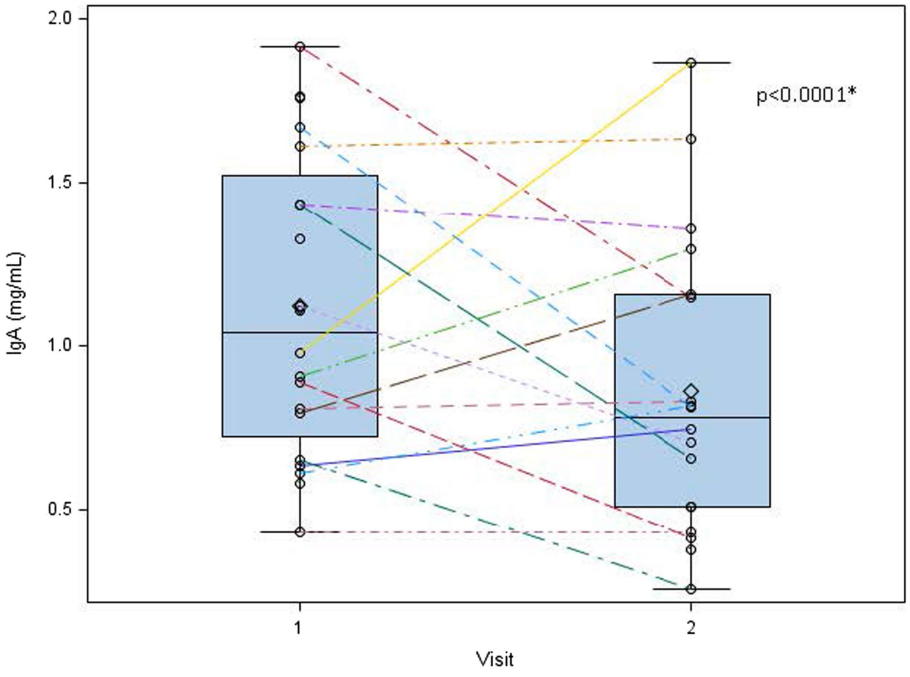

FIGURE 3
Human milk IgA decreases overtime from 1 to 5 weeks postpartum. Human milk IgA was measured at visit 1 (week 1 postpartum) and visit 2 (week 5 postpartum). While significant variation exists in human milk IgA between subjects, we found a general decreasing trend in these measures from week 1 to week 5 postpartum. 0 = mean value, the center line denotes the median value, the box contains the 25th to 75th percentile of the dataset, and the black whiskers mark the minimum and maximum values. *Statistically significant.

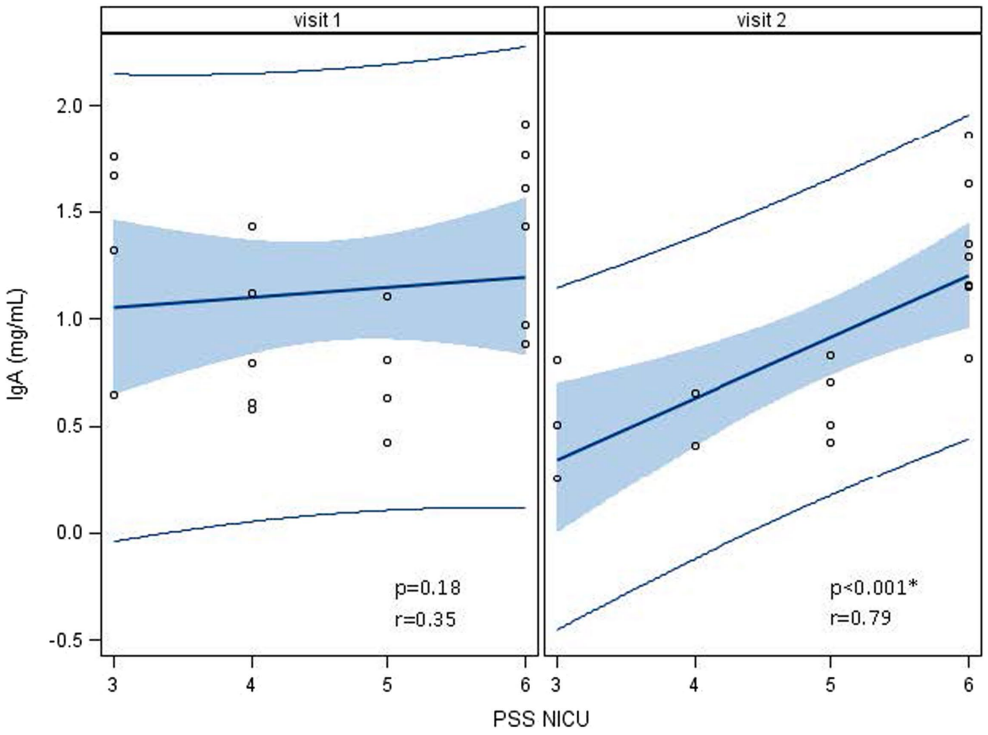

FIGURE 4
Associations between human milk IgA and the Parent Stress Scale: Neonatal Intensive Care Unit (PSS: NICU) over time, visit 1 (week 1) to visit 2 (week 5). At visit 1 (week 1 postpartum), human milk (HM) IgA was not associated with a high maternal stress score on the PSS:NICU. At visit 2 (week 5 postpartum), this association was significant. *Statistically significant.

hospitalization, HM cortisol levels also rose. Other studies have reported associations between maternal stress and depression scores and glucocorticoids in HM and serum (46, 47, 62, 63), while others fail to detect a relationship (64). IgA in HM is more consistently associated with subjective stress measures, as observed in our study, with higher levels of stress on the PSS:NICU scale associated with higher IgA levels in milk (56, 63, 65, 66). Moreover, when analyzed individually, changes in overall stress with hospitalization (PSS:NICU) were positively associated with changes in HM IgA. In addition to being linked to subjective stress measures, concentrations of HM cortisol and IgA are also modifiable. Studies on relaxation, meditation, and laughter have been shown to increase HM IgA and lower HM cortisol (11, 67, 68). These studies, although small and heterogeneous, suggest that alternative factors contribute to the variation in HM cortisol and IgA. This may, in turn, help to explain the variability in these associations between different studies.

Relationships between HM cortisol, IgA, and infant outcomes

Both maternal serum and human milk cortisol appear to have effects on recipient infants. High maternal serum and hair cortisol levels have been associated with delayed lactogenesis 2, lower breastfeeding rates, and milk composition (69–72). Human milk cortisol is associated with infant biometrics (adiposity and head circumference), temperament, and fear reactivity (73–78). We therefore sought to determine if HM cortisol or HM IgA in this highly vulnerable preterm infant population was associated with neurodevelopment. However, our findings indicated no significant association. The findings reported in our study are similar to a study of healthy term infants in whom HM cortisol had no impact on temperament or neurodevelopment (76). We also did not detect any associations between maternal stress and the infant TIMP score.

In this population of preterm infants, we report that HM IgA is negatively associated with the infant illness score (SNAP II), suggesting that mothers with higher concentrations of HM IgA had infants with healthier illness scores. Alterations in HM IgA can have important ramifications for infant immune outcomes. This study is observational and thus cannot establish causality. Furthermore, though the SNAP II score includes variables affected by infant infection (blood pressure, PO2/FiO2, lowest temperature, serum pH, multiple seizures, and urinary output), it is not specific to infectious illness. No association was found between HM IgA and the SNAPPE II, which includes perinatal mortality indicators (such as newborn weight, APGAR score, and being small for gestational age) that are not illness-specific. We therefore postulate that these data support the premise that higher HM IgA may be a protective factor against infant infectious disease. This finding warrants further validation and mechanistic investigation, especially in premature infants.

Strengths and limitations

The strengths of this study included the assessment of an understudied population (premature, hospitalized infants), the

characterization of maternal stress using multiple assessments, the inclusion of multiple specimen types, including saliva and HM, gathered contemporaneously, and the use of validated assessments to evaluate both infant neurodevelopment and illness.

The limitations of the study include the small sample size (which was limited due to the COVID-19 pandemic) and the lack of infant biomarker analysis. This limited sample size reduced our power to detect relationships with a small effect size. Thus, our inability to detect any relationship between maternal stress, HM cortisol, or HM IgA and infant neurodevelopment could represent a true verification of the null hypothesis or reflect our limited power. It could also affect our findings regarding the direction of the association between HM cortisol and reported maternal stress.

The small sample size also limited our ability to control for demographic and medical stressors, such as income, race, marital status, maternal illness, and antenatal steroid use, which may impact breastfeeding outcomes, cortisol levels, or infant neurodevelopment.

We used salivary cortisol as an objective measure of stress instead of serum cortisol, due to its lower circadian variability and the ability to collect the sample at the same time as milk expression on first-morning awakening. However, salivary cortisol levels in the peripartum period may be affected by an impaired adrenal capacity to mount a cortisol response and variable cortisol metabolism, which were not documented (79).

Conclusion

Reported stress in NICU mothers was more tightly correlated with HM than salivary cortisol at 1 week postpartum. Maternal stress was associated with higher HM IgA levels, which, in turn, were associated with lower measures of infant illness.

Therefore, implementing stress reduction techniques for NICU mothers may have significant implications for the health of sick neonates.

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 humans were approved by the Institutional Review Board of the University of Rochester. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants themselves, and participants' legal guardians/next of kin.

Author contributions

CR-C: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. SG: Project administration, Writing – review & editing. HW: Data curation, Formal analysis, Writing – review & editing. SS: Investigation, Writing – review & editing. RM: Methodology, Writing – review & editing. TO'C: Conceptualization, Methodology, Writing – review & editing. KJ: Conceptualization, Methodology, Writing – review & editing. CD'A: Conceptualization, Funding acquisition, Methodology, Writing – review & editing. BY: Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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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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Access to and interest in human milk research opportunities among Black pregnant and postpartum people

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Background: Concerns exist regarding biomedical research participation in marginalized and historically disadvantaged communities.

Objectives: The purpose of this study was to understand critical barriers to participation in human milk research from the perspective of Black pregnant and postpartum people.

Methods: A national sample of Black pregnant and postpartum people (n = 104) was recruited to complete a cross-sectional online survey informed by the Life Course Perspective. Survey questions assessed research experiences and preferences, particularly related to human milk research, knowledge of historical events/policies targeting Black communities, and demographic characteristics. A socio-economic composite score was calculated as an indicator of socio-economic advantage. Survey data were summarized descriptively and potential correlates of research engagement were evaluated.

Results: Most (69%, n =71) respondents reported previous participation in a research study, yet only 8 (8%) reported ever being asked to participate in a breastfeeding/chestfeeding or human milk study, and one respondent was unsure. Despite so few having been asked, 59% (n =61) of respondents indicated they would donate breast/human milk to research if asked. Respondent characteristics associated with prior research participation included having greater socio-economic advantage (p=0.027) and greater knowledge of discriminatory historical events/policies (p<0.001). In contrast, the only respondent characteristic associated with willingness to donate human milk to research was younger age (p=0.002).

Conclusion: Our findings suggest that Black pregnant and postpartum people are interested in biomedical research, specifically human milk and lactation research. However, greater intentionality and targeted recruitment of this underrepresented population is needed to increase diversity among human milk and lactation study samples. Structural and community-based interventions, informed by community members, are needed to address concerns and improve participant engagement.

KEYWORDS

human milk research, lactation research, recruitment, research participation, Black women and birthing people, research opportunities

Introduction

Breastfeeding and the provision of human milk have a significant impact on health for both mothers and infants across their life course (1–3). The benefits noted are short and long term (1–3). Lactating people experience rapid uterine involution and decreased bleeding, greater weight loss, and stronger dyad bonding, and also have reduced risk of depression, uterine cancer, ovarian cancer, breast cancer, cardiovascular disease, and type 2 diabetes later in life (2, 3). Breastfed infants experience fewer infections, specifically gastrointestinal, respiratory, and ear infections, and have lower rates of necrotizing enterocolitis, sudden infant death syndrome, obesity, and type 1 and type 2 diabetes (1–3). Human milk has also been shown to support infant growth and development, a healthy immune system, and gut health (4).

The current national maternal and child health focus on increasing breastfeeding and access to human milk has generated a number of initiatives geared toward investigating how the varied composition of human milk contributes to health. Until recently, human milk composition was thought to be very consistent and uniform across all populations, and that characteristics such as race, age, parity, or diet did not greatly affect milk composition (5). However, recent literature has shown that human milk is a dynamic, bioactive fluid with significant inter-individual variability (6). Human milk provides important nutrients needed for growth and development; however other bioactive components of human milk also play a significant role in shaping infant behavior and neurodevelopment (7).

Studies that include collection and analysis of human milk and other biospecimens are needed to develop a comprehensive understanding of the effect of life course factors on human milk production and composition across all populations. Yet, research shows Black women are less likely to donate biological specimens (8–10) and are underrepresented in biomedical research generally, especially in studies focused on human milk composition (9). A recent review of 28 human milk composition studies conducted from 1980 to 2016 noted that many studies did not disclose participants' race/ethnicity and of those studies that disclosed, the majority of participants self-identified as white and healthy (11).

Concerns exist regarding biomedical research in marginalized and historically disadvantaged communities, particularly when collection of biological specimens is involved and the direct benefit to participants may be low (11). Community engagement prior to recruitment is recommended to ensure that research is conducted responsibly, ethically, and appropriately (12, 13). Reasons for lack of participation include distrust of the healthcare system and ineffective or biased recruitment, as well as unfamiliar procedures and participant burden (8–10). Further, ethical concerns, lack of awareness, economic and geographical barriers, lack of culturally informed recruitment methods, limited diversity among research team members, and previous studies such as the U.S. Public Health Service (USPHS) Syphilis Study at Tuskegee and use of HeLa cells have also been identified as suggested barriers (14). A number of these barriers signify current sociocultural factors and historical factors dating back to slavery and segregation (15, 16). While several barriers to participation in medical research, specifically related to clinical trials have been noted, few studies have reported barriers to participating in human milk research (17). The purpose of this study was to understand critical gaps to participation in human milk research from the perspective of Black pregnant and postpartum people.

Methods

Study design and ethics

This study was designed as part of a larger cross-sectional online survey of Black pregnant and postpartum people and healthcare workers. This analysis focuses specifically on survey data collected from the sample of Black pregnant and postpartum people; analysis of the sample of healthcare workers is reported separately. The study design was informed by the Life Course Perspective, which seeks to examine lives through a structural, social, historical, and cultural lens and provides a framework to examine how personal history and life events impact current health and future decision making (18, 19). The four key elements of the Life Course Perspective framework include: timeline, timing, environment and equity (20). The study protocol was approved by the Institutional Review Board at the University of California, San Francisco (protocol #18–24,803). All participants provided both verbal consent during the eligibility screening and online consent as part of the online survey. All data were collected between November 2020 and February 2021. All participants were compensated for their time with a $50 electronic gift card.

Recruitment and eligibility

Convenience sampling was used to recruit study participants by posting study flyers and advertisements on community bulletin boards, public health programs, hospitals, clinics, and social media platforms (e.g., Facebook, Twitter, and Instagram). Prospective participants were given initial information about the study's purpose, risks, and benefits by the research team. Prior to being sent the online qualitative survey by email, eligibility was confirmed by phone, and consent was obtained through the online survey. Eligibility criteria for the survey of Black pregnant or postpartum people were: (1) self-identify as Black or African American, (2) currently pregnant or parenting a child under the age of 5 years, (3) able to read and write English, (4) based in the US and (5) over 18 years of age.

Online survey

The online survey was conducted using the Qualtrics XM platform and included questions about the participants' demographic characteristics (e.g., age, gender identity, racial/ethnic identity, education, employment status, type of health insurance, income, state of residence), pregnancy and/or birth characteristics, and their experiences with, interest in, and preferences about research participation and engagement, specifically research focused on breastfeeding/chestfeeding and human milk. Questions about participants' knowledge of historical events and policies targeting Black populations (e.g., Jim Crow laws, Tuskegee Syphilis experiments, J. Marion Sims' surgical experiments on enslaved African women, and Henrietta Lacks) were also included. The term "chestfeeding" has recently been introduced to describe feeding a baby from one's chest

and is often preferred by parents who identify as transgender or non-binary (2, 3).

Analysis

Statistical analyses of quantitative survey data were conducted using Stata Statistical Software, release 15 (StataCorp, College Station, TX). Descriptive statistics included means, standard deviations, and ranges for continuous variables, and frequencies and percentages for categorical variables. Continuous variables were checked for normality. Responses were evaluated for differences by four indicators of socio-economic advantage: (1) college degree (yes/no), employment (yes/no), (2) private health insurance (yes/no), and annual income of $50,000 or more (yes/no). A socio-economic composite score was also calculated as the number of indicators of socio-economic advantage that each respondent had (range 0–4). A knowledge composite score was calculated as the number of the four historical events and policies each respondent reported at least some knowledge of (range 0–4). Chi-square or Fisher's exact tests were used for group comparisons on categorical variables as appropriate. Logistic regression was used to estimate odds ratios with 95% confidence intervals for associations between categorical variables. Independent-sample *t*-tests or Wilcoxon's rank sum tests were used to compare two groups on continuous variables as appropriate. *p*-values <0.05 were considered statistically significant.

Description of authors' backgrounds

All authors are trained and/or experienced researchers who worked collaboratively to create the online qualitative survey. Author 1 is an experienced community-based participatory action, qualitative, and mixed methods researcher. Author 2 is an experienced quantitative researcher and data analyst. Authors 3 and 4 are experienced community researchers. Authors 1 and 3 are also experienced International Board-Certified Lactation Consultants (IBCLCS). Author 1 is a Black woman and Associate Professor in Nursing. Author 2 is a White woman, Research Specialist and Data Analyst. Author 3 is a Black woman, IBCLC and Director of a lactation focused non-profit. Author 4 is a Latinx woman and Clinical Research Coordinator. All of the authors bring their own lived experiences and understanding as it relates to the issue of access to research opportunities and interest in human milk research participation, which may affect our analysis and interpretation of the data.

Results

Sample characteristics

Of the 191 people who expressed interest in this study, 109 (57%) were eligible for the survey of Black pregnant and postpartum people, 51 (27%) were eligible for the survey of health care workers, 4 (2%) were not eligible for both survey, and 27 (14%) could not be reached for screening. Of the 109 people eligible for the survey of Black pregnant and postpartum people, 104 (95%) completed the online survey and were included in the analysis. Sample characteristics are summarized in Table 1. All participants identified as Black ($n = 100$) or multi-racial ($n = 4$) and all identified as women. The sample was geographically diverse, representing 23 US states.

Research access, participation, and preferences

Table 2 summarizes the survey respondents' prior experiences with research studies. Most of the women in this sample (69%, $n = 71$) reported having previously participated in a research study. However, only eight respondents (8%) reported ever having been asked to participate in a breastfeeding/chestfeeding or human milk research study, one respondent was unsure, and only three of these nine respondents (33%) reported having participated in such studies. Despite few having been asked, 59% ($n = 61$) of the respondents in this sample indicated that they would donate breast or human milk to a research study if asked.

Table 2 also summarizes respondents' preferences about where, how, and by whom they want to be approached about research studies related to breastfeeding/chestfeeding or human milk. A majority of respondents preferred to be approached about such studies in a healthcare (84%) or community (75%) setting, and preferred recruitment methods were to receive an email about the study (79%), see an advertisement about the study on social media (62%), or learn about it in person (55%). Most respondents were open to learning about breastfeeding/chestfeeding and human milk studies from a range of people, including a lactation support person (88%), doula (78%), or their health care provider (75%).

Table 3 summarizes the experiences of the nine respondents who were or may have been recruited for a prior breastfeeding/chestfeeding or human milk research study. Although the numbers are small, few respondents reported participating in such studies, receiving a breast pump or other supplies as part of a study requesting a human milk sample, or that the research team was diverse or consisted of a Person of Color. Most of these nine respondents (67%) reported receiving strong support from their support system during the time they were approached to participate in a breastfeeding/chestfeeding or human milk research study. For those who were recruited but did not participate in a breastfeeding/chestfeeding or human milk research study, respondents were split as to whether having a different type of support system would have motivated them to participate.

Knowledge of historical events and policies targeting Black populations

Figure 1 summarizes the survey respondents' knowledge of four historical events and policies targeting Black populations. Respondents reported being most knowledgeable about Jim Crow laws and least knowledgeable about Henrietta Lacks and the HeLa cell line (Table 1).

Respondent characteristics associated with research access and participation

Prior participation in a research study was significantly associated with a higher socio-economic composite score ($p = 0.027$, see

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TABLE 1 Sample characteristics ($N = 104$).

Sample characteristic	Statistics
Pregnancy /parenting status	
Pregnant, % (*n*)	25% (26)
Gestation in weeks, mean (SD) [range]	21 (8.8) [8–38]
Parenting a child ≤5 years old, % (*n*)	75% (78)
Age of youngest child in years, mean (SD) [range]	1.2 (1.1) [0–5]
Age in years	
Mean (SD)	32.1 (4.9)
Median [range]	32 [21–49]
Education, % (*n*)	
High school graduate or equivalent	8% (8)
Some college (1–3 years) or technical school	21% (22)
College graduate	34% (35)
Graduate school (Advanced degree)	37% (39)
Employment status, % (*n*)	
Employed for wages	56% (58)
Self-employed	6% (6)
Unemployed/Looking for work	9% (10)
Stay-at-home parent	22% (23)
Student	3% (3)
Unable to work	3% (3)
Prefer not to answer	1% (1)
Health insurance, % (*n*)	
Medicaid	31% (32)
Private insurance	60% (63)
Both medicaid and private Insurance	2% (2)
Tricare	4% (4)
Uninsured	3% (3)
Annual income, % (*n*)	
$0 - $25,000	17% (18)
$25,000 - $50,000	33% (34)
$50,000 - $75,000	18% (19)
$75,000 - $100,000	10% (10)
$100,000 and up	16% (17)
Prefer not to answer / missing	6% (6)
Socio-economic composite*, % (*n*)	*n* = 98*
0	9% (9)
1	20% (20)
2	18% (18)
3	22% (21)
4	31% (30)
Region of residence, % (*n*)	
Northeast	18% (19)
Midwest	11% (11)
South	35% (36)
West	36% (38)
Knowledge of historical events and policies targeting Black populations, mean (SD) [range]**	
Jim Crow laws	3.4 (1.1) [1–5]
Tuskegee Syphilis experiments	2.8 (1.2) [1–5]
J. Marion Sims' surgical experiments on enslaved African women	2.7 (1.3) [1–5]
Henrietta Lacks and the HeLa cell line	2.6 (1.4) [1–5]

*The socio-economic composite is the count of 4 socio-economic indicators (college education, employment, private insurance, and income > $50,000/year) and excludes the 6 participants who did not report an income.
** Knowledge scores ranged from 1 = "Not at all knowledgeable" to 5 = "Most knowledgeable (expert)."

Figure 2). Respondents with at least 3 socio-economic advantages were 4.1 times more likely to have participated in a research study as respondents with fewer than 3 socio-economic advantages (odds ratio = 4.1; 95% CI = 1.6, 10.3; $p = 0.002$). The indicators of socio-economic advantage most strongly associated with prior research participation were having a college degree (78% vs. 43%, $p = 0.001$) and income above $50,000 per year (80% vs. 58%, $p = 0.016$). Being employed or having private insurance were not significantly associated with research participation in this sample.

Prior participation in a research study was also associated with a higher knowledge composite score ($p < 0.001$, see Figure 3). Compared to respondents who reported knowledge of 0–2 of the historical events and policies, those knowledgeable of 3 were 3.8 times more likely to have participated in prior research (odds ratio = 3.8; 95% CI = 1.2, 11.7; $p = 0.023$) and those knowledgeable of all 4 were 10.4 times more likely to have participated in prior research (odds ratio = 10.4; 95% CI = 3.3, 32.7; $p < 0.001$). These associations were attenuated but remained significant when controlling for education (adjusted odds ratio for knowing 3 events/policies = 3.3, 95% CI = 1.02, 10.6; $p = 0.046$; adjusted odds ratio for knowing 4 events/policies = 7.7, 95% CI = 2.4, 25.4; $p = 0.001$). Knowledge of Henrietta Sacks and the HeLa cell line was the historical event most strongly associated with prior research participation, as respondents reporting knowledge of this event were 9.2 times more likely to have prior research participation than those who reported no knowledge of it (odds ratio = 9.2; 95% CI = 3.6, 23.6; $p < 0.001$; education-adjusted odds ratio = 7.2; 95% CI = 2.7, 19.3; $p < 0.001$). Prior participation in research was unrelated to respondents' age, pregnancy status at the time of the survey (pregnant or not), and region of residence.

Unlike prior research participation, willingness to donate human milk to a research study was not associated with respondents' socio-economic composite score ($p = 0.92$, see Figure 2) nor to any of the 4 indicators of socio-economic advantage. Respondents with no socio-economic advantages were just as likely as those with all 4 advantages to be willing to donate human milk to research (56% vs. 59%). Similarly, willingness to donate human milk to a research study was not associated with respondents' knowledge composite score ($p = 0.64$, see Figure 3) nor to knowledge of any of the 4 historical events and policies. Respondents with little knowledge of these events and policies were just as likely as those with more knowledge to be willing to donate human milk to research. Willingness to donate

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TABLE 2 Research experiences, interest, and preferences ($N = 104$).

Survey question	% (*n*)
Have you ever participated in a research study?	
No	28% (29)
Unsure	4% (4)
Yes	69% (71)
Have you ever been asked to participate in a breastfeeding/ chestfeeding OR human milk research study, before today?	
No	91% (95)
Unsure	1% (1)
Yes	8% (8)
If asked, would you donate breast or human milk to a research study?	
No	11% (11)
Unsure	30% (31)
Yes	59% (61)
Missing	1% (1)
Where would you prefer to be approached about such studies? (Check all that apply) [*listed in order of frequency*]	
At a healthcare setting	84% (87)
At a community setting	75% (78)
At a religious setting	20% (21)
Other setting: social media	13% (13)
Other setting: anywhere, stores, library, prenatal yoga, childcare centers, breastfeeding classes, non-profit, word of mouth from family/friends, somewhere I can ask real questions and get real answers	11% (11)
Other setting: online or virtually	6% (6)
Other setting: email, text	3% (3)
How would you prefer to learn about breastfeeding/ chestfeeding or human milk studies you might be eligible for? (Check all that apply) [*listed in order of frequency*]	
By sending an email about the study	79% (82)
By advertising the study on social media (please specify which ones): Instagram, Facebook, Twitter, Reddit, What to Expect, all platforms	62% (64)
In person	55% (57)
By sending a text message about the study	42% (44)
By advertising the study on a website (please specify which ones): hospital, university, or library websites, any website serving the needs of postpartum parents, Baby Center, ROSE, WebMD	16% (17)
By advertising the study on a smartphone app (please specify which ones): Facebook, Instagram, Twitter, Snapchat, What to Expect, The Bump, Peanut, Ovia, Flo, Glow, pregnancy and parenting apps	14% (15)
Some other way (please specify): podcasts, mailings, flyers, parenting magazines, doctor's office, radio, news, trusted community organizations/leaders, word of mouth	8% (8)
Who would you prefer to approach you about participating in breastfeeding/chestfeeding or human milk studies? (Check all that apply) [*listed in order of frequency*]	
A lactation support person	88% (91)
A doula	78% (81)
My healthcare provider	75% (78)
A community health worker	67% (70)
A member of the research team	63% (65)
A public health nurse	62% (64)
Someone else: someone I know/trust, family or friends, another mother/breastfeeding mom, Midwife, WIC staff, community leader	12% (12)

human milk was also unrelated to respondents' pregnancy status and region of residence, but respondents who were willing to donate human milk were significantly younger than those who were unwilling or unsure about donating [30.8 years (SD 4.2) vs. 33.8 years (SD 5.4), $p = 0.002$].

Due to the small numbers of respondents having been asked to participate in a breastfeeding/chestfeeding and/or human milk research study, potential associations between being asked and respondents' socio-economic advantage and knowledge of historical events could not be statistically evaluated in this sample.

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TABLE 3 Breastfeeding/chestfeeding and human milk research experiences among those who were or may have been recruited for such studies ($n = 9$).

Survey questions to those who answered "Yes" or "Unsure" to whether they had ever been asked to participate in a breastfeeding/chestfeeding OR human milk research study before today	% (*n*)
Have you ever participated in a breastfeeding/chestfeeding research study (focused on your experiences and behaviors) before today?	
No	56% (5)
Unsure	11% (1)
Yes	33% (3)
[*If "Yes" or "Unsure"*]	
Was the research team diverse or consist of a person of color?	*n* = 4
No	25% (1)
Unsure	50% (2)
Yes	25% (1)
Have you ever participated in a human milk research study (team asked for breast/human milk sample) before today?	
No	67% (6)
Unsure	–
Yes	33% (3)
[*If "Yes" or "Unsure"*]	
Did the research team provide you with a breast pump and other supplies?	*n* = 3
No	67% (2)
Yes	33% (1)
Was the research team diverse or consist of a Person of Color?	*n* = 3
No	33% (1)
Unsure	33% (1)
Yes	33% (1)
What type of support system did you have during the time you were approached to participate in a breastfeeding/ chestfeeding OR human milk research study?	
Strong support	67% (6)
Moderate support	–
Neutral support	22% (2)
No support	–
Negative support	–
Unsure	11% (1)
[*If "Yes" or "Unsure" if they ever participated in a breastfeeding/chestfeeding OR human milk research study*]	
How supportive was your support system supportive of your decision to participate in the breastfeeding/chestfeeding or human milk study?	*n* = 5
Very supportive	20% (1)
Somewhat supportive	20% (1)
Somewhat unsupportive	40% (2)
Very unsupportive	–
Unsure	20% (1)
[*If "No" or "Unsure" if they ever participated in a breastfeeding/chestfeeding OR human milk research study*]	
Would having a different type of support system have motivated you to participate in a breastfeeding/chestfeeding or human milk research study?	*n* = 7
Definitely yes	–
Probably yes	14% (1)
Probably not	29% (2)
Definitely not	14% (1)
Unsure	43% (3)

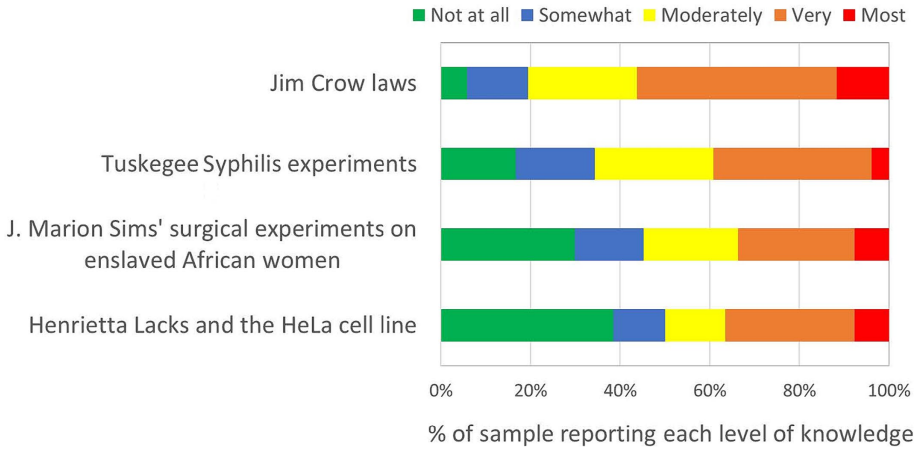

FIGURE 1
Knowledge of four discrimination-related historical events and policies ($n = 104$) on a scale ranging from "Not at all knowledgeable" to "Most knowledgeable (expert)."

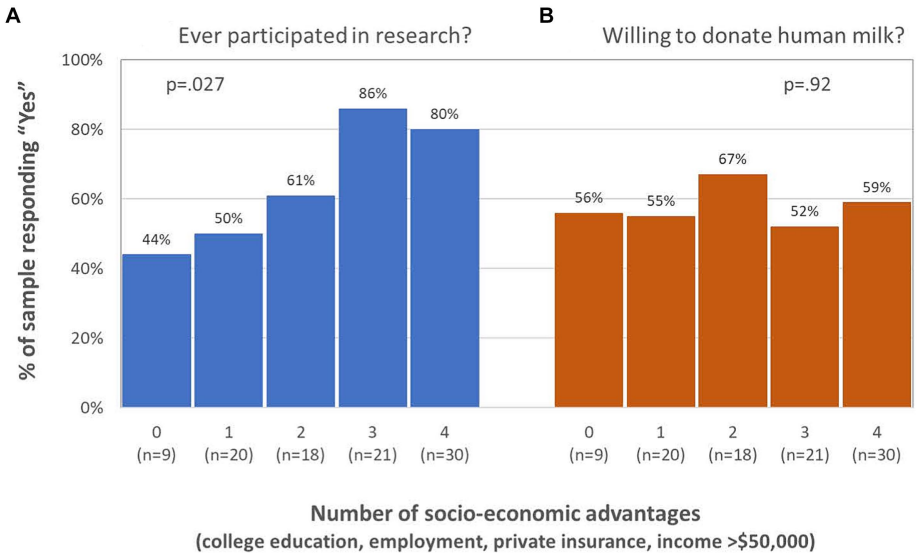

FIGURE 2
Greater socio-economic advantage is associated with **(A)** higher rates of prior research participation ($p = 0.027$), but not with **(B)** willingness to donate human milk to research ($p = 0.92$).

Discussion

We conducted a national, cross-sectional online survey of Black pregnant and postpartum people and found that a majority (69%) of respondents had participated in some type of research study, prior to being recruited for this study. Yet, very few (8%) of our study participants had ever been recruited to participate in breastfeeding/chestfeeding or human milk research studies. Surprisingly, most of our study participants (59%) shared that they would be willing to donate breast or human milk to a research study, if asked. These findings are important as they challenge current negative narratives related to Black women and biomedical and social science research participation (21). There is an erroneous notion that Black women and birthing people are not interested in research. These assumptions are harmful and further exacerbate existing biases and structural barriers. While issues related to perceptions, mistrust, or historical events do contribute to the lack of diversification noted in biomedical research studies, they are not the only drivers (22–24). Findings from our study suggest that one driver often overlooked may be access. Wendler *et al* noted that willingness to participate in health research differed slightly among racially minoritized and white individuals and that efforts to diversify research participation should focus on increasing access to study opportunities and not necessarily changing attitudes and perceptions (25).

Regarding breastfeeding/chestfeeding and human milk research, Palmquist et al. (17) noted that human milk researchers often do not address the ways systemic, structural, and historical factors – such as racism, colonialism, and power and privilege – impact and/or

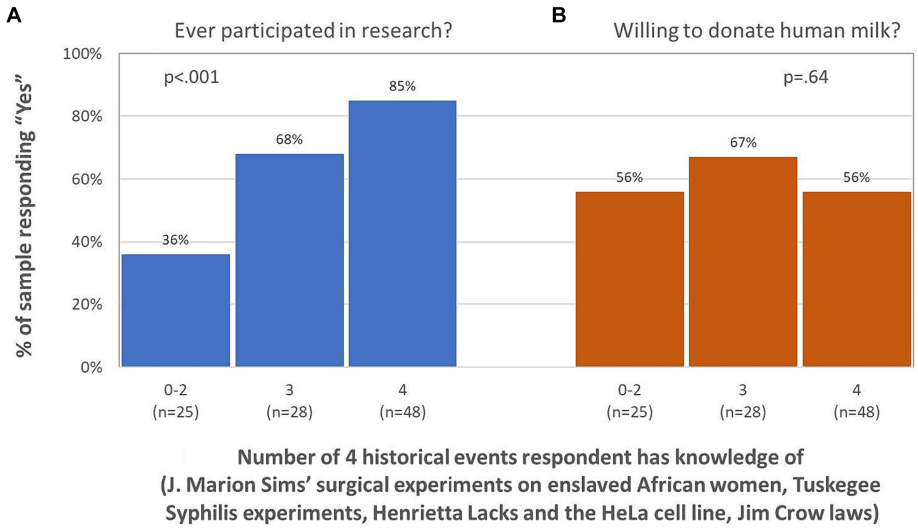

FIGURE 3
Greater knowledge of Black-targeted historical events/policies is associated with **(A)** higher rates of prior research participation ($p < .()OI$), but not with **(B)** willingness to donate human milk to research ($p = 64$).

influence study design, data collection, analysis, and interpretation. Of the few participants in our study who had previously participated in breastfeeding/chestfeeding and human milk research ($n = 3$), only one participant noted that the research team was diverse or consisted of a Person of Color. Further, inequities and disparities noted among the recruitment of diverse study samples in this field may be attributed to the lack of diversity among research teams, limited understanding of root causes of inequities and disparities, and different priorities. Health disparities and inequities are symptomatic of larger societal, historical, and economic policies and practices which perpetuate harm and rooted in structural racism (26). Researchers who share identities with marginalized populations are more likely to pursue research that addresses the needs of these communities, while contributing to the innovation that diverse research teams offer. Moreover, research has shown, minoritized researchers in the health professions are more likely to come from and want to serve historically excluded and minoritized populations and conduct health equity research (27). Strategic measures that address ethical and equitable community-engagement, such as those highlighted by the Breastmilk Ecology: Genesis of Infant Nutrition (BEGIN) Project Working Group (28) and critical investments are needed to address the lack of diversity in the breastfeeding/chestfeeding and human milk research pipeline (17).

Our study also found that prior research participation was associated with greater socio-economic advantage and increased knowledge of historical research activities or events such as the USPHS Syphilis Study at Tuskegee, HeLa cell line, and Marion Sims' inhumane experimentation of enslaved African women, etc. These findings are consistent with previous literature (29, 30). Henderson et al. (29), which focused on identifying Social Vulnerability Indicators and research participant attrition, found that financial-resource strain was associated with participants' inability to full participate and engage in their research study. Durant et al. (30) noted that previous clinical trial participation and not race was a higher predictor of future research participation. Additionally, they also found that knowledge of the Tuskegee Syphilis Study was positively associated with Black study participants' willingness to participate in research (30). This finding along with our data reinforce the importance of knowledge and awareness of past egregious research activities in this population. While interesting, this finding does not negate the lasting impact of mistrust and mistreatment experienced by Black communities (22).

Further, trust was a prominent finding in our study. Regarding research recruitment and engagement about breastfeeding/chestfeeding and human milk research studies, participants overwhelmingly preferred to be engaged in healthcare and/or community settings. These findings align with previous work focused on efforts to increase Black women representation in biomedical research studies (24, 31, 32). Authentic community engagement complimented by cultural humility are critical to the recruitment and retention of Black women and birthing people in breastfeeding/chestfeeding and human milk research studies. In addition, trusted healthcare professionals and individuals, such as Lactation Support Providers (e.g., International Board-Certified Lactation Consultants, Certified Lactation Educators, Certified Lactation Counselors, and Breastfeeding Peer Counselors) and Doulas were identified as the most preferred individuals to discuss breastfeeding/chestfeeding and human milk research studies with Black pregnant and postpartum people in our study, followed by Healthcare Providers. This finding is important as Lactation Support Providers, especially International Board-Certified Lactation Consultants (IBCLCs) can serve as a bridge between research teams and potential participants. IBCLCs have a solid understanding of human milk feeding and lactation and are required to complete an Introduction to Clinical Research course or similar content in order to obtain and/or renew their certification (33, 34). Given that Lactation Support Providers, along with Doulas, are found in both acute care and community-settings, collaborating with these professions may be an important strategy to increasing diversity among breastfeeding/chestfeeding and human milk research study participants.

Limitations

The perspectives presented in this article were from a small sample of Black pregnant and postpartum women. There is a risk of sampling bias, as this convenience sample of women who responded to study flyers and advertisements may differ from the broader population of Black pregnant and postpartum people, particularly those who do not identify as women. Due to the size of the sample, the confidence intervals for the reported associations are large, and small cell sizes precluded some analyses. Future research with a larger sample and/or including qualitative methods is needed to replicate and further elucidate these findings.

Conclusion

Inequities and disparities related to biomedical research participation persist. Thus, it is important to understand barriers and challenges associated with human milk research participation among Black pregnant and postpartum people. Limited discussions are being had in the human milk feeding and lactation landscape regarding increasing diversity among research participants. Greater efforts are needed to increase access to research opportunities, diversity research teams, and build collaborations with trusted community partners. A lack of diversity in human milk research is a missed opportunity for scientific discovery. Additional research is needed to develop recruitment and retention solutions, which are responsive to community concerns and reflect guidance from the communities most impacted by health inequities and disparities.

Data availability statement

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

Ethics statement

This study was approved by the UCSF Institutional Review Board and conducted in accordance with the local legislation and institutional requirements. The study participants provided both verbal consent during the eligibility screening and online consent as part of the online survey.

Author contributions

IA: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing, Project administration. CG: Formal analysis, Writing – original draft, Writing – review & editing. BG-B: Writing – review & editing. GN: Project administration, Writing – review & editing.

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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

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Early enteral nutrition with exclusive donor milk instead of formula milk affects the time of full enteral feeding for very low birth weight infants

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This study investigated the effects of exclusive donor milk or formula in the first 7 days after birth, on the time to full enteral feeding, growth, and morbidity of adverse events related to premature infants. This was a retrospective study carried out from July 2014 to December 2019 at the Department of Neonatology of Shanghai Children's Hospital. All infants with a birth weight < 1,500 g and a gestational age ≤ 32 who received exclusive donor milk or formula in the first 7 days after birth were included in this study. The time to full enteral feeding (defined as 150 mL/kg) in the donor milk group was significantly shorter than in the formula group (18 vs. 22 days, $p = 0.01$). Donated breast milk was also associated with a lower incidence of NEC (4.4 vs. 7%, $p < 0.01$), ROP (3.8 vs. 13.2%, $p < 0.01$), and culture-confirmed sepsis (11 vs. 22.6%, $p < 0.01$). Using donated breast milk instead of current formula milk for early enteral nutrition can shorten the time to full enteral feeding and reduce the incidence of NEC, ROP, and sepsis.

KEYWORDS

full enteral feeding, donor milk, formula milk, premature infants, enteral nutrition

1 Introduction

In recent years, the number of premature infants has been increasing year by year with the changes in the environment and people's lifestyles (1). It has been reported that prematurity accounts for more than 10% of new births each year worldwide and has become one of the leading causes of neonatal death. The survival rate of infants born extremely preterm (gestational age less than 28 weeks) was 62.3% in China (2). Approximately 15% of these preterm babies weigh less than 1,500 g, belonging to very low birth weight (VLBW) (3). Compared to full-term infants, preterm infants have less well-developed organs, weaker immune function, and a significantly higher incidence of neonatal necrotizing enterocolitis (NEC), sepsis, and other related complications. The incidence rate of sepsis varies from 20 to

40%, while the incidence of NEC is approximately 7% (4, 5). Breast milk contains all the nutrients and important antibodies needed for early infant growth, and it is important for improving feeding tolerance, reducing infections, promoting neurological development, and improving long-term prognosis (6–9). It has been suggested that VLBW infants who receive higher amounts of breast milk may have reduced morbidity and mortality (10–14). VLBW infants fed with their own mother's milk have a shorter duration of full enteral feeding and 6–10 times lower NEC incidence (15–18).

After birth, nutritional feeding should be started as soon as possible to enhance enteral feeding tolerance, preferably starting with colostrum, which has a high level of anti-infective properties. However, most preterm infants do not have access to parental breastfeeding during their hospital stay, especially during the first hours of life. Even if the mother starts pumping as soon as possible, she will not provide enough breast milk within 3 days of birth. In recent years, breast milk banks have emerged (19). Qualified donor breast milk has become an important source of enteral nutrition for preterm infants due to the similar nutritional composition and functions as mother's milk, greatly satisfying the demand for early breastfeeding of preterm infants (20, 21). Breast milk banking has changed the feeding pattern of preterm babies to some extent (22).

Our department has established a breast milk bank since June 2016 to provide breastfeeding support to hospitalized premature infants in the neonatal department and advocates the early use of donated breast milk for enteral nutrition (mother's milk is unavailable). In this article, we investigate the effect of using exclusive donor breast milk or formula in the first 7 days after birth on the time of full enteral feeding, growth, and morbidity of adverse events related to premature infants.

2 Materials and methods

2.1 Design and setting

This was a retrospective study on the effect of donor milk feeding within 7 days after birth on preterm infants. Preterm infants with gestational age ≤ 32 weeks or birth weight < 1,500 g admitted to the neonatal intensive care unit (NICU) of Shanghai Children's Hospital from July 2014 to December 2019 were retrospectively included in the study. Inclusion criteria: admission within 24 h after birth, hospitalization for more than 2 weeks, and exclusive donor milk or formula feeding within 7 days after birth. Exclusion criteria included dysplasia of the digestive tract, severe congenital heart disease (excluding atrial septal/ventricular septal defect, patent ductus arteriosus, and patent foramen ovale), systemic metabolic diseases, mixed feeding or exclusive mother milk breastfeeding within 7 days after birth.

Infants received exclusive pasteurized donor milk or preterm formula (Nestle preNAN) during the first 7 days of life. After 7 days, infants received their mother's breast milk if their own mother's milk was available. After full enteral feeding, some infants in the donor milk group may be fed with preterm formula, as donated breast milk should be used preferentially for low gestational age infants. The numbers for infants with full enteral feeding in the donor milk group and formula group were 175 and 204, respectively. All parents were informed that their infant would receive donor milk and provided informed consent. This study has been approved by the Ethics Committee of Shanghai Children's Hospital.

2.2 Standardized feeding regimen

The enteral feeding protocol for preterm infants weighing less than 1,500 g follows the guidelines published by the American Academy of Pediatrics and the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition. All infants with very low birth weight received a gastric catheter during the first hour of life. At the same time, enteral nutrition (EN) was started within 24 h of birth in clinically stable preterm infants. Enteral feeding was advanced by approximately 20 mL/kg/day based on the infant's feeding tolerance and total enteral feeding was defined as 150 mL/kg of enteral intake (23). If infants were breastfed, human milk fortifier (PreNAN, Nestle) was added at 100 mL/kg/day of EN, first at half-strength for 48 h and then at full strength. Once total enteral nutrition was achieved, parenteral nutrition (PN) was discontinued and central venous catheterization was removed if there was no need for continuous intravenous medication. During the transition phase, a "nutrition-based" transition program was implemented, aimed at maintaining target kcal and protein intakes. Parenteral glucose, amino acid, and lipid prescriptions were adjusted daily to maintain a goal energy intake of 100–120 kcal/kg/day (PN + EN) and protein intake of >3 g/kg/day, with parenteral lipids providing ≤50% of the parenteral energy. Serum glucose and triglycerides were maintained within acceptable limits.

2.3 Data collection and definitions

Basic information and clinical data were collected from the electronic medical record system. Basic information collected included gender, gestational age, birth weight, and mode of birth. Clinical data included time to start oral feeding, time to reach full enteral feeding, Apgar score, amount of enteral nutrition at 14 days, hospital stay, and weekly weight. The main complications of premature infants include NEC, intracranial hemorrhage (grade 3–4), bronchopulmonary dysplasia (BPD), late-onset sepsis (LOS), and the retinopathy of prematurity (ROP). The diagnostic and grading criteria for BPD were based on the definition of Jensen in 2019 (24). Respiratory support is still required at the corrected age of 36 weeks and the severity of respiratory support is graded based on the situation. NEC was defined using Bell staging criteria, stage 2 or greater (25). ROP is diagnosed based on fundus screening. Sepsis was defined as a positive blood culture and a C-reactive protein level>10 mg/L (4). C-reactive protein levels in patients' serum were measured using the QuikRead go instrument with the QuikRead go hsCRP kit (Highly Sensitive Particle Enhanced Turbidimetric Method).

2.4 Statistical methods

The data were statistically analyzed using SPSS 25.0 statistical software. Normally distributed data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and two-sample *t*-tests were used to compare the two groups. Non-normally distributed data were expressed as medians (interquartile spacing) [M (P25, P75)], and the Wilcoxon test was used

for comparison between groups. The enumeration data were expressed as a percentage (%), and comparisons between groups were using the χ^2 test or Fisher exact probability method. $p < 0.05$ indicated a statistically significant difference. Adjusted OR and its confidence intervals (95% CI) were determined using a logistic regression model and a value of p less than 0.05 was considered significant. Multivariate logistic analysis was adjusted with gestational age, sex, birth weight, mode of birth, antibiotics, antenatal steroids, and Apgar score at 5 min.

3 Results

3.1 Characteristics of the sample

A total of 394 preterm infants fulfilled the study criteria. Two hundred and twelve (53%) infants received exclusive formula and 182 (47%) were fed exclusive donor milk during the first 7 days of life. There were no statistically significant differences in birth weight, gestational age, sex, birth way, multiple birth rate, 5 min Apgar score, small for gestational age (SGA), maternal antibiotics use, and maternal steroid use between the two groups ($p > 0.05$). As shown in Table 1.

TABLE 1 Characteristics of study infants.

	Formula group	Donor milk group	*p*-value
N	212	182	
Birth weight (g)	1,214 ± 345	1,183 ± 296	0.53
Gestational age (w)	29.3 ± 2.0	29.1 ± 2.8	0.91
Male sex (%)	124 (58.5%)	93 (51.1%)	0.14
Cesarean section (%)	154 (72.6)	129 (70.9)	0.70
multiple births (%)	63 (29.7)	65 (35.7)	0.21
Apgar score at 5 min < 7 (%)	41 (19.3)	39 (21.4)	0.61
SGA (%)	38 (17.9)	23 (12.6)	0.15
Maternal antibiotics (%)	150 (70.7)	119 (65.4)	0.25
Maternal steroids (≥1 does) (%)	156 (73.6)	118 (64.8)	0.06

SGA, Small for gestational age.

3.2 Enteral feeding and growth

A total of 15 children in both groups died before reaching total enteral nutrition (eight in the formula group and seven in the donor milk group) and were therefore not included in the calculation of the time to reach full enteral feeding. The causes of death can be found in Supplementary Table S1. As expected, enteral feeds were initiated within 24 h. The amount of enteral nutrition on day 14 is 111.2 ± 13.4 in the formula group and 116.8 ± 17.2 in the donor milk group. Infants in the donor milk group achieved full enteral feeding 5 days earlier than those in the formula group, with a median of 13 days [interquartile range (IQR) 16–21], compared to a median of 18 days (IQR 22–28, $p = 0.01$) in the formula group. Infants in the donor milk group stayed 7 days shorter in the NICU than infants in the formula group, with a median of 50 days (IQR 47–53, $p = 0.01$) vs. a median of 43 days (IQR 40–47, $p = 0.12$).

There was no statistically significant difference in birth weight, 7-day weight, and 14-day weight between the two groups, but there was a difference in the discharge weight. Infants in the donor milk group weighed 2,532 ± 823 g and the weight in the formula group was 2,205 ± 754 g ($p = 0.04$). The absolute weight changes in individual birth weights were shown in Supplementary Figure S1. The weight of both groups of infants did not show a significant decrease in the first 7 days, but both showed a significant increase after 14 days. The weight gain at discharge was different between the two groups, with the formula group showing a greater increase. The difference in discharge weight may be related to the later discharge of the formula group (Table 2).

TABLE 2 Enteral feeding and body growth of the whole population.

	Formula group	Donor milk group	*p*-value
	N = 212	*N* = 182	
Onset of feeds (h)	22 (16–30)	18 (13–27)	0.45
Amount enteral feeding on day 14 (mL/kg)	111.2 ± 13.4	116.8 ± 17.2	0.33
Full enteral feeding (d)	18 (9–108)	13 (10–98)	0.01
Hospitalized days (d)	50 (9–145)	43 (1–142)	0.12
Birth weight (g)	1,214 ± 345	1,183 ± 296	0.53
Weight day 7 (g)	1,182 ± 355	1,217 ± 305	0.69
Weight day 14 (g)	1,473 ± 468	1,391 ± 414	0.28
Weight at discharge (g)	2,532 ± 823	2,205 ± 754	0.04
Died before reached full enteral feeding	8 (3.8%)	7 (3.8%)	0.84

3.3 Morbidities related to premature infants

In the whole population, 17 infants (8%) in the formula group developed NEC compared to six infants (3.3%) in the donor milk group ($p = 0.04$). Forty-eight infants (22.2%) in the formula group had culture-proven sepsis compared to 20 infants (11.5%) in the donor milk group ($p < 0.01$). Despite the differences in infectious outcomes, including NEC and culture-proven sepsis, we observed no differences in the rates of intubation, bronchopulmonary dysplasia, or intraventricular hemorrhage between the two groups (Table 3). In addition, the incidence of retinopathy of prematurity (ROP) significantly decreased in the donor milk group. After adjusting for the relevant confounders, exclusively formula feeding (OR 3.48, 95% CI 2.13–8.06, $p = 0.04$) in the first 7 weeks was significantly associated with a higher incidence of NEC, compared with the donor milk feeding. Similarly, exclusively formula feeding in the first 7 weeks was independently associated with an increased risk of ROP (OR 3.95, 95% CI 0.78, 9.05, $p < 0.01$) or culture-proven sepsis (OR 2.53, 95% CI 1.18–7.48, $p < 0.01$) compared with the donor milk feeding.

Further, we grouped preterm infants according to gestational age (GA) <28 weeks and >28 weeks to observe whether early breast milk feeding would be beneficial for extremely premature infants, as shown in Table 4. We found 241 premature infants (61.2%) with GA >28 weeks and 153 premature infants (38.8%) with GA<28 weeks. The

TABLE 3 Outcomes of different groups.

	Formula group	Donor milk group	OR* (95% CI)	*p*-value
N	212	182		
Intubated	45 (21.2)	41 (22.5)	1.04 (0.31–3.03)	0.76
NEC	17 (8%)	6 (3.3%)	3.48 (2.13–8.06)	0.04
IVH III + IV	19 (9%)	11 (6%)	0.12 (0.02, 0.90)	0.62
BPD	33 (15.6%)	23 (12.6%)	0.16 (0.05–0.36)	0.41
ROP	28 (13.2%)	7 (3.8%)	3.95 (0.78, 9.05)	<0.01
Culture-proven sepsis	47 (22.2%)	21 (11.5%)	2.53 (1.18–7.48)	<0.01

Multivariate logistic analysis was adjusted with gestational age, sex, birth weight, mode of birth, antibiotics, antenatal steroids, and Apgar score at 5 min. The Donor milk group was the reference group. NEC, Necrotizing enterocolitis; IVH, Intraventricular hemorrhage; BPD, Bronchpulmonary dysplasia; ROP, Retinopathy of prematurity; OR, Odds ratio; and CI, Confidence interval. *Multivariate analysis with adjusted OR using logistic regression.

TABLE 4 Prevalence of preterm infant comorbidities in different gestational weeks.

	GA > 28 weeks (*N* = 241)			GA < 28 weeks (*N* = 153)		
	Formula group	Donor milk group	*p*-value	Formula group	Donor milk group	*p*-value
N	140	101		72	81	
Intubated	25 (17.9%)	19 (18.8%)	0.85	20 (27.8%)	22 (27.2%)	0.93
NEC	9 (6.4%)	4 (4%)	0.40	8 (11.1%)	2 (2.5%)	0.03
IVH III + IV	8 (5.7)	2 (2)	0.15	11 (15.3)	9 (11.1)	0.45
BPD	21 (15)	12 (11.9)	0.49	12 (16.7)	11 (13.6)	0.59
ROP	17 (12.1)	3 (3)	0.11	11 (15.3)	4 (4.9)	0.03
Culture-proven sepsis	29 (21)	12 (11.9)	0.07	18 (25)	9 (11.1)	0.02

NEC, Necrotizing enterocolitis; IVH, Intraventricular hemorrhage; BPD, Bronchpulmonary dysplasia; ROP, Retinopathy of prematurity.

mobility of almost all adverse events was increased in preterm infants with GA<28 weeks compared to GA> 28 weeks, except for the donor milk group where there was a decrease in the incidence of NEC and sepsis. It can be found that early feed with donor milk has a more pronounced protective effect on preterm infants younger than 28 weeks in terms of the occurrence of NEC, ROP, and sepsis.

3.4 Feeding at discharge

Preterm infants received exclusive formula or donor milk feeding within 7 days after birth. After 7 days, infants received donor milk or formula until their mother's breast milk was available. If their mother's breast milk was unavailable, they received donor milk or formula until they were discharged from the hospital. 26.4% of infants in the donor milk group were still fed—donor milk at discharge. 47.7% of infants in the formula group and 62.1% in the donor milk group received their mother's own milk at discharge. The type of milk at the start of enteral nutrition did influence the type of feeding at discharge (Table 5).

4 Discussion

In this retrospective study, we show that the intake of donor human milk during the first 7 days of life is associated with shorter full enteral feeding time and decreased morbidity of adverse events related to premature infants. The results of this study are consistent with the findings of previous studies, which showed that compared with formula, donated breast milk could significantly reduce the incidence of NEC and the time of full enteral feeding (26–29). We further substantiate and expand the pool of evidence suggesting a relationship between the donated breast milk the premature infants received and the lower risk of ROP or sepsis. In addition, we use donated breast milk exclusively within 7 days after birth to better illustrate the role of donated breast milk in early enteral nutrition for premature infants. These studies demonstrated that human milk intake is particularly important in the first few days of life. Feeding preterm infants with donor breast milk (rather than formula when the mother's breast milk is not available) is associated with shortened time to full enteral feeding and reduced incidence of NEC, ROP, and sepsis. And it has a more pronounced protective effect on preterm infants with GA<28 weeks.

Breast milk is rich in immune active factors and low osmotic pressure has a good protective effect on the fragile intestinal barrier of very/ultra preterm infants. Donor milk has also been shown to promote feeding tolerance. Meta-analyses have shown that donor milk reduces the incidence of feeding intolerance compared to formula for preterm infants (30). An international survey of enteral feeding practices for very low birth weight infants also found that wards with donated breast milk sources can better establish total enteral feeding (31). As a result, the American Academy of Pediatrics recommends donor milk as a supplement or replacement for very low birth weight infants when parental breast milk is insufficient or contraindicated (32). The emphasis on donor milk feeding in the

TABLE 5 Type of feeding at discharge.

	Formula group	Donor milk group	*p*-value
	N = 212	*N* = 182	
Mother's own milk	58 (27.4%)	63 (34.6%)	0.12
Donor milk	0	16 (8.8%)	<0.01
Formula	97 (45.8%)	34 (18.7%)	<0.01
Mother's own milk + Donor milk	0	22 (12.1%)	<0.01
Mother's own milk + Formula	43 (20.3%)	28 (15.4%)	0.21
Donor milk + Formula	0	10 (5.5%)	<0.01
Died before discharge	14 (6.6%)	9 (4.9%)	0.48

standardized enteral feeding protocol developed in conjunction with the availability of a breast milk bank in our hospital has contributed to a significantly higher rate of early donor milk use in very low birth weight infants, which may have been an important reason for the shortened time to achieve total enteral feeding.

In our study, we generally started enteral feeding within 24 h. Previous studies have found that the structural and functional integrity of the gastrointestinal tract in preterm infants is closely related to early enteral feeding. Postnatal fasting can cause thinning of the intestinal mucosa, flattening of the villi, and translocation of intestinal bacteria (33). Therefore, delayed enteral nutrition may be detrimental (34). Although early enteral feeding is a low-calorie, low-volume feeding, it facilitates the maintenance of higher gastrointestinal hormone levels and promotes continued maturation of intestinal motility, microbiome development and gastrointestinal function in preterm infants, thus improving tolerance of early enteral feeding (35–39). Therefore, the early use of donor breastfeeding advances the time of initiation of enteral feeding and facilitates the establishment of enteral nutrition.

This study found that early exclusive donor breastfeeding was associated with a reduced incidence of NEC, sepsis, and ROP in preterm infants. Many studies have also confirmed that donated breastfeeding can reduce the incidence of nosocomial infection, sepsis, and NEC (29, 40, 41). Quigley et al. (14) found that donor milk reduced NEC and helped improve neurodevelopment in a study of 1809 preterm infants fed with donor milk and formula. Villamor-Martínez et al. (42) also showed that donor milk feeding may prevent bronchopulmonary dysplasia. The neurodevelopmental benefits of donor breast milk for preterm infants are now quite clear, with meta-analyses showing that breastfed infants have higher levels of cognitive development compared to formula-fed infants and that the neurodevelopmental system of preterm infants is stimulated by certain nutrients in breast milk. In addition, the improved growth, health, and development can be attributed to lower inflammation among breast milk fed infants. Konnikova et al. found that the anti-inflammation and immunomodulating components of breast milk help to protect the preterm infant against infections, meningitis, and late-onset sepsis (43–45). Konnikova et al. found that breast milk can reduce the inflammatory condition of the intestine, thereby reducing NEC. They have shown that tryptophan and bifidobacterium in breast milk produce a metabolite response that acts as an anti-inflammatory by inhibiting aryl hydrocarbon receptor transcription factors that stimulate the IL-8 response (46). Breast milk contains many protective anti-inflammatory components that prevent excessive inflammation until the infant can develop its mature anti-inflammatory mechanisms.

In previous clinical work, we have not focused specifically on the relationship between donor breastfeeding and retinopathy of prematurity. In this study, we specifically observed that donated breast milk was more beneficial in reducing the incidence of retinopathy of prematurity in preterm infants. These results were consistent with existing research findings. Hylander et al. (47) showed that after excluding other confounding factors, breastfed very low birth weight infants had a lower incidence of neonatal retinopathy than formula-fed infants, suggesting that breastfeeding is also important for the neurological development of preterm infants. Another study found that any amount of human milk intake is strongly associated with protection from all ROP and severe ROP (48). They hypothesized that the antioxidant properties of breast milk and high levels of dodecahexaenoic acid are the reasons for the protective effect on ROP.

The occurrence of increased NEC, sepsis, or ROP with preterm formula may be related to the following reasons. Preterm formulas do not retain the immune components and active substances found in breast milk, such as lactoferrin, lysozyme, lipase, immunoglobulins, cytokines, and oligosaccharides, which promotes the development of bodies, helps with the maturation of the infant's immune system (10, 43) and protects against infections. The composition of preterm formulas affects milk osmolality and the renal load, the latter may be a further factor in determining fluid intakes in preterm infants with limited renal concentration ability and excretory capacity (49, 50), thus affecting the water-electrolyte balance of the preterm infants. Furthermore, the osmotic pressure of milk affects intestinal permeability and inflammation, which can cause incomplete digestion of formula and increase the incidence of NEC (51, 52).

It is interesting to note that donating milk on admission increases the proportion of human milk feeding at discharge (62.1 vs. 47.7%), indicating that donating milk affects the feeding type at discharge. This is related to the promotion of breastfeeding through written, online, and social propaganda since the establishment of our breast milk bank, as well as the implementation of hospital breastfeeding guidance, emphasizing the importance of breast milk for the growth and development of premature infants and newborns.

There are limitations to this study. It is a single-center retrospective study, which has inherent limitations except for a small sample size. The data are influenced by the level of detail in medical records. We therefore selected indicators for analysis that are present in medical records.

5 Conclusion

In this study, we show that the intake of exclusive donor milk during the first 7 days of life is associated with shorter full enteral feeding time and decreased morbidity of adverse events related to premature infants, illustrating the importance of early donor milk feeding.

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 humans were approved by Ethics Committee of Shanghai Children's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MW: Data curation, Methodology, Software, Writing – original draft. XG: Resources, Validation, Writing – review & editing. LY: Data curation, Methodology, Software, Writing – original draft. FS: Data curation, Software, Writing – review & editing. DL: Data curation, Formal analysis, Investigation, Writing – review & editing. QF: Resources, Validation, Writing – review & editing. TZ: Writing – review & editing. XY: Validation, Supervision, Visualization, Writing – review & editing.

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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.2024.1345768/full#supplementary-material

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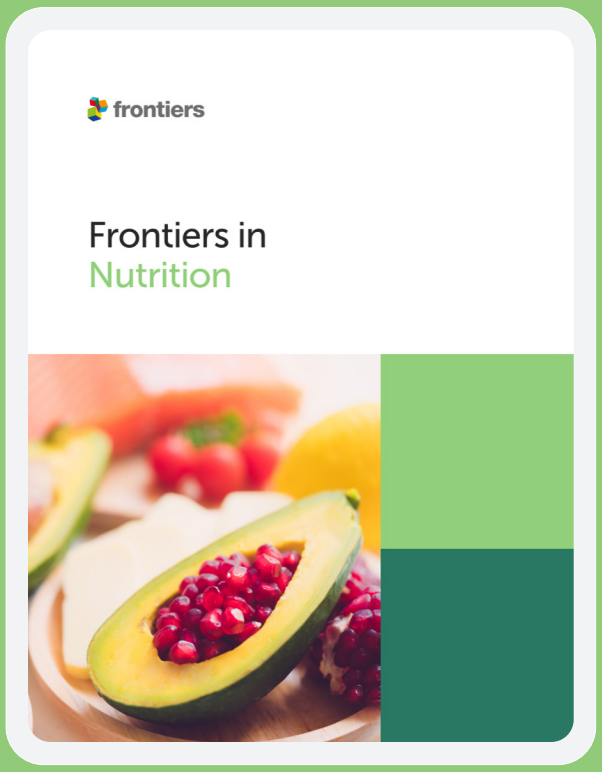

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