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Macronutrient concentrations in human milk beyond the first half year of lactation: a cohort study

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ABSTRACT

Objective Human milk composition is dynamic. While extensive research has focused on its macronutrient concentrations during the first 6 months of lactation, limited research exists for extended lactation periods. This study aims to examine the nutritional composition of human milk during these longer lactation phases.

Design A retrospective longitudinal cohort study performed within the National Dutch Human Milk Bank.

Participants We selected donors who had provided milk donations at least once after the 6-month postpartum mark.

Main outcome measures The Miris Human Milk Analyser was used to analyse macronutrient concentrations in the milk samples. Linear mixed models were used for longitudinal analysis of these concentrations, factoring in time variables established for six sequential lactation periods.

Results We analysed 820 milk samples from 86 women, collected between 5 weeks and 28 months postpartum. Initially, milk protein concentrations dropped over the first 8 months of lactation (diff = −0.19 g/dL, p<0.001) and stabilised between 8 and 18 months before increasing again by 0.21 (95% CI 0.06–0.21) g/dL. Carbohydrate concentrations remained steady throughout the study period. Fat concentrations were stable for the first 8 months but saw an increase afterwards. Post 18 months, the fat content saw a rise of 1.90 (95% CI 1.59–2.21) g/dL. The caloric density mirrored the pattern of the fat concentrations.

Conclusion The nutritional content of human milk does not decrease after 6 months of lactation. Therefore, human milk banks may accept donations from mothers up to 2 years post-birth.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Previous research has emphasised the changing nutritional composition of human milk in the first 6 months post-birth with limited study of its composition beyond this period. Existing literature shows discrepancies due to varied lactation durations and measurement techniques leaving a significant gap in understanding extended breastfeeding.

WHAT THIS STUDY ADDS

⇒ Our study, with the largest sample size and most consecutive samples per donor to date, found that human milk maintains stable macronutrient content over 2 years of lactation, supporting its potential suitability for donation to preterm infants.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These insights could influence guidelines for human milk banks, enhancing their donation acceptance policies to better serve neonatal intensive care units.

INTRODUCTION

Human milk is universally acknowledged as the ideal source of nutrition for infants.[1–3] Its composition is uniquely dynamic, adapting to fulfil the evolving nutrition needs of a developing child.[4 5] Previous research demonstrated that protein concentrations decrease during the first 6 months post-birth[6] while fat content can increase.[7 8] However, there is limited knowledge about the composition of human milk beyond the first 6 months of lactation.[9–12] These data come, however, from relatively few women and showed inconsistent results.

The WHO advises that infants should be exclusively breastfed for the first 6 months, supplemented by other food but still breastfeeding for at least 2 years.[13] Given the current lack of understanding about the nutritional content of human milk during prolonged breastfeeding, it is crucial to fill this knowledge gap.

This information is also crucial for donor human milk (DHM) banks which depend on human milk altruistically donated by mothers who produce an excess of milk and might lactate for lengthy periods. When a mother's milk is unavailable or insufficient, donated human milk becomes the preferred alternative to preterm formula for very preterm infants.[14–16] Currently, there is no consensus or established guidelines from the European Milk Bank Association (EMBA) or the Human Milk Banking Association of North America (HMBANA) regarding the duration a woman should be allowed to donate milk to a bank during extended lactation or whether this milk, even with multinutrient fortification, is nutritionally less suitable for preterm infants.[17]

This study aims to measure the macronutrient composition after the first 6 months of lactation. The results could guide human milk banks in establishing their acceptance policies.

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METHODS

Study design

This study is a retrospective, longitudinal assessment conducted by the Dutch National Human Milk Bank, housed in the Amsterdam University Medical Centre. It used an existing database containing donor information from December 2017 to July 2023. The National Milk Bank currently serves all neonatal intensive care units in the Netherlands offering DHM for extremely premature infants (those with a gestational age of less than 30 weeks) whose mothers cannot produce adequate milk. On average, our facility receives milk from 25 to 40 active donors simultaneously.

Significant variation exists among donors regarding their postpartum donation timeline, duration and amounts. All potential milk bank donors must complete a rigorous approval process which includes serologic testing and a comprehensive questionnaire. It covers topics such as smoking habits, maternal body mass index (BMI), alcohol and drug usage, diet and medication. Simultaneously, data about the child including their sex and gestational age are collected.

Donating breast milk is entirely voluntary for the mothers involved. Every participant whose milk was used in this study provided approval via a non-objection form. The form allowed the non-interventional investigation of data and milk used for quality assurance metrics, not given to premature infants (NL37296.029.11).

Inclusion criteria

We focused on the macronutrient composition during extended lactation. Therefore, we selected only donors from our database who provided milk at least once beyond the first 6 months postpartum. Our analysis included all available samples from these qualifying mothers even those obtained within the first 6 months after birth.

Procedures

When donating, women were instructed to express the total contents of a breast using either a breast pump or by hand instead of expressing the fore or hindmilk separately. Donated milk batches were stored at −20°C for no more than 3 months before further processing. Multiple batches from the same donor were combined into a single pool of up to 2000 mL which was then divided into smaller bottles and pasteurised. A small 10 mL aliquot was saved for quality assurance and macronutrient content analysis. This analysis was performed using a commercial human milk analyser with mid-infrared technology (Miris, Uppsala, Sweden) equipped with 2013 software. The analyser measures concentrations of protein, carbohydrates and fat based on spectral content. Energy content is calculated by applying Atwater factors to the measured concentrations of these macronutrients.

The Miris Human Milk Analyser measures both lactose and oligosaccharides in calculating the carbohydrate concentration. It measures true protein by assessing total nitrogen, then adds 20% to account for non-protein nitrogen resulting in an estimate of crude (total) protein content.[18] This study only reports the crude protein concentration.

Before analysing the macronutrient concentrations in each pool, the milk was first homogenised using the Miris Ultrasonic Processor and then warmed to the ideal measurement temperature range of 35–40°C. Each sample was tested in duplicate with 3 mL of milk being applied to the analyser's injector for each evaluation. If the results from these two analyses showed a discrepancy greater than 0.4 g/100 mL in protein concentration, a third run would be done on the same sample using another 3 mL portion. The average value of the results was then calculated.

Statistical analyses

Milk from different pump dates was merged into one 2 litre pool for analysis with a corresponding average pump date calculated for each analysis date. If multiple measurements were conducted within the same week, the mean macronutrient concentrations for that week were calculated. We determined the number of weeks between the average pump date and the birth date.

For statistical analysis, we initially segregated lactation into six distinct periods (0–2 months; 2–4 months; 4–8 months; 8–12 months; 12–18 months; and>18 months). Multiple samples from the same donor could be analysed within each period.

We performed statistical analyses using SPSS V.28 for Windows (SPSS). Mean values±SD were calculated for all samples from each donor during each lactation period. We used linear mixed models (LMMs) to analyse the time-dependent variations in breast milk's fat, protein, carbohydrates and energy content. LMMs help deal with longitudinal data by correlating measurements at different times within a donor via a random intercept at the donor level. These analyses were adjusted for gestational age at birth and maternal BMI. We included these covariates as there is evidence suggesting that gestational age can affect breast milk protein concentration and that BMI is associated with breast milk fat concentration and energy content.[19 20] We considered a p value of<0.05 to be statistically significant in all analyses.

RESULTS

Our study included 86 women who had donated milk at least once to our milk bank after 6 months postpartum. From these donors, milk was collected between 5 weeks and 28 months postpartum and 820 pools of approximately 2 litre were created, of which samples were analysed for macronutrients. Thus, on average, we measured macronutrients in samples from nearly 10 2-litre pools per donor, but this ranged from 1 to 48 pools. The volume of individual donated bottles from each donor ranged from 50 to 200 mL, and all samples combined into a single pool were collected over several weeks or up to 2 months. These 820 samples were divided into six different lactation periods: 0–2 months (25 samples, 8 donors), 2–4 months (98 samples, 20 donors), 4–8 months (381 samples, 63 donors), 8–12 months (175 samples, 45 donors), 12–18 months (91 samples, 12 donors), and>18 months (50 samples, 6 donors).

On beginning their donations, the women had an average age of 37.4 years with an SD of 3.7 and an average BMI of 25.0 kg/m^2, with an SD of 4.7. The average gestational age at childbirth was 38.1 weeks, with a SD of 3.7.

The concentrations of macronutrients as well as the energy content of all milk samples are depicted in figure 1A–D with average values for each lactation period given in table 1. The statistical analysis of these results shows a gradual decrease in protein concentration from the first 2 months of lactation persisting until the 8th month. After this, the protein concentration stabilises until the 18th month at which point it begins to rise again (see online supplemental table 1). For instance, during the second to fourth month, the average protein concentration in milk was about 0.12 g/100 mL less than that of the first 2 months after birth, featuring a 95% CI between −0.18 and −0.06.

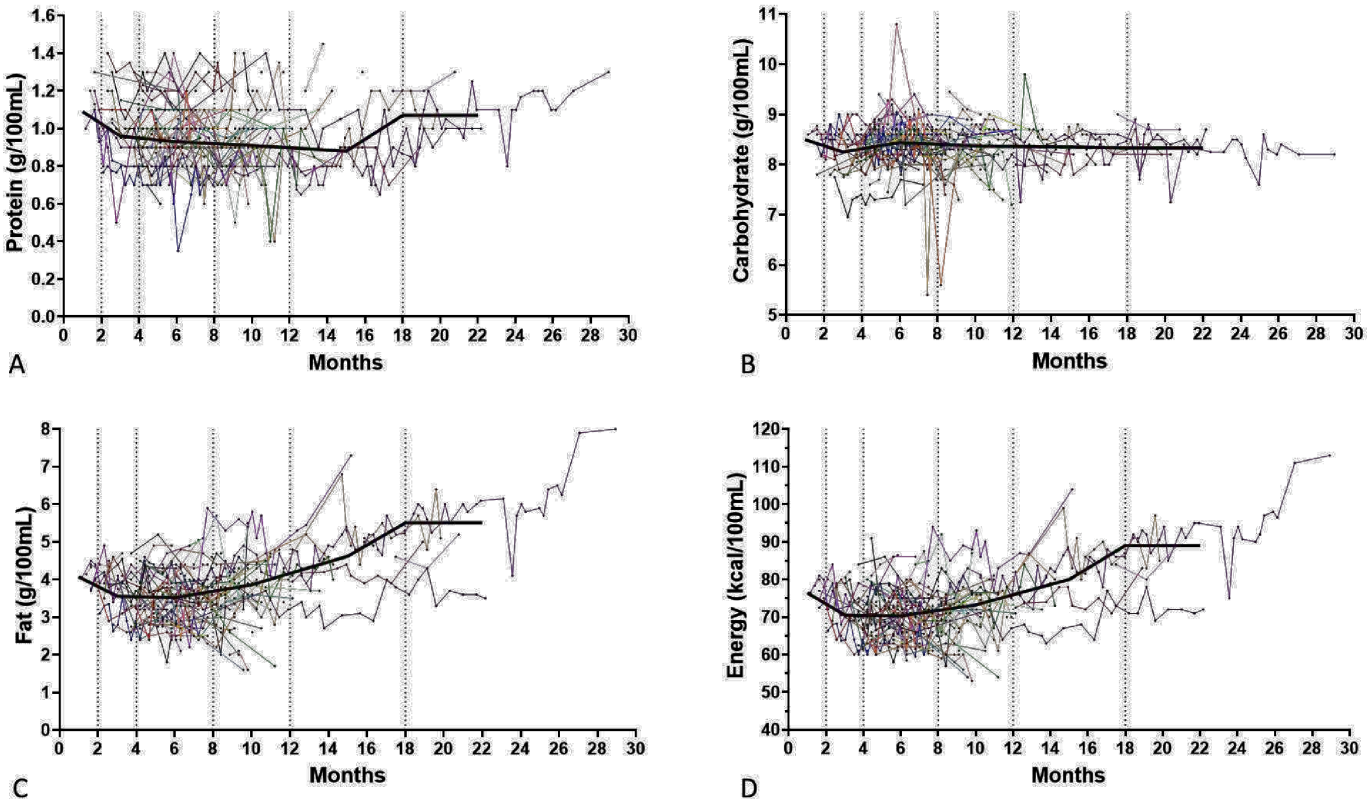

Figure 1 A–D Longitudinal macronutrient concentrations in months postpartum in donated human milk samples (n=86 donors, N=820 samples). A: crude protein concentration; B: carbohydrate concentration; C: fat concentration; D: energy content. The grid lines on the x-axis indicate the lactation periods defined in this study and used for the statistical analyses. Bold trendlines represent the average values.

The carbohydrate concentration in the expressed milk remained stable throughout the 28-month study period with no significant changes (online supplemental table 2).

The fat concentration in milk stayed consistent for the initial 8 months but saw a substantial rise from the 8th month of lactation through the end of our study (online supplemental table 3). Given the fat's high caloric density, the energy content also began increasing after 8 months of lactation, continuing to rise even after 2 years (online supplemental table 4).

DISCUSSION

This research project aimed to measure changes in macronutrient composition during an extended lactation period. Besides fundamental scientific interest, our findings could help human milk bank policymakers decide on the eligibility of potential donors throughout prolonged lactation.

The study demonstrates that human milk protein levels follow a U-shaped path, dipping between 8 and 18 months after birth. Carbohydrate concentrations, on the other hand, remain notably consistent through a lactation period extending to 28 months. It was also observed that fat concentration and energy content were stable in the first 8 months but started to increase steadily from the 8th month, continuing beyond 2 years of lactation.

While the protein-to-energy ratio (PER) shows a general decline during the first year of life, there are small variations over time. Specifically, the PER decreases from approximately 1.4 g/100 kcal during the 0–2 month period to around 1.1 g/100 kcal by 12–18 months. However, after 18 months, the PER slightly rises again reaching approximately 1.2 g/100 kcal.

Understanding which donors are appropriate is crucial. Our findings indicate that human milk, even when lactated for up to 2 years, maintains sufficient macronutrient richness, making it a

Table 1 Mean concentrations of crude protein, fat, carbohydrates and energy content in each of the lactation periods

	N	n	Protein (g/100 mL)	Carbohydrate (g/100 mL)	Fat (g/100 mL)	Energy (kcal/100 mL)
0–2 months	25	8	1.09±0.15	8.50±0.30	4.07±0.36	76.4±3.5
2–4 months	98	20	0.96±0.17	8.25±0.45	3.56±0.67	70.5±6.6
4–8 months	381	63	0.93±0.16	8.44±0.61	3.52±0.70	70.3±7.4
8–12 months	175	45	0.91±0.18	8.37±0.46	3.86±0.82	73.2±7.7
12–18 months	91	12	0.88±0.19	8.35±0.48	4.62±0.78	80.0±7.7
>18 months	50	6	1.07±0.14	8.33±0.46	5.51±0.99	89.0±9.5

Values presented as mean±SD.
N: total number of samples included in the lactation period; n: total number of donors included in the lactation period.

suitable donation for very preterm infants. The sample size for women who donated milk beyond 24 months post-birth was too low to form recommendations for prolonged lactation, yet there was no observable pattern of declining nutrient content. There are relatively few studies exploring the macronutrient composition of human milk during extended lactation. In one such study with 137 samples, the macronutrient content of breast milk was examined in mothers who breastfed beyond 18 months.[9] The findings revealed that this milk had higher protein and fat content but lower carbohydrate levels compared with milk samples taken before the 12th month of breastfeeding. However, between the 24th and 48th months of lactation, the concentrations of these macronutrients in human milk remained consistent. It is worth noting, though, that this study found wide variations in nutrient concentrations. For instance, around 10% of the samples displayed a protein concentration exceeding 2.0 g/100 mL with several even surpassing 3.5 g/100 mL—measurements notably higher than our observations.

Another study examined the changes in the composition of human milk over time, specifically between the 11th and 17th months after childbirth.[10] The study involved 131 samples from 19 mothers. It was found that among the macronutrients, only protein content showed a gradual, minor increase during this period. In a secondary analysis, these samples were compared with another 33 samples from women who had been lactating for a median of 4 months. This comparison revealed a slight increase in protein content in the milk of women with longer lactation periods. However, there was no noticeable difference in the content of fat and lactose between early (median of 4 months) and late (median of 14 months) lactation.

The study of Michaelson *et al* reported results similar to ours.[11] They found that protein concentration initially declined up to 8 months, then increased until the 17th month. The carbohydrate concentration showed only a minimal decrease (0.3 g/dL) over the entire 17 months period. Additionally, fat concentration started to increase from the 4th month.

The findings from various studies investigating long-term macronutrient composition in human milk including those from our present study show some discrepancies. This could stem from the differences in lactation periods analysed and the measurement techniques employed in each study such as mid-infrared technology, nuclear magnetic resonance, bicinchoninic acid assays based on the biuret reaction and tandem mass spectrometry. However, our study boasts the largest sample size and the greatest number of consecutive samples per donor which lends substantial credibility to our observed results.

The Miris analyser employed in this study is unable to differentiate human milk oligosaccharides (HMOs) from other carbohydrates such as lactose. HMOs constitute about 10% of the total carbohydrate content. As such, the determination of caloric density in human milk is slightly inflated due to the inclusion of these indigestible oligosaccharides in the carbohydrate assessment as HMOs do not contribute directly to caloric availability.[21] In a 2-year longitudinal study by Plows *et al* (n=28), HMO concentration changes over time were examined.[22] Their results suggested that HMO concentrations did not consistently increase or decrease during the lactation period. Conversely, another study demonstrated a gradual decline in HMOs during lactation with different HMOs predominating at various lactation stages.[23] The study of Perrin *et al*, however, demonstrated an increase in HMO content between 11 and 17 months postpartum.[10] Therefore, a study methodology that separately measures HMO and lactose concentrations would be instrumental in providing a more detailed understanding of carbohydrate concentrations during extended lactation periods.

Our research primarily focused on the nutritional aspects of breast milk overlooking its bioactive components like antibodies, enzymes and hormones which are vital for infant immunity and growth.[24 25] While most studies examined these bioactive components for the first 6 months of breastfeeding, there is a notable research gap for extended lactation periods. Understanding how these components persist and change over time is crucial for assessing the long-term benefits of breastfeeding.[5 26] Future research should examine both bioactive components and macronutrients in breast milk beyond the initial breastfeeding months.

This study is among the pioneers in exploring the nutritional composition of human milk longitudinally over extended lactation periods. We considered only mothers who had breastfed for more than 6 months to study the impacts of long-term lactation. An advanced statistical technique helped us evaluate differences over time while factoring in baseline confounders. Extra strengths of our research included analysing samples obtained from adjacent expression moments, effectively minimising pre-analytical expression conditions. We also measured nutrient concentrations longitudinally using on average nearly 10 pooled samples per donor. To enhance the statistical power of our analysis, we grouped data into larger time periods to increase the sample size within each interval. Our rationale for doing so was that we did not anticipate significant fluctuations in macronutrient levels from month to month, especially not during periods of prolonged lactation. Instead, we expected a more longitudinal trend of increase or decrease, with only a few periodic variations. This study, however, bears a few limitations. First, it had a comparatively small sample size, notably lacking in lactating mothers beyond 18 months. Second, limited information about aspects such as sociodemographic traits and dietary habits of the donors could potentially impact the results observed.[27 28] Furthermore, a lack of specifics concerning the timing and amounts of milk pumping, whether morning, afternoon or evening could potentially overlook variations in milk constituents throughout the day, lending to confounded results. Yet, to mitigate this variability, milk from multiple adjacent donation batches was combined into a single pool. Finally, the design may be seen as a limitation as all the milk donors were from a single country where nutritional habits and living conditions are relatively uniform. On the other hand, there is a wide range of dietary patterns and socioeconomic and cultural backgrounds of the donors which could suggest that our findings may still be generalisable to other populations.

The WHO recently updated its guidelines on the recommended duration of breastfeeding, advocating for exclusive breastfeeding for the first 6 months of a baby's life, followed by complementary feeding along with breastfeeding for 2 years or more.[13] Our findings support the WHO's recommendations as it offers adequate nutritional macronutrient value during these 2 years provided the mother ensures an adequate intake of essential micronutrients such as vitamin D, vitamin A, iron and other micronutrients and vitamins through a varied diet or supplements.

Currently, the EMBA and the HMBANA provide no guidelines on how extended lactation periods impact the nutritional composition of DHM or the suitable milk donation period.[17] The results of this study could contribute to creating detailed

directives for human milk banks, particularly in developing their acceptance policies.

CONCLUSION

Our research highlights the consistent nutritional composition of expressed human milk over a 2-year lactation period. This finding suggests that mothers could potentially donate milk to human milk banks throughout the entire second year of lactation.

Contributors JM and JIAL conducted the research. JM, JIAL and JWRT performed statistical analyses. JM, CHPVDA, JBvG and BJvK interpreted the data and wrote the paper. All authors critically revised the manuscript. JM served as the guarantor, assuming primary responsibility for the overall content, including the integrity of the research, access to the data and the decision to publish. All authors read and approved the final manuscript.

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Competing interests JM, JIAL, JWRT, BJvK, AS: All report no conflict of interest. JBvG: Member of the Dutch National Health Institute and founder and director of the Dutch National Human Milk Bank. CHPVDA: Reports receipt of speakers and consultancy honoraria from Nestlé Nutrition Institute, Nutricia Early Life Nutrition and Baxter.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the institutional review boards of VU University Medical Center Amsterdam. (NL37296.029.11). Every participant whose milk was used in this study provided approval via a non-objection form. The form allowed the non-interventional investigation of data and milk used for quality assurance metrics, not given to premature infants (NL37296.029.11).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement No data are available. The data used in this study were acquired from the National Dutch Human Milk Bank. Due to ethical considerations aimed at preserving the anonymity of the study participants, individual data cannot be shared. For these reason, data cannot be made available.

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REFERENCES

1 Li R, Ware J, Chen A, *et al*. Breastfeeding and post-perinatal infant deaths in the United States, A national prospective cohort analysis. *Lancet Reg Health Am* 2022;5:100094.
2 de Weerth C, Aatsinki A-K, Azad MB, *et al*. Human milk: From complex tailored nutrition to bioactive impact on child cognition and behavior. *Crit Rev Food Sci Nutr* 2023;63:7945–82.
3 Victora CG, Bahl R, Barros AJD, *et al*. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet* 2016;387:475–90.
4 Italianer MF, Naninck EFG, Roelants JA, *et al*. Circadian Variation in Human Milk Composition, a Systematic Review. *Nutrients* 2020;12:2328.
5 Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am* 2013;60:49–74.
6 Zhang J, Zhao A, Lai S, *et al*. Longitudinal Changes in the Concentration of Major Human Milk Proteins in the First Six Months of Lactation and Their Effects on Infant Growth. *Nutrients* 2021;13:1476.
7 Gidrewicz DA, Fenton TR. A systematic review and meta-analysis of the nutrient content of preterm and term breast milk. *BMC Pediatr* 2014;14:216.
8 Saarela T, Kokkonen J, Koivisto M. Macronutrient and energy contents of human milk fractions during the first six months of lactation. *Acta Paediatr* 2005;94:1176–81.
9 Czosnykowska-Łukacka M, Królak-Olejnik B, Orczyk-Pawiłowicz M. Breast Milk Macronutrient Components in Prolonged Lactation. *Nutrients* 2018;10:1893.
10 Perrin MT, Fogleman AD, Newburg DS, *et al*. A longitudinal study of human milk composition in the second year postpartum: implications for human milk banking. *Matern Child Nutr* 2017;13:e12239.
11 Michaelsen KF, Skafte L, Badsberg JH, *et al*. Variation in macronutrients in human bank milk: influencing factors and implications for human milk banking. *J Pediatr Gastroenterol Nutr* 1990;11:229–39.
12 Shenker NS, Perdones-Montero A, Burke A, *et al*. Metabolomic and Metataxonomic Fingerprinting of Human Milk Suggests Compositional Stability over a Natural Term of Breastfeeding to 24 Months. *Nutrients* 2020;12:3450.
13 WHO guideline for complementary feeding of infants and young children 6-23 months of age. Geneva WHO Guidelines Approved by the Guidelines Review Committee; 2023.
14 Quigley M, Embleton ND, McGuire W. Formula versus donor breast milk for feeding preterm or low birth weight infants. *Cochrane Database Syst Rev* 2019;7:CD002971.
15 Eidelman AI, Schanler RJ, Johnston M, *et al*. Breastfeeding and the Use of Human Milk. *Pediatrics* 2012;129:e827–41.
16 Embleton ND, Jennifer Moltu S, Lapillonne A, *et al*. Enteral Nutrition in Preterm Infants (2022): A Position Paper From the ESPGHAN Committee on Nutrition and Invited Experts. *J Pediatr Gastroenterol Nutr* 2023;76:248–68.
17 Weaver G, Bertino E, Gebauer C, *et al*. Recommendations for the Establishment and Operation of Human Milk Banks in Europe: A Consensus Statement From the European Milk Bank Association (EMBA). *Front Pediatr* 2019;7:53.
18 Perrin MT, Festival J, Starks S, *et al*. Accuracy and Reliability of Infrared Analyzers for Measuring Human Milk Macronutrients in a Milk Bank Setting. *Curr Dev Nutr* 2019;3:nzz116.
19 Burianova I, Bronsky J, Pavlikova M, *et al*. Maternal body mass index, parity and smoking are associated with human milk macronutrient content after preterm delivery. *Early Hum Dev* 2019;137:104832.
20 Leghi GE, Netting MJ, Middleton PF, *et al*. The impact of maternal obesity on human milk macronutrient composition: A systematic review and meta-analysis. *Nutrients* 2020;12:934.
21 Groh-Wargo S, Valentic J, Khaira S, *et al*. Human Milk Analysis Using Mid-Infrared Spectroscopy. *Nutr Clin Pract* 2016;31:266–72.
22 Plows JF, Berger PK, Jones RB, *et al*. Longitudinal Changes in Human Milk Oligosaccharides (HMOs) Over the Course of 24 Months of Lactation. *J Nutr* 2021;151:876–82.
23 Thum C, Wall CR, Weiss GA, *et al*. Changes in HMO Concentrations throughout Lactation: Influencing Factors, Health Effects and Opportunities. *Nutrients* 2021;13:2272.
24 Duijts L, Jaddoe VWV, Hofman A, *et al*. Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy. *Pediatrics* 2010;126:e18–25.
25 Agostoni C, Braegger C, Decsi T, *et al*. Breast-feeding: A commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr* 2009;49:112–25.
26 Carr LE, Virmani MD, Rosa F, *et al*. Role of Human Milk Bioactives on Infants' Gut and Immune Health. *Front Immunol* 2021;12:604080.
27 Petersohn I, Hellinga AH, van Lee L, *et al*. Maternal diet and human milk composition: an updated systematic review. *Front Nutr* 2023;10:1320560.
28 Miliku K, Duan QL, Moraes TJ, *et al*. Human milk fatty acid composition is associated with dietary, genetic, sociodemographic, and environmental factors in the CHILD Cohort Study. *Am J Clin Nutr* 2019;110:1370–83.

Table 1. Differences in crude protein concentration (g/100 mL) for each lactation period.

	0-2 months			2-4 months			4-8 months			8-12 months			12-18 months		
	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*
2-4 months	-0·12	-0·18--0·06	**<0·001**	..	..	..	..	..	..	..	..	..	..	..	..
4-8 months	-0·17	-0·23--0·11	**<0·001**	-0·05	-0·08--0·02	**0·001**	..	..	..	..	..	..	..	..	..
8-12 months	-0·19	-0·26--0·13	**<0·001**	-0·07	-0·11--0·04	**<0·001**	-0·02	-0·05-0·00	0·072	..	..	..	..	..	..
12-18 months	-0·19	-0·26--0·13	**<0·001**	-0·07	-0·11--0·03	**0·001**	-0·02	-0·05-0·01	0·237	0·00	-0·03-0·04	0·917	..	..	..
>18 months	0·02	-0·05-0·09	0·614	0·14	0·09-0·19	**<0·001**	0·19	0·15-0·23	**<0·001**	0·21	0·06-0·26	**<0·001**	0·16	0·05-0·27	**<0·001**

Values are differences between lactation periods compared by linear mixed models analysis adjusted for gestational age and maternal BMI. Each time period was tested against the preceding periods. Bold font indicates statistical significance (P <0·05).

Table 2. Differences in carbohydrate concentration (g/100 mL) for each lactation period.

	0-2 months			2-4 months			4-8 months			8-12 months			12-18 months		
	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*
2-4 months	-0·04	-0·27-0·20	0·754	..	..	..	..	..	..	..	..	..	..	..	..
4-8 months	0·07	-0·16-0·29	0·569	0·10	-0·01-0·22	0·084	..	..	..	..	..	..	..	..	..
8-12 months	0·01	-0·22-0·25	0·913	0·05	-0·08-0·18	0·468	-0·05	-0·15-0·02	0·273	..	..	..	..	..	..
12-18 months	-0·04	-0·30-0·21	0·738	-0·01	-0·17-0·16	0·940	-0·11	-0·24-0·02	0·101	-0·06	-0·19-0·08	0·415	..	..	..
>18 months	-0·10	-0·37-0·17	0·487	-0·06	-0·25-0·13	0·541	-0·16	-0·32-0·01	0·060	-0·11	-0·27-0·05	0·191	-0·05	-0·22-0·11	0·527

Values are differences between lactation periods compared by linear mixed models analysis adjusted for gestational age and maternal BMI. Each time period was tested against the preceding periods. Bold font indicates statistical significance (P <0·05).

Table 3. Differences in fat concentration (g/100 mL) for each lactation period.

	0-2 months			2-4 months			4-8 months			8-12 months			12-18 months		
	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*
2-4 months	0·02	-0·24-0·29	0·863	..	..	..	..	..	..	..	..	..	..	..	..
4-8 months	0·06	-0·21-0·32	0·674	0·03	-0·10-0·17	0·612	..	..	..	..	..	..	..	..	..
8-12 months	0·35	0·08-0·62	**0·012**	0·32	0·17-0·48	**<0·001**	0·29	0·18-0·40	**<0·001**	..	..	..	..	..	..
12-18 months	1·06	0·77-1·35	**<0·001**	1·04	0·85-1·23	**<0·.001**	1·01	0·86-1·15	**<0·001**	0·72	0·56-0·87	**<0·001**	..	..	..
>18 months	1·90	1·59-2·21	**<0·001**	1·88	1·66-2·09	**<0·001**	1·84	1·66-2·02	**<0·001**	1·56	1·37-1·74	**<0·001**	0·85	0·67-1·04	**<0·001**

Values are differences between lactation periods compared by linear mixed models analysis adjusted for gestational age and maternal BMI. Each time period was tested against the preceding periods. Bold font indicates statistical significance (P <0·05).

Table 4. Differences in energy content (kcal/100 mL) for each lactation period.

	0-2 months			2-4 months			4-8 months			8-12 months			12-18 months		
	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*	Diff.	95% CI	*p*
2-4 months	-0·20	-3·05-2·65	0·891	..	..	..	..	..	..	..	..	..	..	..	..
4-8 months	-0·27	-3·06-2·52	0·849	-0·05	-1·47-1·37	0·943	..	..	..	..	..	..	..	..	..
8-12 months	0·21	-0·69-5·10	0·136	2·42	0·77-4·08	**0·004**	2·48	1·33-3·63	**<0·001**	..	..	..	..	..	..
12-18 months	8·61	5·48-11·7	**<0·001**	8·82	6·80-10·9	**<0·001**	8·88	7·29-10·5	**<0·001**	6·43	4·78-8·08	**<0·001**	..	..	..
>18 months	17·0	13·7-20·3	**<0·001**	17·2	14·9-19·5	**<0·001**	17·3	15·3-19·2	**<0·001**	14·8	12·8-16·8	**<0·001**	8·53	6·53-10·5	**<0·001**

Values are differences between lactation periods compared by linear mixed models analysis adjusted for gestational age and maternal BMI. Each time period was tested against the preceding periods. Bold font indicates statistical significance (P <0·05).