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Longitudinal assessment of vitamin concentrations in donor human milk

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ABSTRACT

Donor human milk (DHM) is the preferred alternative for preterm infants when mother's own milk is unavailable, though its nutrient composition varies with lactation stage. This study evaluated temporal changes in vitamin concentrations in DHM. A total of 189 raw milk samples were collected from 72 donating mothers at three postpartum periods: 2–12, 13–16, and 17–24 weeks. Thirteen vitamins, including B-group vitamers, vitamin C, and vitamin D, were quantified using LC-MS. Significant time-dependent variations were found for several B vitamins. Thiamine increased markedly from 29.5 ng/mL at 2–12 weeks to 45.2 ng/mL at 17–24 weeks ($p = 3.5E-09$), while thiamine monophosphate decreased from 404 to 240 ng/mL ($p = 5E-04$). Pyridoxal levels rose from 77.3 to 97.8 ng/mL ($p = 3.4E-02$). In contrast, vitamin C (22.6 μg/mL) and vitamin D (0.9 ng/mL) remained stable throughout lactation. These results demonstrate measurable shifts in vitamin composition of DHM, particularly in thiamine-related compounds and selected B vitamins, while vitamins C and D show temporal stability. Continuous monitoring of DHM's nutritional profile is recommended, as such variations may influence its ability to meet preterm infants' nutritional needs. Further studies should address the clinical significance of these findings.

1. Introduction

Human milk (HM) plays a pivotal role in infant nutrition during the first 6 – 12 months of life, providing the key macro- and micronutrients required for healthy development. HM promotes optimal infant growth and is associated with a reduced risk of future metabolic disorders such as obesity and type II diabetes (Borer, 2025). The World Health Organization (WHO) recommends exclusive or predominant breastfeeding up to 6 months and promotes breastfeeding continuity for up to 2 years of age complemented by other foods, whilst other organizations such as the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) advise exclusive breastfeeding up to 4 months and predominant up to 6 months. If a mother of a preterm infant is unable to provide her own milk, donor human milk (DHM) is recommended (Tyebally Fang et al., 2021; WHO, 2022; European Society for Paediatric Gastroenterology, 2024). Although much attention is given to the proper macronutrient composition of DHM, there is a lack of systematic analysis in the literature regarding the vitamin composition of DHM, as well

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as the availability of standardized methods of its analysis (Gates et al., 2023; Bala, 2024; Fu et al., 2024). To the extent that they exist, such studies often examine individual vitamins, the deficiencies of which may result from, for example, the donor's diet (vitamin C, B12) (Castro et al., 2021; Greco et al., 2025).

Vitamins are essential micronutrients. Most, except for vitamin D and niacin (B3), cannot be synthesized in the human body and, therefore, should be provided through the diet. There are eight B vitamins: thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6) biotin (B7), folic acid (B9), and cobalamin (B12) along with their various vitamers, which express identical biological activity (Gregory et al., 2005). B vitamins are vital for energy metabolism (B1, B2, B6, B7), amino acid synthesis (B1, B6, B7, B9), DNA synthesis and repair (B3, B12) and neural physiology (B1, B6, B9) (Dror and Allen, 2018; Gibney, 2009; Hanna et al., 2022). Thiamine deficiency is most abundant in Asian countries (Smith et al., 2021) and its deficiency in children leads to dry beriberi, characterised by limb numbness and motor impairment (Gomes et al., 2021); neuropathies leading to language delay and motor system dysfunction (Fattal-Valevski et al., 2009); and pulmonary hypertension and impaired cardiac function (Andersen et al., 2013). B_2 deficiency is associated with dermatological and neurological disorders (Dror and Allen, 2018). B3, a product of tryptophan metabolism, exist as two biologically similar vitamers – nicotinic acid and nicotinamide, both essential for cellular metabolism (Gibney, 2009). B5 is a critical coenzyme and cofactor, involved in the function of approximately 4% of mammalian enzymes, and plays an essential role in energy metabolism (Kennedy, 2016). It is found at concentrations 50 times higher in the brain than in blood plasma, contributing to the synthesis of cholesterol, phospholipids, and fatty acids, as well as neurotransmitters, particularly acetylcholine, and is believed to play a key role in the myelination process (Ismail et al., 2020; Uchida et al., 2015).

Vitamin C is an important cofactor for monooxygenases or dioxygenases, playing a key role as an antioxidant. It supports extracellular collagen stability by maintaining its hydroxylation (Peterkofsky, 1991). Vitamin C is well known for its immunomodulatory effects, enhancing leukocyte activity, antibody production, and interferon synthesis (Dror and Allen, 2018). Vitamin C deficiency leads to scurvy, marked by impaired collagen synthesis, fragile blood vessels, and tissue damage.

Vitamin D plays a crucial role in calcium and phosphate homeostasis and is essential for proper bone mineralization and skeletal development. Its biologically active form, 1,25-dihydroxyvitamin D (1,25 $(OH)_2D_3$), regulates intestinal absorption of calcium and phosphate and supports bone calcification (Bouillon et al., 2003), thereby contributing to longitudinal growth and dental enamel formation (Simmer and Fincham, 1995). In addition, vitamin D has important immunomodulatory functions, including the regulation of inflammatory responses and stimulation of antimicrobial peptide production (Mora et al., 2008). Deficiency of vitamin D in infancy may lead to rickets, a condition characterized by impaired bone mineralization, skeletal deformities, and growth disturbances (Holic, 2007).

Although vitamins in human milk have been studied for decades, most available reports focus on individual vitamins or small vitamin groups, frequently using heterogeneous analytical approaches and cross-sectional sampling designs. Comprehensive, standardized multi-vitamin profiling of donor human milk across defined lactation stages remains limited. This gap is particularly relevant in the context of milk banking, where understanding temporal variation in micronutrient composition is essential for nutritional evaluation and clinical use. Therefore, the present study aims to provide a longitudinal, multi-vitamin assessment of donor human milk using a validated tandem mass spectrometry (LC-MS/MS) platform, enabling integrated analysis of vitamin dynamics across lactation. Given these considerations, we aimed to investigate the content and temporal variation of 13 vitamins in DHM, using tailored liquid chromatography coupled with LC-MS/MS quantification methods. High method specificity and sensitivity allowed the generation of broad view of vitamin profiles and their time-dependent changes.

2. Materials and methods

2.1. Study design and the collection of human milk samples

The study included three populations of human milk donors: from Poland, Italy, and the Netherlands, recruited from six human milk banks (four in Poland, one in Italy, and one in the Netherlands) between February 2020 and May 2022. Inclusion criteria involved being qualified as a donor at one of the study human milk banks according to the European Milk Bank Association guidelines (Weaver et al., 2019) and providing written informed consent to participate in the study. Ethical approval for the study was obtained from the local ethics committees in all participating countries. In Poland, the Bioethical Commission of Warsaw Medical University accepted information about conducting the study without reservation. (Decision no. AKBE/39/2020). Separate ethical approvals were also obtained for the Italian population (Decision no. 2021.0597) and the Dutch population (Decision no.169849 del 8.3.2021). DHM samples were collected at three time points: 2–12 weeks (A), 13–16 weeks (B), and 17–24 weeks (C) weeks postpartum. A total of 189 samples from 72 mothers were collected: 72 at time point A, 63 at time point B, and 54 at time point C. Recruitment timelines varied between countries due to logistical factors, including ethical approvals, staffing or location changes, and most importantly, disruptions related to the COVID-19 pandemic, all of which likely influenced the recruitment outcomes. The recruitment process and sample numbers are presented in Fig. 1.

2.2. Human milk collection and general handling

Human milk (HM) samples were collected in the morning between 8.00 and 10.00 AM. Donors were instructed to express 5–10 mL of fore- and hind milk (10–20 mL in total). Foremilk and hindmilk obtained during each collection were thoroughly mixed to create a single homogeneous composite sample prior to freezing and subsequent analysis, to minimize intra-feed variability in milk composition. Then, samples were immediately frozen at −20°C and stored until further analysis.

Before analysis, samples were thawed at 4°C overnight and sonicated for 30 min in a water bath, then transferred to 15 mL polypropylene conical centrifuge tubes and vortexed for 30 min.

2.3. LC-MS quantification of B vitamins

Stock solutions (1 mg/mL) were prepared using standards for11 B-vitamin vitamers and 9 of their respective isotopically labelled analogues (full details in Supplementary Information).

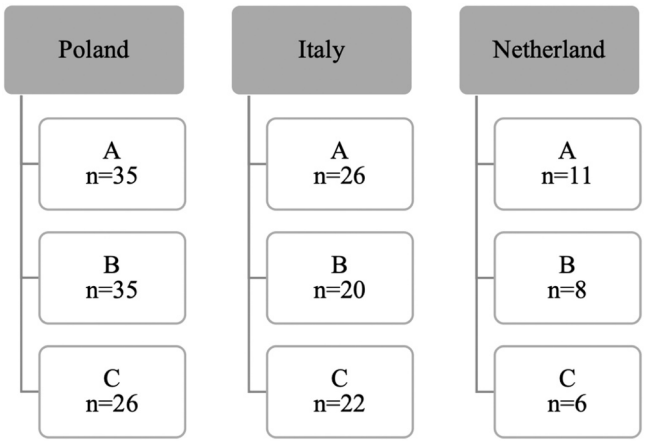


Fig. 1. Number of donors who provided milk samples at each stage of the study from Poland, Italy, and the Netherlands at three different time points: (A) 2–12 weeks, (B) 13–16 weeks, and (C) 17–24 weeks.

For analysis, 50 μL of the pre-treated HM was transferred to a 1.5 mL sample tube and mixed with 250 μL of internal standard (IS) solution in HPLC grade methanol (MeOH) containing 0.1% formic acid to initiate protein precipitation (thiamine-$^{13}C_3$, riboflavin-$^{13}C_4^{15}N_2$, nicotinic acid-$^{13}C6$, nicotinamide-$^{13}C_6$, pantothenic acid-$^{13}C_3^{15}N$, biotin-d_4, folic acid-d_4, pyridoxal-d_3, pyridoxine-d_3 at 2 ng/mL). Next, samples were vortexed at 1 500 RPM for 10 min and further centrifuged at 14 000 RPM for 10 min. Subsequently, the aliquot was transferred to a new tube and evaporated to dryness under a nitrogen stream in a water bath held at 50°C. Samples were reconstituted in 200 μL of 20 mM ammonium formate with 0.2% NH_4OH solution and vortexed for 10 min. Lipids were extracted by adding 400 μL of hexane, vortexing at 1 500 RPM for 10 min followed by centrifugation at 14 000 RPM for 10 min. The hexane layer was discarded and 150 μL of aqueous phase was transferred to a chromatographic polypropylene vial.

Quantification of B vitamers was conducted using a Waters Acquity Ultra Performance Liquid Chromatography system coupled with Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separation was achieved using an ACQUITY UPLC BEH C8 (1.7 μm, 2.1 mm × 100 mm) column. The column oven was set to 50°C. Mobile phase A was 20 mM ammonium formate + 0.1% NH_4OH in Milli-Q grade water, and mobile phase B was LC-MS grade methanol. The initial flow was set to 0.35 mL/min with 0% phase B kept for 1 min, then increased to 20% phase B over the next 4 min and to 90% phase B for the next 2 min, for a total run time of 9 min. The mass spectrometer operated in a positive electrospray ionization mode (ESI+), with a capillary voltage of 0.7 kV, cone voltage of 20.0 V, desolvation temperature 600°C, desolvation gas flow 800 L/hr and cone gas flow 200 L/hr.

2.4. LC-MS quantification of vitamin C

For vitamin C analysis, 25 μL of pre-treated HM was placed in a new 1.5 mL sample tube. 100 μL of 10 mM tris(2-carboxyethyl) phosphine (TCEP) and 100 μL of 3% meta-phosphoric acid containing IS (vitamin C-$^{13}C_6$ at 5 μg/mL) were added and mixed for 1 min. The samples were incubated at room temperature for 30 min. The mixture was then centrifuged and transferred to a chromatographic polypropylene vial.

The instrumentation consisted of a Waters Acquity UPLC coupled with a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separation was performed using a Waters HILIC column (1.7 μm, 2.1 mm × 50 mm) kept in a column oven at 25°C. Mobile phase A consisted of 15 mM ammonium formate in Mili-Q grade water and mobile phase B was acetonitrile (LC-MS grade). The flow rate of the mobile phase was set to 0.5 mL/min. The total time of separation was 2.0 min. Vitamin C was detected in negative ESI mode (ESI-). Mass spectrometer parameters were capillary voltage 2.2 kV, cone voltage 25.0 V, desolvation temperature 650°C, desolvation gas flow 800 L/hr and cone gas flow 150 L/hr.

2.5. LC-MS quantification of vitamin D

Solid-phase extraction (SPE) was performed using Oasis HLB SPE plates (30 μm, 10 mg sorbent, Waters). Plates were conditioned with 500 μL of 0.1% formic acid, next with 500 μL of MeOH and lastly with 500 μL of 0.25% NH_4OH solution. 500 μL samples and calibrators were loaded on a preconditioned plate. To each, 500 μL of IS solution in MeOH with 0.25% NH_4OH was added (25-OH-D_3-d_6 at 5 ng/mL). The plate was vortexed for 10 s at 500 RPM, elution was performed through SPE using a Positive Pressure processor (Waters). Eluate was discarded. The loaded plate was washed sequentially with 500 μL of 5% MeOH, and 500 μL of 60% MeOH. Freshly prepared 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD, 1 mg/mL) in a 1:1 mixture of ACN and ethanolamine (EA) was used as derivatizing and eluting solution. Samples were eluted from SPE with 75 μL derivatizing solution and collected on 96 well plate. The solution was left for an hour to derivatize in a room temperature.

Chromatographic separation was performed using a Waters ACQUITY UPLC I-Class system with an ACQUITY UPLC CSH Fluoro-Phenyl (1.7 μm, 2.1 mm×100 mm) analytical column (Waters) at 70°C. Mobile phase A consisted of 0.1% formic acid in Mili-Q grade water and mobile phase B of 0.1% formic acid in LC-MS grade MeOH. The total run time was 4 min. MS operated in ESI+ , tune parameters were: capillary voltage 2.5 kV, cone voltage 50.0 V, desolvation temperature 600°C, desolvation gas flow 1000 L/hr, and cone gas flow 250 L/hr.

2.6. Method development and validation

Best mass transitions in multiple-reaction-monitoring (MRM) mode were selected using direct infusion of pure analyte solutions to mass spectrometer at stepped collision energies to achieve maximum abundance of daughter ions. Each analyte had its favorite cone energy selected, for which the highest signal of the parental ion was detected. In order to assess calibration curve linearity, three calibration curves were prepared in different ranges, according to the biological availability of the analyte class, and were tested using weighted linear regression (weighing factor 1/x). Limit of detection (LOD) and limit of quantification (LOQ) were determined as analyte concentration for which the signal-to-noise ratio was at least 3 and 10, respectively. Repeatability and reproducibility (precision) were determined by calculating the relative standard deviation (RSD) for five repetitions of QC samples at three different levels (low, medium and high), on the same day by the same analyst and on the other day by the same analyst respectively. Accuracy was assessed by calculating the mean concertation of five QC repetitions (low, medium, high) divided by the nominal QC value. Matrix-matched recovery was measured by spiking the post-extraction solution and calculated by subtracting the baseline concentration from the total measured concentration and divided by the spiked concentration value. The validation criteria are summarized in the Table 1. Detailed information on the method parameters are outlined in the Supplementary Materials.

2.7. Statistical analysis

Data analysis was performed using GraphPad Prism version 10.4.2. One-way ANOVA was used ($p = 0.05$) to test for differences in vitamin concentrations. False Discovery Rate adjustment, using the two-stage linear step-up Benjamini, Krieger and Yekutieli procedure was performed, with a Q-value of 5%. Post hoc Tukey's HSD tests (α=0.05) were performed to compare vitamin levels across milk collection time points (A, B, and C). Spearman nonparametric correlation assay (two-tailed, 95% confidence interval) was performed to assess association between variables. Bubble, violin, and correlation matrix plots were generated using the same software.

3. Results

Table 2summarizes the mean and median concentrations of selected vitamins at three measured time points. Pantothenic acid (vitamin B5)

Table 1
Summarized acceptance criteria for validation of vitamins B, C and D quantification.

Linearity range	Vitamins B 1 – 2000 ng/mL Vitamin C 0.1 – 100 μg/mL Vitamin D 0.1 – 40 ng/mL
Linearity coefficient (R^2)	$\geq$ 0.950
LOD	S/N ratio at least 3
LOQ	S/N ratio at least 10
Precision	< 15% for low QC < 10% for medium QC < 5% for high QC
Recovery	> 80%
Accuracy	90 – 110%

Table 2
Results for vitamin concentrations in donor human milk samples (n = 189).

Vitamins[1]	Time A (2–12 weeks)		Time B (13–16 weeks)		Time C (17–24 weeks)	
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)
Thiamine (B_1)	29.5 ± 13.8	29.2 (19.1–37.2)	40.8 ± 13.2	41.0 (33.4–42.7)	45.2 ± 15.2	45.5 (35.3–55.4)
Thiamine monophosphate (B_1)	404 ± 218	413 (241–630)	267 ± 197	273 (144–414)	240 ± 199	197 (112–433)
Total B_1	440.68 ± 212.18	450.21 (279,56 – 580.56)	284.03 ± 136.79	292.09 (178.53 – 350.89)	284.76 ± 195.42	237.38 (152.46 – 347.07)
Riboflavin (B_2)	72.8 ± 77.4	48.5 (35.2–58.8)	100 ± 107	60.2 (39.1–81.8)	111 ± 134	60.0 37.8–89.7
Nicotinic acid (B_3)	8.9 ± 20.2	2.7 (1.6 – 5.0)	20.1 ± 96.3	2.5 (1.0–5.8)	7.8 ± 22.9	1.9 (1.2–6.1)
Nicotinamide (B_3)	486 ± 313	407 (296 – 570)	437 ± 235	385 (286–552)	469 ± 275	414 (282–557)
Total B_3	495 ± 316	410 (302–556)	457 ± 233	390 (297–621)	476 ± 275	418 (293–582)
Panthotenic acid (B_5)	2143 ± 802	2016 (1621–2585)	2064 ± 800	1853 (1529–2421)	2064 ± 787	1950 (1532–2327)
Pyridoxamine (B_6)	10.6 ± 9.5	8.7 (0.0–14.2)	27.3 ± 72.7	14.7 (9.0–22.0)	15.4 ± 18.4	10.0 (6.8–16.0)
Pyridoxine (B_6)	0.2 ± 1.2	0.0 0.0 – 0.0	7.1 ± 52.2	0.0 (0.0–0.1)	0.1 ± 0.7	0.0 (0.0 – 0.0)
Pyridoxal (B_6)	77.3 ± 43.1	66.9 (49.6–91.3)	102 ± 46.4	95.6 (66.4–114)	97.8 ± 47.0	92.1 (65.0–98.1)
Total B_6	87.9 ± 47.3	78.2 (53.1–110)	129 ± 88.5	112 (79.7–136)	113 ± 57.9	100 (72.8–138)
Biotin (B_7)	7.1 ± 6.2	5.6 (3.8–7.6)	25.4 ± 109	5.6 (3.5–7.6)	7.2 ± 7.0	5.3 (3.0–6.9)
Folic acid (B_9)	4.9 ± 6.4	2.5 (0.0–7.4)	4.6 ± 7.8	0.0 (0.0–8.1)	4.9 ± 8.3	0.5 (0.0–5.5)
Vitamin C	22.6 ± 15.2	19.0 (10.9–33.8)	25.7 ± 16.1	24.2 (13.2–33.8)	25.3 ± 13.9	23.2 (13.2–38.5)
25-OH-D3 (D)	0.9 ± 0.6	0.8 (0.5–1.4)	0.9 ± 0.5	0.9 0.6–1.2	0.8 ± 0.5	0.8 (0.5–1.2)

[1]All B vitamins and 25-hydroxyvitamin D3 concentrations are expressed in ng/mL, whereas vitamin C is in μg/mL.
Total levels of niacin (B_3) were calculated as the sum of nicotinic acid and nicotinamide, whereas total levels of B_6 were calculated as a sum of pyridoxamine and pyridoxal, excluding pyridoxine

was the most abundant vitamin, showing the highest mean concentration among all B vitamins in DHM, with a mean concentration of 2143 ng/mL (±802) at weeks 2–12 and remaining relatively stable for the next 12 weeks with a mean concentration of 2064 ng/mL (±800) at the 13–16 weeks, and 2064 ng/mL (±787) at 17–24 weeks. In contrast, folic acid exhibited among the lowest concentration, with mean levels of 4.9 ng/mL (±6.4) at time point A, 4.6 ng/mL (±7.8) at time point B and 4.9 ng/mL (±8.3) at C. Pyridoxine, a vitamer of vitamin B6, was undetectable in most DHM samples, with mean concentrations below the quantification limit (0.2 ng/mL at time point A and 0.1 ng/mL at time point C).

Vitamin C concentrations remained relatively stable, with mean values of 22.6 μg/mL (±15.2) at time point A and 25.3 μg/mL (±13.9) at time point C. Similarly, 25-hydroxyvitamin D3 levels remained very stable, with mean concentrations of 0.9 ng/mL (±0.6) at time point A and 0.8 ng/mL (±0.5) at time point C.

Five B vitamins showed significant variation over the tested time periods, as determined by one-way analysis of variance ANOVA: thiamine (p = 3.49E-09), thiamine monophosphate (TMP) (p = 1.32E-05), pyridoxamine (p = 1.26E-02), pyridoxal (p = 4.18E-03) and folic acid (p = 2.15E-02). After applying false discovery rate (FDR) correction, using the Benjamini, Krieger and Yekutieli method (Benjamini et al., 2006), three vitamins, thiamine (p = 3.49E-09), TMP (p = 1.32E-05) and pyridoxal (p = 4.18E-03) were found as significant discoveries. Vitamin C (p = 4.27E-01; p-adj=5.03E-01) and 25-hydroxyvitamin D3 (p = 8.11E-01; p-adj=7.10E-01), did not show significant changes across the three time points. Results of the ANOVA statistic and FDR adjustment are summarized in Table 3.

Multiple comparisons using Tukey's HSD post hoc test were applied

Table 3
Results of the ANOVA performed on concentrations of 13 vitamins in donor human milk samples collected at three lactation time points (A, B, and C) between weeks 2 and 24 postpartum. Both raw and FDR-adjusted p-values are reported.

Vitamin	*p*-value	*p*-adj
Thiamine (B_1)	3.49E-09	3.66E-08
Thiamine monophosphate (B_1)	1.32E-05	6.93E-05
Riboflavin (B_2)	1.08E-01	2.27E-01
Nicotinic acid (B_3)	4.31E-01	5.03E-01
Nicotinamide (B_3)	5.87E-01	6.16E-01
Panthotenic acid (B_5)	6.11E-01	7.10E-01
Pyridoxamine (B_6)	1.26E-02	2.14E-01
Pyridoxine (B_6)	3.35E-01	5.03E-01
Pyridoxal (B_6)	4.18E-03	1.46E-02
Biotin (B_7)	6.05E-01	3.08E-01
Folic acid (B_9)	2.15E-02	7.92E-01
Vitamin C	4.27E-01	5.03E-01
25-OH-D3 (D)	8.11E-01	7.10E-01

to the three significantly differing B vitamins (Fig. 2). Thiamine concentration increased between weeks 2 and 24, with significant differences between time points A and B (p.adj= 1.57E-05) and A and C (p.adj=9.18E-09). In contrast, TMP decreased over the same period and significantly differed between A and B (p.adj=4.95E-04) and A and C (p.adj=4.90E-05). Pyridoxal concentration showed an upward trend, with a significant increase between A and B (p.adj=5.91E-03) and A and C (p.adj=3.46E-02). Thiamine and TMP concentrations displayed opposite temporal trends across the three time points (Fig. 3).

Spearman correlation coefficient test was performed to establish

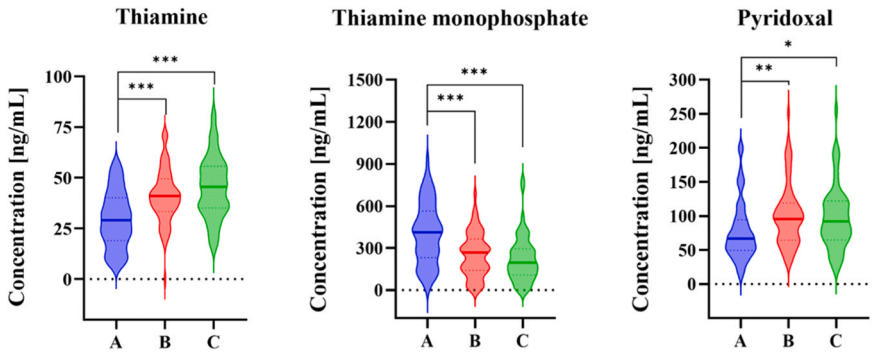


Fig. 2. Violin plots representing thiamine, thiamine monophosphate and pyridoxal levels across the three time points A, B and C. Significance levels are indicated with asterisks *p = 0.05, **p = 0.01, ***p = 0.001.

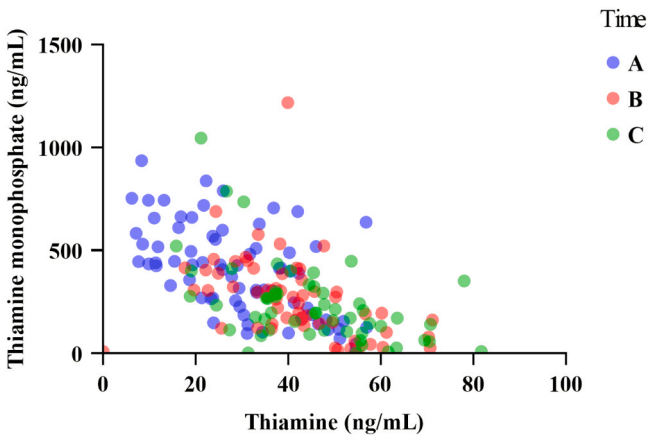


Fig. 3. Time-dependent variation of thiamine and thiamine monophosphate concentrations (ng/mL). Each dot represents an individual sample.

correlation between variables. A graphical representation of the correlation heat map is shown in Fig. 4. Thiamine and TMP showed moderate negative correlation ($r = -0.62$, $p < 0.0001$), suggesting that the decrease in TMP is associated with a corresponding increase in free thiamine in DHM.

4. Discussion

In this study, we analyzed the concentration and temporal trends of multiple vitamins in DHM across the first six months of lactation. We found that thiamine concentrations increased over time, accompanied by a progressive decrease in TMP. Thiamine exists in multiple forms, including its phosphorylated derivatives, such as TMP, thiamine diphosphate (TDP), and thiamine triphosphate (TTP), with TTP being a key enzyme cofactor in energy metabolism and mitochondrial function, especially in the pentose phosphate pathway (PPP) (Zhao et al., 2002). Thiamine is absorbed from the intestinal lumen in phosphorylated forms, which are then dephosphorylated by enzymes, such as thiamine pyrophosphatase (Rindi and Laforenza, 2000). We observed that a gradual decrease of TMP was accompanied by a simultaneous increase in free thiamine in HM over lactation. This observation is consistent with the findings by Wang et al. (2025) who also reported a negative correlation between thiamine and TMP over lactation. Our thiamine concentrations for mature milk were higher than those reported by Wang et al. (2025) but aligned with those described by Ren et al. (2015). The progressive decrease of TMP in HM, due to its dephosphorylation or hydrolysis into free thiamine, has been described also by Hampel et al. (2016). TMP is a product of TTP dephosphorylation to free thiamine, rather than a substrate in its synthesis. Interestingly, TMP levels are higher than free thiamine in cerebrospinal fluid (Tallaksen et al., 1992), and cellular uptake of TMP is mediated by tissue-specific carriers, which impact cellular thiamine status (Zhao et al., 2002).

Riboflavin (vitamin B2) is crucial for the synthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are key coenzymes for cellular energy metabolism. Our study showed relatively high and stable concentrations. Earlier data from Roughead and McCormick (1990), showed higher levels of HM riboflavin. However, the authors collected samples only from 5 participants and used much less sensitive method of flavins detection, which compared to LC-MS methods, has much lower specificity and sensitivity, suggesting that the high levels of riboflavin in their report resulted from high method uncertainty. Riboflavin concentration in HM mainly relates to maternal intake. Regional dietary differences likely explain the discrepancies in HM riboflavin concentration reported in various studies, such as Sakurai et al., (2005) who described lower levels of riboflavin in HM among Japanese mothers.

Niacin (vitamin B3), levels from our study were lower than those reported by Ren et al. and Redeuil et al. (2019). Differences in niacin status were previously described by Hampel et al. (2012), who showed significant variation of nicotinamide levels among samples of HM collected from various geographic areas (Cameroon, Malawi, China, India, and the USA). Authors speculated that those differences arise not only from the geographical origin and the obvious diet variations, but from food fortification as well (Hampel et al., 2012). This agrees with the investigation by Gibson et al. (2020) who showed a positive association between vitamin B3 dietary intake and its prevalence in HM. Variation in HM niacin levels also arise from methodological inconsistencies in measurement and reporting. Thus, our findings highlight the need for standardised approach to HM nutrient analysis (Gibson et al., 2020).

Pantothenic acid (vitamin B5) levels in our study were relatively consistent with the few data already described by the literature (Johnston et al., 1981; Song et al., 1984; Romera et al., 1996, Coryell & Harris, 1945; Ren et al., 2015) and confirmed that vitamin B5 is abundant in HM and generally sufficient for infant needs.

Ren et al. (2015) also measured the total vitamin B6 content, expressed as the sum of pyridoxal, pyridoxine, and pyridoxamine. Their measurement is not consistent with the results of our study. They report total levels of B6 at 62.7 (40.9, 93.9) ng/mL for mature milk at 180 days lactation, where we detected 10.0 ng/mL (6.8, 16.0 ng/mL) for pyridoxamine and 92.1 ng/mL (65.0, 98.1 ng/mL) for pyridoxal. Pyridoxine was undetected in > 50% of the samples and was excluded from calculating the total levels of B6 in DHM. This generated a median total B6 level of 36.4 ng/mL (10.4, 94.8 ng/mL) for DHM between 17 – 24 weeks of lactation, making the results more adjusted and reliable. Vitamin B6 is an important HM nutrient, and its low levels were associated with impaired infant behaviour, increased crying, and poor response to aversive stimuli (Rahmanifar et al., 1993). It was also found

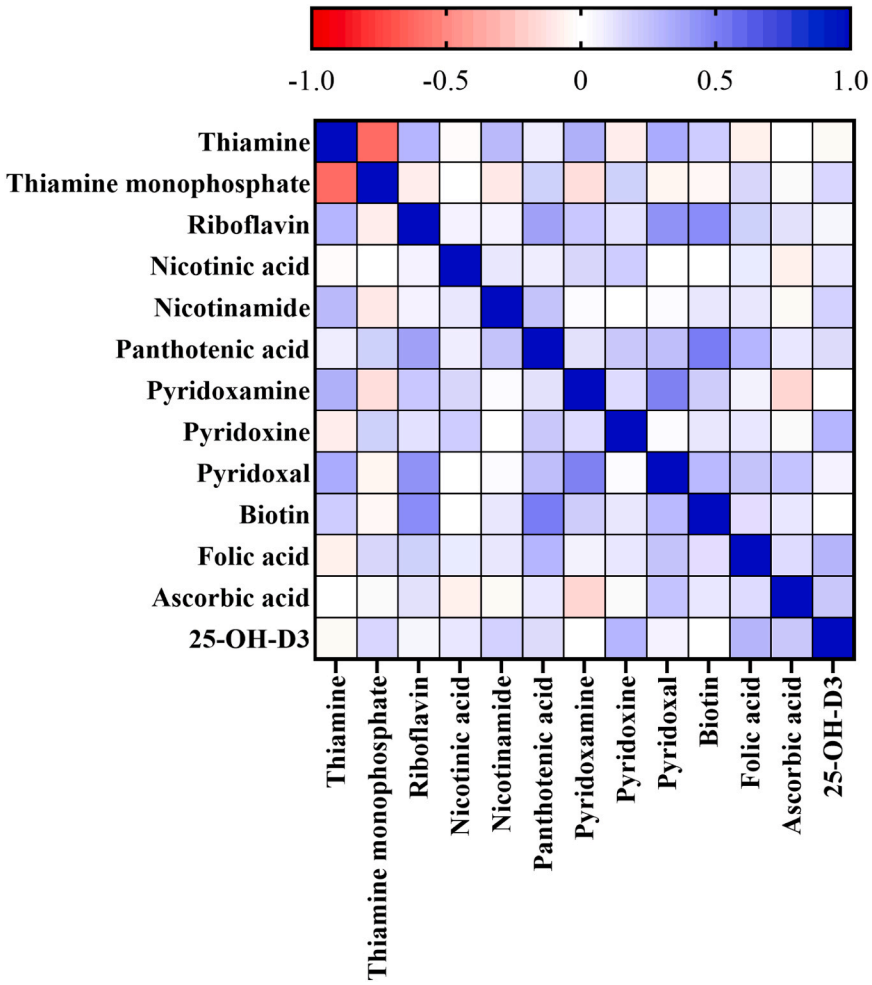


Fig. 4. Correlation matrix using Spearman ranking. The red color indicates a negative correlation, while the blue positive one. The more intense the color, the stronger the correlation. Thiamine and thiamine monophosphate are negatively correlated (-0.6) p = 2.79E-21.

that high levels of B6 in HM were significantly positively correlated with increased performance assessment in Brazelton's Neonatal Behavioral Assessment Scale (NBAS) (Ooylan et al., 2002).

Biotin, vitamin B7, is found in HM at relatively low levels. Our findings align with Ren et al. (2015), who reported mean biotin concentrations of 2.6 ng/mL in transitional milk, and 9.6 and 6.9 ng/mL in mature milk (31–100 and 101–300 days postpartum respectively). Here, we report a steady biotin concentration across 180 days of lactation postpartum, with a median of 5.6 (3.8, 7.5) ng/mL at 2–12 weeks, 5.6 (3.5, 7.6) ng/mL in mature milk 13 – 16 weeks postpartum and 5.3 (3.0, 6.9) ng/mL 17–24 weeks postpartum. Mock *et al.* (1997) showed the time-dependent distribution of biotin and its two analogues, bisnorbiotin, and biotin sulfoxide, to vary significantly. They have proven the increase in biotin and decrease in bisnorbiotin to be most remarkable in the first days of lactation, especially during the progression from colostrum to transitional milk. They have also suggested the importance of balance between biotin and its derivatives, as it was proven previously for both bisnorbiotin and biotin sulfoxide to inhibit free biotin absorption in the intestine (Said et al., 1987). Additionally, Gibson et al. reported a median of biotin 6.4 (4.7–7.9) μg/L in milk of Indonesian mothers at 5 months postpartum (Gibson et al., 2020).

Folic acid (FA), vitamin B9, is found in several chemical forms including the most predominant 5-methyltetrahydrofolate. An increasing trend is observed in HM maturation from colostrum up to 3 months in lactation, leading to a slight decrease and stabilization up to 6 months postpartum (Dror and Allen, 2018). Folate supplementation was found to elevate only free FA concentration in HM but no increase in other folate species was detected, and the biological significance of this phenomenon on infant health is unknown (Khambalia et al., 2006; Houghton et al., 2006; West et al., 2012). Interestingly, the levels of folate concentrations alter throughout the day, becoming higher as the day progresses (Udipi et al., 1987). A study by Han et al. (2009) showed no significant correlation between FA levels and infant weight, and length (Han et al., 2009). Our study shows relatively low and stable levels of FA throughout lactation, with mean of 4.9 ng/mL, 4.6 ng/mL, and 4.9 ng/mL respectively for the three lactation time points. This might result from the quantification of only FA, which is the supplemented form of vitamin B9 from fortified foods and quickly converted to folates. Even though the unmetabolized form of FA is more readily absorbed using the intestinal reduced folate carrier (RFC) (Milman, 2012), it is immediately converted by liver reductases to become different species of tetrahydrofolate, including 5,10-methyltetrahydrofolate and 5-methyltetrahydrofolate, which show greatest biological significance (Pertiwi et al., 2022). Page et al. (2017) have studied the distribution of unmetabolized folic acid (UMFA) and folates in HM. Interestingly, they have found that increased FA intake of $>$ 400 μg/daily might result in elevation of the UMFA in HM, with a gradual decrease in tetrahydrofolates, which are bioactively more relevant. They suggest the intake of FA to be managed at recommended levels of up to 400 μg/day as increased levels of UMFA might inhibit the activity of reductases leading to downregulation of tetrahydrofolates production. They also showed that UMFA makes around 25% of the total folate in HM when 0–400 μg of FA is consumed daily (Page et al., 2017). The study by Xue et al. (2017) reported an increase in total folate levels

ranging from an average of 7.3 ng/mL in transitional milk up to 23.3 ng/mL in mature milk 121 – 240 days postpartum (Xue et al., 2017). Su et al. (2022) reported total folate concentrations of 12.9 ng/mL in colostrum, 54.7 ng/mL 40–45 days postpartum, and 56.8 ng/mL 200–240 days postpartum. They have appointed 0.6 ng/mL, 0.0 ng/mL, and 1.2 ng/mL of UMFA for the mentioned time points respectively. This agrees with our data, proving that UMFA is scarce in human milk (Su et al., 2022).

Daily intake of vitamin C for an infant was estimated to be 30 – 35 mg per day, with an average absorption rate of 85% (Lawrence & Lawrence, 2016). It was reported that maternal vitamin C supplementation does not significantly increase its levels in HM (Sneed et al., 1981; Byerley & Kirksey, 1985). An increasing trend in vitamin C levels of HM was observed in our study across 24 weeks of lactation postpartum but of an insignificant nature (p-adj=5.03E-01). The lowest levels of 19.0 μg/mL (10.9–33.8) were quantified for time 2 – 12 weeks and increased up to 24.2 μg/mL (13.2–33.8) between 13 – 16 weeks, and then stabilized over time to 23.2 μg/mL (13.2–38.5) between 17 and 24 weeks postpartum. Ahmed et al. (2004) measured vitamin C levels to be on average 35.2 μg/mL, 32.5 μg/mL, and 30.3 μg/mL in colostrum (2nd day postpartum), transitional (2nd week postpartum) and mature HM samples (4th week postpartum) respectively. A study by Hoppu et al. (2005) reported increased levels of vitamin C among mothers with no food restrictions of up to 64 μg/mL as compared to mothers with food hypersensitivity (experiencing adverse reactions to various foods, here to citrus fruits, nuts and vegetables) of 55 μg/mL. Significantly increased risk of skin atopy in infants was related to low vitamin C levels in HM ($p = 0.038$) (Hoppu et al., 2005). It is worth noting, that quantification of true vitamin C levels in HM samples is largely impacted by the storage conditions. Buss et al. (2001) measured a one-third decrease after 1 month of freezer conditions and two-thirds up to two months in the freezer (Buss et al., 2001).

Vitamin D exists in two major forms: vitamin D2 (ergocalciferol), derived primarily from fungal sources, and vitamin D3 (cholecalciferol), synthesized endogenously in humans and other animals. Vitamin D_2 is produced from its provitamin, ergosterol, through exposure to ultraviolet B (UV-B) radiation and is commonly used in dietary supplements. Vitamin D3 is synthesized in the skin from 7-dehydrocholesterol following UV-B exposure. Although the biologically active forms of vitamin D are the dihydroxylated metabolites of both vitamin D2 and D3, circulating 25-hydroxyvitamin D is widely used as the most reliable marker of vitamin D status in plasma (Saenger et al., 2006; Mazahery & von Hurst, 2015). In the present study, only 25-hydroxyvitamin D3 (25-OH-D3) was quantified in DHM. We observed no significant changes in 25-OH-D3 concentrations across the three assessed lactation stages, with median levels of 0.8 ng/mL (2.0 nM) at 2–12 weeks, 0.9 ng/mL (2.3 nM) at 13–16 weeks, and 0.8 ng/mL (2.0 nM) at 17–24 weeks postpartum.

Jones et al. (2025) validated an LC-MS/MS method for the quantification of vitamin D metabolites in human milk from Gambian women, reporting concentrations of both cholecalciferol and 25-hydroxyvitamin D at later stages of lactation (around 14 weeks postpartum). In that study, cholecalciferol was the predominant vitamin D form in milk, whereas 25-hydroxyvitamin D was present at substantially lower concentration, allowing direct comparison with our measured analyte. Their previous review further summarized global data on vitamin D metabolites in HM and highlighted considerable geographic variability (Jones et al., 2023). Available evidence (Jones et al., 2023) indicates that average cholecalciferol concentrations in human milk typically remain below 10 nM (4.01 ng/mL), with reported values ranging from 0.1 to 7.8 nM (0.04–3.12 ng/mL). An exception was described by Ferreiro-Vera et al. (2013), who reported notably higher cholecalciferol concentrations (mean ~16 nM; 6.41 ng/mL) in a small cohort of Spanish mothers, suggesting a potential influence of UV-B exposure on milk vitamin D content. The relationship between maternal sun exposure and vitamin D status has been further explored by Dawodu et al. (2015), who examined associations between maternal and infant serum 25-hydroxyvitamin D concentrations and sun exposure indices across different geographic locations. Their findings demonstrated that higher sun exposure was generally associated with improved maternal vitamin D status and, consequently, higher infant serum 25-hydroxyvitamin D levels. Nevertheless, human milk alone is often insufficient to meet infant vitamin D requirements, particularly when maternal sun exposure is limited, underscoring the continued importance of dietary fortification and supplementation strategies to prevent vitamin D deficiency and rickets in infants. As cholecalciferol is generally more abundant than 25-hydroxyvitamin D in human milk, simultaneous quantification of both forms would provide a more comprehensive assessment of vitamin D delivery to the infant and should be considered in future studies.

The vitamin profile of DHM differs from that of raw milk due to processing steps required to ensure safety for preterm infants. Holder method (HoP), the most used method in milk banks, heats milk at 62.5°C for 30 min. While effective in eliminating pathogens, HoP can affect certain bioactive components. Most B vitamins (B1, B2, B3, B12) and vitamin C remain stable, though B6 shows moderate losses (11–16%). Notably, HoP appears to increase levels of active cholecalciferol (vitamin D3), likely by promoting conversion from its precursor form. These findings suggest that treatment duration, rather than temperature alone, influences nutrient retention (Peila et al., 2016; Wesolowska et al., 2019; Davis et al., 2025).

The findings of this study should be interpreted considering both its methodological strengths and inherent limitations. Although milk samples were collected at identical lactation stages across all centres, unequal sample distribution between countries and milk banks within each stage precluded robust geographic comparisons. Nevertheless, inclusion of samples from multiple regions increases the external relevance of the dataset, enhancing the generalizability of findings related to vitamin concentrations in DHM. Additionally, the use of advanced liquid chromatography-mass spectrometry (LC-MS) provided high sensitivity and specificity in quantifying multiple vitamins, allowing for a comprehensive analysis of the vitamin profile in DHM. The relatively large sample size of 189 milk samples collected at three postpartum time points further improves the reliability of the results and enables assessment of temporal variations in vitamin concentrations. However, the study also presents some limitations. Differences in recruitment procedures and ethical frameworks between participating countries may have introduced population heterogeneity, potentially affecting the consistency of the findings. Moreover, the study's reliance on specific collection and handling protocols for milk samples could contribute to pre-analytical variability that could impact the vitamin levels measured. For instance, the freezing and thawing processes, as well as the time taken from collection to analysis, may influence the stability of certain labile vitamins.

5. Conclusions

This study demonstrates that vitamin composition of donor human milk undergoes distinct, vitamin-specific changes across lactation, with pronounced shifts observed for selected B vitamins and relative stability of vitamins C and D. By applying a longitudinal, multi-vitamin analytical approach, our findings contribute to a more detailed understanding of micronutrient dynamics in DHM during the first six months of lactation. These data provide a valuable reference for nutritional evaluation of DHM and may inform future strategies aimed at optimizing its use in preterm and vulnerable infant populations. Future research integrating additional micronutrients and clinical outcomes will be essential to fully elucidate the nutritional significance of temporal variation in DHM.

CRediT authorship contribution statement

Mariusz Radkiewicz: Writing – review & editing, Writing – original draft, Resources, Methodology, Formal analysis. **Tonetto Paola:**

Investigation, Conceptualization. **Molas Aleksandra:** Investigation, Data curation. **Sottemano Stefano:** Investigation, Data curation. **Agnieszka Bzikowska-Jura:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Sinkiewicz-Darol Elena:** Investigation, Data curation. **Królak-Olejnik Barbara Krolak-Olejnik:** Data curation, Conceptualization. **van Goudoever Johannes:** Investigation, Data curation. **Gawronska Małgorzata:** Data curation, Conceptualization. **Gawel Paweł:** Investigation, Data curation. **Drazkowska Izabela:** Investigation, Data curation. **C.H.P. van den Akker:** Investigation, Data curation. **Gandino Serena:** Investigation, Data curation. **Wesolowska Aleksandra:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Bertino Enrico:** Investigation, Conceptualization.

Research data for this article

Given the sensitive nature of the questions, participants were informed that all raw data would be kept confidential and not shared.

Ethical statement

The study was conducted in accordance with ethical standards and approved by the relevant institutional review boards (approval numbers: AKBE/39/2020, 2021.0597, 169849 del 8.3.2021). Informed consent was obtained from all participants prior to their inclusion in the study. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Agnieszka Bzikowska-Jura reports financial support was provided by National Agency for Academic Exchange. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2026.109210.

Data availability

Data will be made available on request.

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