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A micronutrient supplement during preconception and pregnancy increases human milk vitamin D but not B vitamin concentrations

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Abstract

Background & Aims: Optimal maternal vitamin status during pregnancy and lactation is essential to support maternal and infant health. For instance, vitamin D_3 is involved in infant bone development, and B vitamins are involved in various metabolic processes, including energy production. Through a double-blind randomized controlled trial, we investigated the effects of maternal supplementation from preconception throughout pregnancy until birth on human milk (HM) concentrations of vitamin D_3 and B vitamins. In addition, we aimed to characterize longitudinal changes in milk concentrations of these vitamins.

Methods: Both control and intervention supplements contained calcium, iodine, iron, β-carotene, and folic acid, while the intervention also contained zinc, vitamins B_2, B_6, B_{12}, and D_3, probiotics, and myo-inositol. HM samples were collected across 4 time points from 1 week to 3 months post-delivery from mothers in Singapore (n=158), and 7 time points from 1 week to 12 months in New Zealand (n=180). HM vitamin D was quantified using supercritical fluid chromatography and B vitamins with mass spectrometry. Potential intervention effects on HM vitamins D_3, B_2, B_6, and B_9, as well as other B-vitamins (B_1 and B_3) concentrations were assessed using linear mixed models with a repeated measures design.

Results: Over the first 3 months of lactation, HM 25-hydroxyvitamin D_3 concentrations were 20% (95% CI 8%, 33%, P=0.001) higher in the intervention group, with more marked effects in New Zealand. There were no observed intervention effects on HM concentrations of vitamins B_1, B_2, B_3, B_6, and B_9. In New Zealand mothers, longitudinally, vitamin D_3 concentrations gradually increased from early lactation up to 12 months, while vitamins B_1 and B_2 peaked at 6 weeks, B_3 at 3 weeks, and B_6 and B_9 at 3 months.

Conclusions: Maternal supplementation during preconception and pregnancy increased HM vitamin D, but not B-vitamin, concentrations in lactation. Further studies are required to examine the discrete

benefits of vitamin D supplementation starting preconception vs during pregnancy, and to further characterize the effects of supplementation on later offspring health outcomes.

Clinical Trial registration: Registered at ClinicalTrials.gov on the 16 July 2015 (identifier NCT02509988); Universal Trial Number U1111-1171-8056. This study was academic-led by the EpiGen Global Research Consortium.

Keywords: human milk, pregnancy, supplement, vitamin B, vitamin D

Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
5MeTHF	5-methyltetrahydrofolic acid
aMD	adjusted mean difference
CI	confidence interval
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HM	human milk
LLoQ	lower limit of quantification
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate;
NiPPeR trial	The Nutritional Intervention Preconception and During Pregnancy to Maintain Healthy Glucose Metabolism and Offspring Health trial
NMN	nicotinamide mononucleotide
NR	nicotinamide riboside
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine-5'-phosphate
SD	standard deviation
TMP	thiamine monophosphate
TPP	thiamine pyrophosphate

Introduction

Vitamin D_3 is important for maternal and infant health during pregnancy and lactation (1). Vitamin D_3 is synthesized from sun exposure (conversion of 7-dehydrocholesterol in the skin by ultraviolet B rays) or can be obtained from dietary or supplemental sources (1,2). The precursor vitamin D_3 (cholecalciferol) is converted into 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver and then activated to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in the kidneys and human placenta (2,3). To support fetal development, the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ increases during pregnancy (1), plasma $1,25(OH)_2D_3$ levels increasing by 2-fold by 12 weeks of gestation, compared with pre-pregnancy values (3,4). The daily recommended vitamin D intake for pregnant women varies internationally, with 400 IU daily recommended in several settings (5–7). However, pregnancy vitamin D deficiency (serum $25(OH)D_3 < 50$ nmol/L) and insufficiency (serum $25(OH)D_3 < 75$ nmol/L) are frequently reported worldwide (8), estimated to be 33% and 69% in the United States (9), 24% and 65% in Canada (10), and 35.1% and 28.3% in the UK (11), respectively. Inadequate vitamin D status during pregnancy has been linked to adverse pregnancy outcomes such as preeclampsia (12), gestational diabetes mellitus (13), and an increased risk for caesarean section delivery (14).

During pregnancy, maternal serum $25(OH)D_3$ is positively correlated with cord blood $25(OH)D_3$ concentrations (15). Maternal vitamin D deficiency increases the risks of infant vitamin D deficiency which is associated with infantile rickets (16). Globally, the prevalence of infantile vitamin D deficiency rickets is growing (17), and higher rates of vitamin D deficiency were reported among mothers of rachitic infants (97%, n=38, median infant age 13.5 months) compared to mothers of non-rachitic infants (52%, n=50, median infant age 13.0 months) (18). Gestational vitamin D supplementation has been shown to not only improve maternal vitamin D status at the time of delivery but also reduce the risk of infantile rickets (19). Gestational vitamin D supplementation has also been associated with other health benefits for the offspring, such as a reduced risk of infantile eczema (20) and higher childhood bone mineral density (21). Infants 0–12 months require 200–400 IU vitamin D

per day (22,23). At birth, infant vitamin D status was highly correlated with maternal circulating levels, both of which were increased with maternal supplement during pregnancy in a dose-dependent manner (24). Infant vitamin D stores acquired from the mother in utero are depleted by about 8 weeks of age (25), after which, human milk (HM), sunlight exposure, and supplementation become the main sources of vitamin D for infants. Vitamin D content in HM is reported to be lower in winter than in summer (26) and in mothers with darker skin than in mothers with lighter skin (27). Vitamin D deficiency in infants can be prevented and treated with direct supplementation. Among breastfed infants, prophylactic supplementation was observed to lower the incidences of vitamin D deficiency (28,29). Conversely, when managing mothers particularly at risk of vitamin D deficiency/insufficiency, prophylactic treatment during pregnancy and/or lactation will benefit both the mother and the infant. A high dose (e.g., 4000 IU or 6400 IU per day) of vitamin D supplementation in the mother alone during lactation was shown to increase maternal circulating vitamin D levels, leading to increased HM vitamin D concentrations, and adequate infant vitamin D status (30–32). Further, infant vitamin D status achieved through maternal supplementation alone was similar to that of infants who received direct oral supplementation (32). This suggests that vitamin D supplementation solely in the mother can achieve adequate vitamin D status for both mothers and infants at risk of vitamin D deficiency or insufficiency.

B vitamins function as coenzymes in various biological processes such as macronutrient metabolism and energy production, and infant deficiency may lead to various health consequences. For example, vitamin B_1 (thiamine) deficiency is associated with infantile beriberi (33), and B_2 (riboflavin) with anaemia, growth retardation and dermatologic abnormalities (33). B_3 (niacin) is a precursor of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) coenzymes; deficiency in infants has been associated with pellagra (34). B_6 deficiency in infants is associated with neurological and behavioural abnormalities (35). Vitamin B_9 (folate) plays a role in DNA synthesis and cell growth, and low status in infants has been associated with a reduced growth

rate (36). HM B vitamin content is directly associated with maternal status (37) and previous studies showed maternal supplementation during lactation increased HM levels of vitamins B_1, B_2, and B_6 (38,39). HM vitamin B_9 concentration is unaffected by maternal status or supplementation, but B_9 supplementation is recommended to prevent deficiency in mothers during breastfeeding (36,40). Currently, there is limited knowledge on the effects of maternal micronutrient supplement taken before lactation on HM vitamin concentrations. This study aimed to examine the effects of the intervention supplement (taken before and during pregnancy but not after delivery) on HM concentrations of vitamins D and B, and their vitamer constituents. Moreover, we aimed to describe the longitudinal changes of these vitamins in HM during the first year of lactation.

Material and Methods

Study design

The detailed NiPPeR study protocol (ClinicalTrials.gov, identifier: NCT02509988, Universal Trial Number U1111-1171-8056; registered on 16 July 2015) has been published (41). Briefly, in a double-blind, randomised trial, the effects of a nutritional supplement taken from preconception and during pregnancy on maternal pregnancy and infant outcomes were investigated. The primary outcome of gestational glycaemia was no different between the intervention and control groups (42). **Table 1** shows the micronutrient contents of the control and intervention supplements. The control supplement comprised micronutrients present in supplements commonly used during pregnancy (calcium, iron, iodine, folic acid and β-carotene); in addition, the NiPPeR intervention supplement contained vitamins B_2, B_6, B_{12}, and D_3, as well as zinc, myo-inositol and probiotics. The study supplements were packaged as a powder in sachets and were taken twice daily, as a drink reconstituted with water. Adherence to the study supplements was assessed by sachet counting (42). The study was conducted in Southampton (UK), Singapore, and Auckland (New Zealand) with ethics approval from the UK Health Research Authority National Research Ethics Service Committee South Central Research Ethics Committee

(15/SC/0142), the Singapore National Healthcare Group Domain Specific Review Board (2015/00205) and the New Zealand Northern A Health and Disability Ethics Committee (15/NTA/21). All participants provided written informed consent. The procedures followed the ethical standards of the responsible institutional or regional committees on human experimentation, and in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Table 1. Detailed nutrient composition of the intervention and control drinks in the NiPPeR study.

Group	Nutrient	Intervention	Control	Daily dose	UNIMMAP formulation	% RDA*
Minerals	Calcium (as calcium-L-lactate)	✓	✓	150 mg	1,000 mg #	15%
	Iodine (as potassium iodide)	✓	✓	150 µg	150 µg	100%
	Iron (as ferric pyrophosphate)	✓	✓	12 mg	30 mg	40%
	Zinc (zinc glycinate chelate)	✓	×	10 mg	15 mg	67%
Vitamins	A (β-carotene)	✓	✓	720 µg	800 µg RAE	90%
	B_2 (Riboflavin)	✓	×	1.8 mg	1.4 mg	128%
	B_6 (Pyridoxine)	✓	×	2.6 mg	1.9 mg	137%
	B_9 (Folic acid)	✓	✓	400 µg	400 µg	100%
	B_{12} (Cobalamin)	✓	×	5.2 µg	2.6 µg	200%
	D_3 (Cholecalciferol)	✓	×	400 IU (10 µg)	400 IU (10 µg) #	200%
Other	Myo-inositol	✓	×	4 g	n/a	n/a
	Lactobacillus rhamnosus †	✓	×	>1 × 10^9 CFU	n/a	n/a
	Bifidobacterium animalis ssp. *lactis* §	✓	×	>1 × 10^9 CFU	n/a	n/a

* %RDA calculated as daily dose in the supplement divided by the UNIMMAP formulation

RDA during pregnancy according to the reference nutrient intake for the UK (5), recommended dietary allowance for Singapore (6), and recommended daily intake for New Zealand (7). %RDA calculated as daily dose in the supplement divided by the UNIMMAP formulation.

† NCC 4007 (CGMCC 1.3724)

§ NCC 2818 (CNCM I-3446)

Abbreviations: CFU, colony-forming units; n/a, not applicable; RAE, retinol activity equivalent; RDA, recommended daily intake; UNIMMAP, United Nations International Multiple Micronutrient Antenatal Preparation (43).

Study participants

Participants were recruited by self-referral from the community after study information was distributed through local and social media advertisements. The key inclusion criteria were women aged 18-38 years who were planning to conceive within 6 months. The full inclusion, exclusion and withdrawal criteria have been reported previously (41) and are provided in **Supplementary Table 1**.

Human milk sample collection

HM samples were collected from participants willing to provide samples in Singapore (from July 2016 to March 2019) and New Zealand (from May 2017 to November 2019). HM samples were not collected if the mother had ceased breastfeeding, her milk supply was low, or there were complications with milk expression. Samples were collected at 1 week ± 3 days, 3 weeks ± 5 days, 6 weeks ± 5 days, and 3 months ± 10 days (4 time points); in New Zealand, there were additional HM collections at 6 months ± 14 days, 9 months ± 14 days, and 12 months ± 14 days (7 time points in total). The opportunity to collect and assay HM samples only arose when follow-ups of mothers and infants post-delivery were already underway. As the recruitment of participants in NZ was ahead of that in Singapore, the collection of early HM samples from some women in NZ was not possible. Practical constraints centred on infrastructure for the collection and processing of samples precluded collection at the UK site and collection beyond 3 months in Singapore. Mothers were asked to refrain from breastfeeding for 2 hours prior to sample collection from the unilateral breast from where samples would be collected, allowing for a full breast to be emptied at the time of collection. Whole HM samples were collected in the morning from a single breast using an Ameda Lactaline breast pump (Ameda Inc, Murarrie, Australia). The breast was pumped for 15 minutes until fully emptied under the supervision of trained staff. Soon after collection, HM samples were homogenised and then stored at -80ºC until analyses. The total number of samples analysed at each time point is provided in **Figure 1**.

The number of participants with longitudinal samples to 3 months of lactation is summarised in **Supplementary Table 3**, and to 12 months of lactation in New Zealand in **Supplementary Table 4**.

Human milk vitamin D quantification

Quantitative analysis of vitamin D in HM was carried out as previously published (44). After thawing and homogenising by vigorous shaking at 40°C, a 200 µL portion was submitted to ethanolic protein precipitation. After liquid-liquid organic extraction and derivatization, sample extracts were analysed by supercritical fluid chromatography-tandem mass spectrometry. Calibration curves were created with each series of analyses (20 samples). Two QCs (low and high) were created by spiking a pooled HM sample (naturally containing vitamin D_3 and 25(OH)D_3) to yield approximately 200 and 400 ng/L of each of the metabolites, respectively, for inclusion in each analytical series.

Human milk vitamin B quantification

HM B-vitamers analyses and quantification were performed at NEOTRON SpA (Modena, Italy). A detailed description of the applied methodology has been published previously (45,46). Briefly, 200 µL of HM were exposed to methanolic protein precipitation. After evaporation, reconstitution and filtration, sample extracts were analysed by reversed-phase liquid chromatography combined with tandem mass spectrometry. In each analytical sequence, unknown samples were quantified with a matrix-matched calibration containing 7 calibration standards, and 9 QC samples (3 at low level (corresponding to 7.5 × STD1), 3 at mid-level (corresponding to 40 × STD1) and 3 at high level (corresponding at 150 × STD1)). The content of each vitamer (individual molecule) was calculated individually.

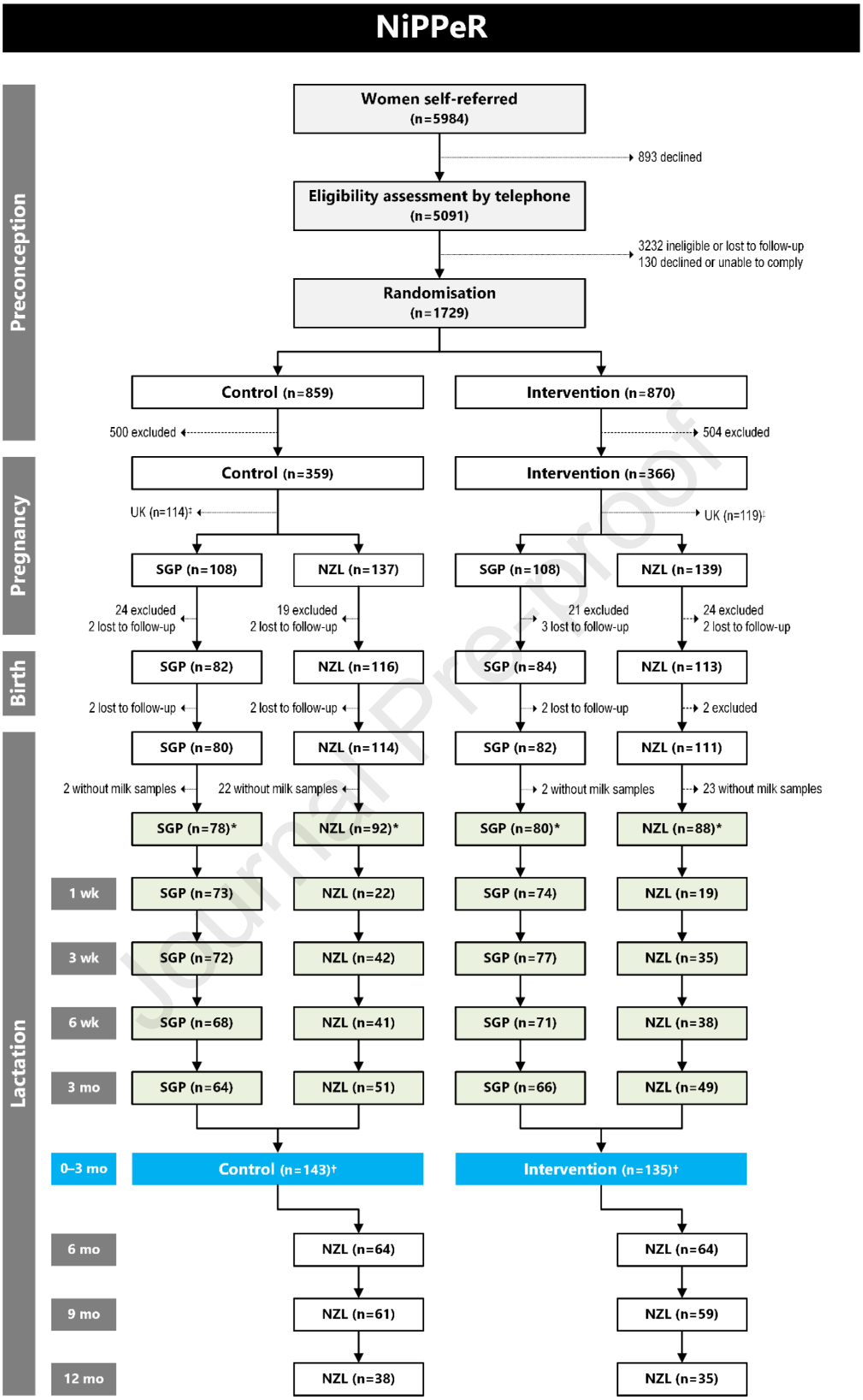


Figure 1. CONSORT diagram with the numbers of human milk (HM) samples analysed for vitamin concentrations in the NiPPeR study.

Reasons for exclusion during the preconception phase have been published previously (42), while reasons for exclusion during pregnancy and birth in Singapore (SGP) and New Zealand (NZL) are provided in **Supplementary Table 2**. The numbers in this figure correspond to the number of samples analysed for B vitamins. Of these, 42 samples could not be analysed for vitamin D due to insufficient volume. ‡ There were no HM samples collected in the United Kingdom (UK), so all participants from that site were excluded from this diagram. * Number of participants who provided at least one HM sample during 12 months of lactation. † Number of participants who provided at least one HM sample during the first 3 months of lactation.

Statistical analysis

Participant characteristics (categorical) between the control and intervention groups were compared using Fisher's exact tests. Vitamer concentration measurements below the lower limit of quantification (LLoQ) were assigned a value of 0.5 × LLoQ (**Supplementary Table 5**). To minimise the removal of values from the dataset, we adopted a conservative approach defining extreme values (i.e., outliers) as measurements outside the range of mean ± 5 * standard deviations (SD). There were no values < (mean – 5*SD), but there were values > (mean + 5*SD) for some vitamers that were classified as extreme (i.e., >99.99997th percentile) and removed from analyses (**Supplementary Table 5**). Further, it was not possible to undertake reliable statistical analysis on six vitamers (i.e., vitamin D_2, 25(OH)D_2, nicotinic acid, pyridoxamine, pyridoxine, and folic acid) as a large proportion of their values (>50%) were below the LLoQ.

B-vitamers of the same B vitamin group were summed together to give the total HM concentrations for vitamins B_1, B_2, B_3, B_6, and B_9 (**Table 2**). For all outcomes, data were log-transformed to approximate a normal distribution.

Table 2. List of vitamin groups and their vitamer constituents.

Vitamin Group	Vitamer
–	25(OH)D_3
–	Vitamin D_3
–	25(OH)D_2 [1]
–	Vitamin D_2 [1]
Vitamin B_1	Thiamine TMP TPP
Vitamin B_2	FAD FMN Riboflavin
Vitamin B_3	Nicotinamide NMN NR Nicotinic acid[1]
Vitamin B_6	Pyridoxal PLP Pyridoxamine[1] PMP Pyridoxine[1]
Vitamin B_9	Folic acid[1] 5MeTHF
–	NAD
–	NADP

[1] Large proportion of the values below the lower limit of quantification.
Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 5MeTHF, 5-methyltetrahydrofolic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NAD, nicotinamide adenine dinucleotide; NAPD, nicotinamide adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine-5'-phosphate; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate.

Potential intervention effects on HM vitamin concentrations were only examined on the samples collected in the first 3 months of lactation in Singapore and New Zealand. In sensitivity analyses, models were run for the subgroup of participants who provided consecutive samples across the 4 time points in the first 3 months. These outcomes were assessed using linear mixed models with a repeated measures design. Parameters included in models were randomisation group, visit, their interaction term (group*visit), and study site, as well as adherence to the study supplements, maternal pre-pregnancy body mass index, and gestational age as continuous variables. Additionally, for 25(OH)D_3 and vitamin D_3, season at the time of HM collection was included as a covariate (with the four seasons defined according to the meteorological criteria (47)); for these outcomes, the interaction between randomisation group and season was also tested, with the interaction term removed if non-significant. The participant's study ID was also included as a random factor to account for the multiple

measurements on the same individual (non-independence). If the group*visit interaction term was statistically significant, between-group comparisons were only reported per visit. A group*site interaction was also included in the model to test for differential responses to treatment in the two sites, and subsequently removed if not statistically significant. In addition, overall differences in B vitamin concentrations (across all participants in Singapore and Auckland) at 1 week, 3 weeks, 6 weeks, and 3 months were compared using the previously described linear mixed models based on repeated measures, with *P*-values adjusted for multiple comparisons by the Bonferroni method (48).

Lastly, subgroup analyses were also performed to examine potential intervention effects over the first 3 months of lactation separately for Singapore and New Zealand. Temporal changes in HM vitamins from 1 week to 12 months of lactation were plotted and reported for the New Zealand site only, as samples from the later time points were unavailable in Singapore. These were also examined in a subgroup of New Zealand participants who provided HM samples for at least five out of six time points between 3 weeks and 12 months.

Study outcomes are reported as the back-transformed least-square means (i.e., adjusted means) for each group or the adjusted mean difference (aMD) between groups, and their respective 95% confidence intervals (CI). Note that the aMD for back-transformed values represent proportional differences between intervention and control groups. Statistical analyses were carried out using R version 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria) and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). All tests were two-sided; statistical significance was maintained at $P<0.05$ without adjustments for multiple comparisons (unless stated otherwise), and with no imputation of missing values.

Results

Study population

Of 387 participants in Singapore and New Zealand who continued to the postpartum stage of the study, 338 (87.3%) provided at least one HM sample during the study period (**Figure 1**). Maternal demographics and pre-pregnancy BMI characteristics were similar in control and intervention groups (**Table 3**). In Singapore, most participants were of Chinese ethnicity, while in New Zealand most were White Caucasians. Adherence to the study control and intervention supplements was high and averaged approximately 87% consumption for both groups. The mean (± SD) duration of supplementation was similar between groups: 405 ± 105 and 393 ± 98 days in the control and intervention groups, respectively. Passive smoking during pregnancy was more common among controls than in the intervention group (19.4% vs 9.5%, respectively; *P*=0.013). Other pregnancy and birth outcomes were similar between the two groups overall (**Table 3**) and within each site (**Supplementary Table 6**).

Impact of preconception and pregnancy intervention on human milk vitamin D

Over the first 3 months of lactation, 25(OH)D_3 concentrations were 20% higher in the intervention group compared to the control group (*P*=0.001, **Table 4**). When this was examined at individual visits, the differences between the groups were most evident at 1, 3, and 6 weeks of lactation, the intervention group being higher than the control group by 26%, 30% and 16%, respectively (**Figure 2A**). This intervention effect was also reflected in vitamin D_3 concentrations (**Supplementary Figure 1A**). Maternal smoking during pregnancy (predominantly passive) was not associated with HM 25(OH)D_3 or vitamin D_3 concentrations and did not alter the overall outcome (data not shown). For completeness, we have performed sensitivity analyses adjusting for maternal baseline serum 25(OH)D_3 level, but the observed intervention effects on HM 25(OH)D_3 and vitamin D_3 were unchanged.

The interaction term between randomisation group and season was not statistically significant for either vitamin D_3 (P=0.59) or 25(OH)D_3 (P=0.89), indicating that the intervention effect was independent of season at the time of HM sample collection. In addition, there was also no interaction between randomisation group and site for either outcome (P=0.24 and P=0.29, respectively), and thus, no evidence of differential treatment effects at the two sites. Nonetheless, since women in Singapore (latitude 1.3° N) and Auckland (≈37° S) experience different levels of sunlight exposure throughout the year (47) and are ethnically and culturally distinct, it was of interest to examine potential intervention effects on HM vitamin D_3 separately for each site. In Singapore, 25(OH)D_3 levels in the control group were higher than their New Zealand counterparts (**Table 4**), and while an intervention effect on HM 25(OH)D_3 in Singapore was detected at week 1 [intervention 131 (95% CI 118, 145) ng/L vs control 113 (95% CI 101, 125) ng/L, P=0.049], it was subsequently attenuated [week 3: intervention 137 (95% CI 124, 152) ng/L vs control 120 (95% CI 108, 133) ng/L, P=0.08] (**Figure 2B**), and there were no observed effects on vitamin D_3 (**Supplementary Figure 1B**). In New Zealand, 25(OH)D_3 concentrations were higher in the intervention group by 36% over the first 3 months of lactation compared to the control group (P=0.001, **Table 4**). The difference between the groups was most evident at 3 weeks [intervention 166 (95% CI 137, 199) ng/L vs control 100 (95% CI 85, 118) ng/L, P<0.0001] and at 6 weeks [intervention 157 (95% CI 132, 187) vs control 115 (98, 135) ng/L, P=0.011] (**Figure 2C**). A similar pattern was observed for vitamin D_3 concentrations (**Supplementary Figure 1C**) at the same time points. The intervention effect on HM 25(OH)D_3 and vitamin D_3 was also present in the subgroup of mothers who provided all 4 consecutive samples in the first 3 months (data not shown).

Table 3. Baseline and perinatal characteristics of participants in the NiPPeR study who provided at least one human milk sample during 12 months of lactation.

	Overall (n = 338)	
	Control	**Intervention**
n	170 (50.3%)	168 (49.7%)
Duration of supplementation (days)	404.6 ± 105.0	393.1 ± 98.0
Adherence (%)	87.4 ± 11.2	86.9 ± 13.4
Ethnicity		
Caucasian	70 (41.2%)	67 (39.9%)
Chinese	70 (41.2%)	69 (41.1%)
South Asian	10 (5.9%)	10 (6.0%)
Malay	10 (5.9%)	10 (6.0%)
Other	10 (5.9%)	12 (7.1%)
Age at delivery (years)	31.9 ± 2.9	32.4 ± 3.2
BMI (kg/m^2)	24.4 ± 5.2	23.4 ± 4.4
BMI status		
Underweight or normal weight	100 (58.8%)	103 (61.3%)
Overweight	41 (24.1%)	48 (28.6%)
Obesity	29 (17.1%)	16 (9.5%)
Missing	–	1 (0.6%)
Highest level of education		
Bachelor's degree or higher	137 (80.6%)	136 (81.0%)
Lesser qualification [1]	33 (19.4%)	32 (19.0%)
Household income quintile		
5 (lowest)	4 (2.4%)	1 (0.6%)
4	12 (7.1%)	16 (9.5%)
3	44 (25.9%)	43 (25.6%)
2	60 (35.3%)	55 (32.7%)
1 (highest)	44 (25.9%)	43 (25.6%)
Missing	6 (3.5%)	10 (6.0%)
Smoking during pregnancy		
None	134 (78.8%)	148 (88.6%)
Passive	33 (19.4%)	16 (9.6%)
Active	3 (1.8%)	3 (1.8%)
Missing	–	1 (0.6%)
GDM		
No GDM	126 (74.1%)	125 (74.4%)
GDM	42 (24.7%)	43 (25.6%)
Missing	2 (1.2%)	–
Mode of delivery		
Vaginal delivery	125 (73.5%)	119 (70.8%)
Caesarean section	44 (25.9%)	49 (29.2%)
Missing	1 (0.6%)	–
Gestational age (weeks)	39.1 ± 1.6	39.2 ± 1.5
Preterm	14 (8.2%)	11 (6.5%)
Term or post-term	156 (91.8%)	157 (93.5%)
Parity		
Primiparous	114 (67.1%)	95 (56.5%)
Multiparous	56 (32.9%)	73 (43.5%)
Infant sex		
Male	76 (44.7%)	79 (47.0%)
Female	94 (55.3%)	89 (53.0%)

Data are n (%) or mean ± standard deviation (SD). The adherence to the study protocol was determined by sachet counting. The duration of supplementation was calculated by counting the number of days between randomisation and delivery. Body mass index (BMI) status was defined using ethnic-specific thresholds: for Asians, underweight or normal weight <23.0 kg/m^2, overweight 23.0–27.49 kg/m^2, and obesity ≥27.5 kg/m^2; for non-Asians, underweight or normal weight <25.0 kg/m^2, overweight 25.0–29.99 kg/m^2, and obesity ≥30.0 kg/m^2. Gestational diabetes mellitus (GDM) was defined by the International Association of Diabetes and Pregnancy Study Groups criteria (49). Gestational age was determined using a pre-specified algorithm as previously described (50), with preterm birth defined as <37 weeks of gestation, and term or post-term births as ≥37 weeks of gestation. [1] Including incomplete and complete high school qualifications and other tertiary level qualifications below a bachelor's degree (e.g., diploma or certificate).

Table 4. Comparisons in average vitamin D concentrations in human milk (HM) over the first 3 months of lactation in the intervention and control groups.

Vitamin D (ng/L)	Intervention	Control	aMD	*P*-value
25(OH)D_3	141 (130, 152)	118 (109, 126)	1.20 (1.08, 1.33)	**0.001**
Singapore	132 (122, 144)	119 (110, 129)	1.11 (0.99, 1.24)	0.074
New Zealand	157 (137, 180)	116 (102, 131)	1.36 (1.13, 1.63)	**0.001**
Vitamin D_3	133 (109, 162)	89 (74,108)	1.49 (1.13, 1.95)	**0.005**
Singapore	133 (104, 171)	106 (83, 136)	1.26 (0.88, 1.79)	0.205
New Zealand	136 (99, 188)	67 (50, 89)	2.04 (1.33, 3.14)	**0.001**

Data are the least-square mean (i.e., adjusted mean) for each group or the adjusted mean difference (aMD), and respective 95% confidence intervals derived from repeated measures analyses, adjusted for visit, an interaction term (group*visit), study site, adherence, maternal pre-pregnancy body mass index, gestational age at birth, and season at HM sample collection. All data have been log-transformed to approximate a normal distribution and then back-transformed, so the aMD represents a proportional difference between the groups (i.e., intervention vs control). Bold font indicates a statistically significant difference between groups at $P<0.05$.

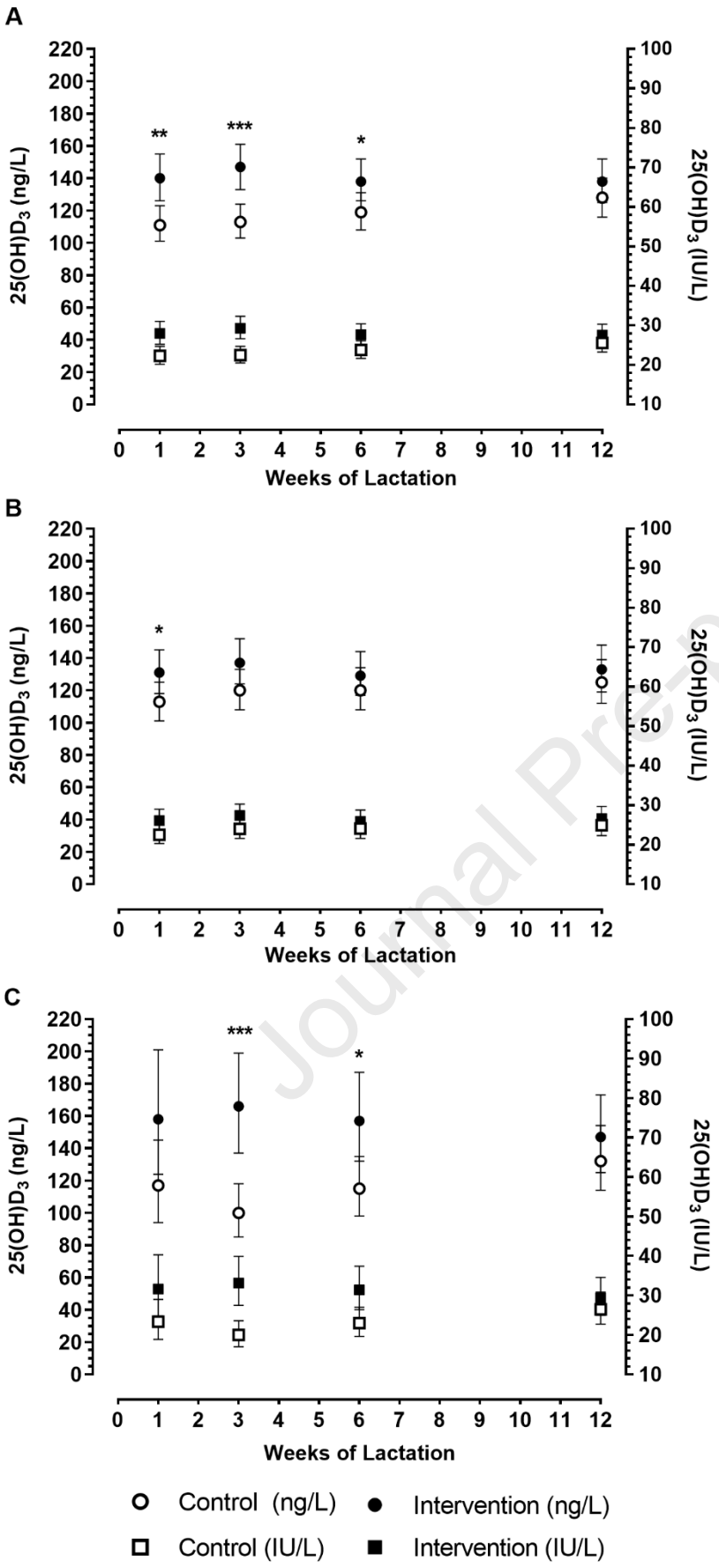


Figure 2. 25(OH)D_3 concentrations in human milk of control and intervention groups in the NiPPeR study during the first 3 months of lactation: (A) Overall, (B) Singapore, and (C) New Zealand. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for visit, an interaction term (group*visit), study site, adherence, maternal pre-pregnancy body mass index, gestational age at birth, and season at human milk sample collection; error bars represent the respective 95% confidence intervals. **P*<0.05, ***P*<0.01, and ****P*<0.001 for the difference between intervention and control groups at a given time point.

Impact of preconception and pregnancy intervention on human milk B vitamin concentrations

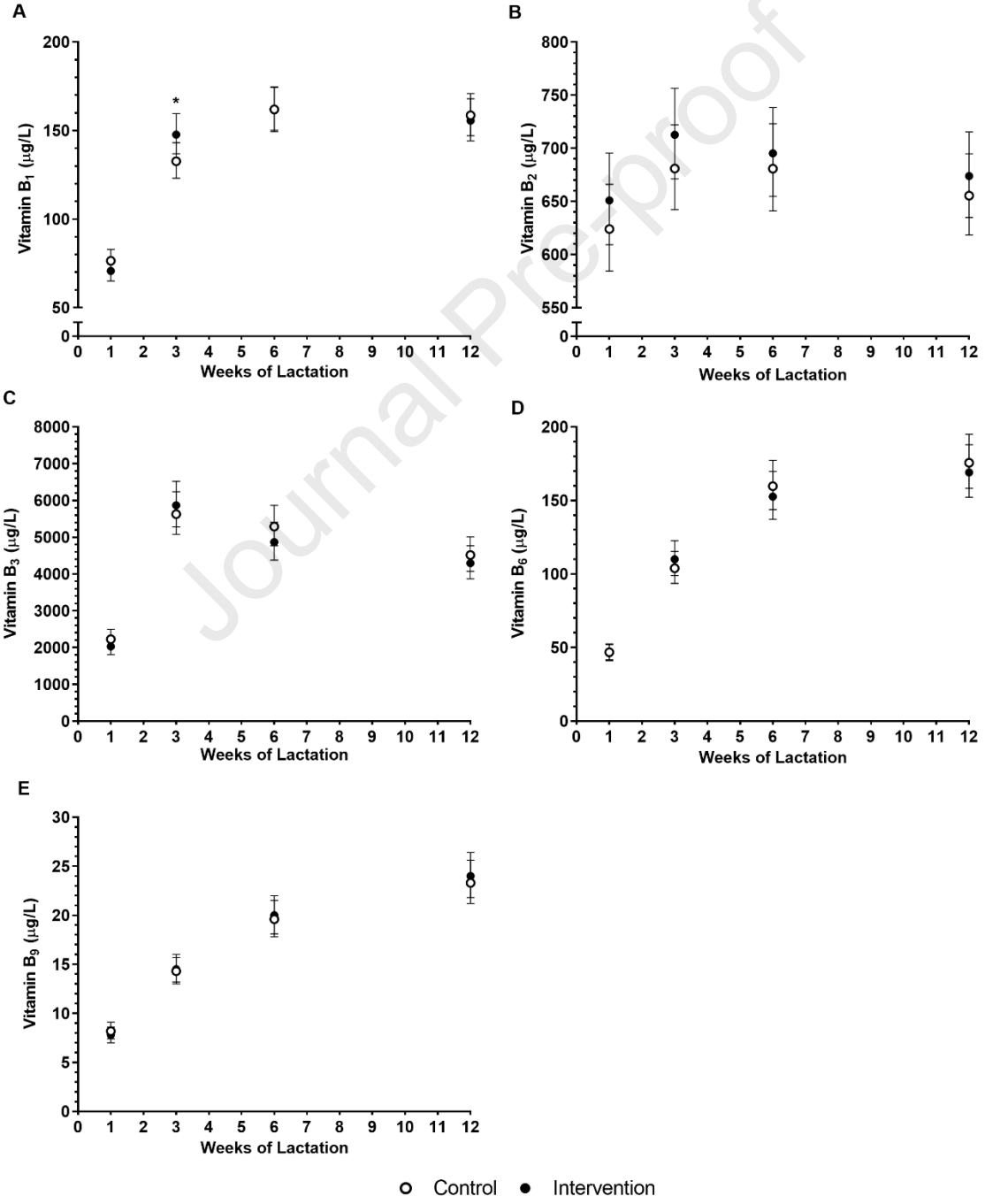
Overall, the total HM concentrations of vitamins B_1, B_2, B_3, B_6, and B_9 were similar between the control and the intervention groups across the first 3 months of lactation (**Table 5**) and at individual time points (**Figure 3**). Also, the mean B-vitamers concentrations over the first 3 months did not differ between the control and intervention groups (**Supplementary Table 7**). In analyses stratified by site, the mean vitamin B and B-vitamer concentrations over the first 3 months did not differ between the control and intervention groups within each site (data not shown). Maternal smoking during pregnancy (predominantly passive) was not associated with HM vitamin B concentrations and did not alter the overall outcome (data not shown). Note that there were marked differences in B vitamin concentrations between visits (P<0.0001 for all B vitamins); thus, all HM samples collected at 3 weeks, 6 weeks, and 3 months had higher B vitamin concentrations than the week-1 sample (all adjusted p-values <0.01), the only exception being the B_2 sample at 3 months (**Figure 3**). Lastly, for completeness, we ran sensitivity analyses adjusting for the respective maternal baseline serum B vitamin levels, but our findings were unchanged.

Table 5. Comparisons in average vitamin B concentrations in human milk (HM) over the first 3 months of lactation in the intervention and control groups.

Vitamin B (µg/L)	Intervention	Control	aMD	P-value
B_1	Significant group*visit interaction P=0.012			
B_2 *	683 (656, 711)	660 (634, 686)	1.03 (0.98, 1.09)	0.228
B_3	3975 (3686, 4286)	4162 (3867, 4480)	0.95 (0.86, 1.06)	0.384
B_6 *	107 (99, 116)	108 (100, 117)	0.99 (0.89, 1.10)	0.865
B_9 #	15.3 (14.2, 16.4)	15.2 (14.2, 16.3)	1.00 (0.91, 1.10)	0.972

* Only present in the intervention drink. # Present in both control and intervention drinks. Data are the least-square mean (i.e., adjusted mean) for each group or the adjusted mean difference (aMD), and respective 95% confidence intervals, derived from repeated measures analyses, adjusted for visit, an interaction term (group*visit), study site, adherence, maternal pre-pregnancy body mass index, and gestational age at birth. Where a statistically significant group*visit interaction was present, it was necessary to interpret potential intervention effects on a per-visit basis. All data have been log-transformed to approximate a normal distribution and then back-transformed, so the aMD represents a proportional difference between the groups (i.e., intervention vs control). Comparisons in average HM B-vitamer concentrations over the first 3 months of lactation are shown in **Supplementary Table 7**.

Figure 3. Vitamin B concentrations in human milk of control and intervention groups in the NiPPeR study, during the first 3 months of lactation: (A) B_1, (B) B_2, (C) B_3, (D) B_6, and (E) B_9. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for visit, an interaction term (group*visit), study site, adherence, maternal pre-pregnancy body mass index, and gestational age at birth; error bars represent the respective 95% confidence intervals. *P<0.05 for a difference between intervention and control groups at a given time point.



Changes in human milk vitamins over time in New Zealand (0-12 months)

Analyses of the HM samples collected in New Zealand showed that HM vitamin concentrations changed dynamically from early lactation to 12 months post-delivery. In both control and intervention groups, 25(OH)D_3 concentrations gradually increased (**Figure 4A**). In the control group, 25(OH)D_3 concentrations increased from 116 (95% CI 92, 147) ng/L at 1 week to 185 (95% CI 153, 224) ng/L at 12 months. Similarly, in the intervention group, 25(OH)D_3 concentrations increased from 159 (95% CI 123, 207) ng/L to 181 (95% CI 149, 220) ng/L from 1 week to 12 months of lactation. This pattern was also reflected in HM vitamin D_3 which gradually increased over the same period (**Supplementary Table 8**).

Total vitamin B_1 concentrations in New Zealand increased from 1 week to 6 weeks of lactation and then remained constant until 12 months (**Figure 4B**). Similarly, thiamine monophosphate (TMP) concentrations peaked at 6 weeks, then continued to decrease until 12 months, while thiamine concentration gradually increased from 1 week to 12 months of lactation (**Supplementary Table 9**). At 1 week, TMP contributed the most to total HM vitamin B_1, at 85.4%; at 12 months, thiamine contributed the most at 54.6% (**Figure 5A**).

Total vitamin B_2 concentrations increased in early lactation, peaking at 6 weeks, followed by a nadir at 6 months, then increasing from 6 to 12 months (**Figure 4C**). Flavin adenine dinucleotide (FAD) concentrations were highest at 1 week and lowest at 6 months. Riboflavin concentration gradually increased from 1 week to 9 months of lactation (**Supplementary Table 9**). During this time, the FAD contribution to total HM vitamin B_2 decreased from 90.6% to 70.1%, while the riboflavin contribution increased from 6.2% to 27.0% from 1 week to 9 months of lactation **(Figure 5B**).

Total vitamin B_3 concentration was highest at 3 weeks, fell to lower concentrations at 6 months, then remained stable from 6 to 12 months of lactation (**Figure 4D**). Throughout the 12 months of lactation studied nicotinamide mononucleotide (NMN) was the dominant form of HM vitamin B_3 (**Supplementary Table 9**), its contribution ranging from 78.1% to 85.1% (**Figure 5C**).

Total vitamin B_6 concentration increased from 1 week to 6 months then remained stable thereafter until 12 months of lactation (**Figure 4E**). Pyridoxal concentrations reached a maximum at 6 months and pyridoxal 5'-phosphate (PLP) concentration peaked at 3 months of lactation (**Supplementary Table 9**). In early lactation, PLP was the predominant vitamin B_6 vitamer, contributing 50.6%, while pyridoxal contributed 43.0%; by 12 months of lactation, this ratio shifted, PLP decreasing to 14.5% and pyridoxal increasing to 83.6% (**Figure 5D**).

Finally, total vitamin B_9 concentrations increased in early lactation, reaching a peak at 3 months, followed by a steep decrease from 3 months to 6 months, thereafter remaining constant from 6 to 12 months of lactation (**Figure 4F**). This pattern was also reflected in 5-methyltetrahydrofolic acid (5MeTHF) (Supplementary Table 9), the predominant contributor to total HM vitamin B_9 throughout the 12 months studied, with a contribution ranging from 62.2% to 73.3% (**Figure 5E**). The patterns of temporal changes in HM vitamins were similar when assessed in a subset of New Zealand women who provided at least 5 out of 6 samples between 3 weeks and 12 months (data not shown).

Figure 4. Vitamin concentrations in human milk from control and intervention groups in New Zealand in the NiPPeR Study, during the first 12 months of lactation: (A) 25(OH)D_3, (B) B_1, (C) B_2, (D) B_3, (E) B_6, and (F) B_9. Data are the least-squares means (i.e., adjusted means) for each group adjusted for visit, a group*visit interaction term, adherence, maternal pre-pregnancy body mass index, and gestational age at birth, with season at sample collection also included in the model for 25(OH)D_3. Error bars represent the respective 95% confidence intervals. *P<0.05 and **P<0.01 for the difference between intervention and control groups at a given time point.

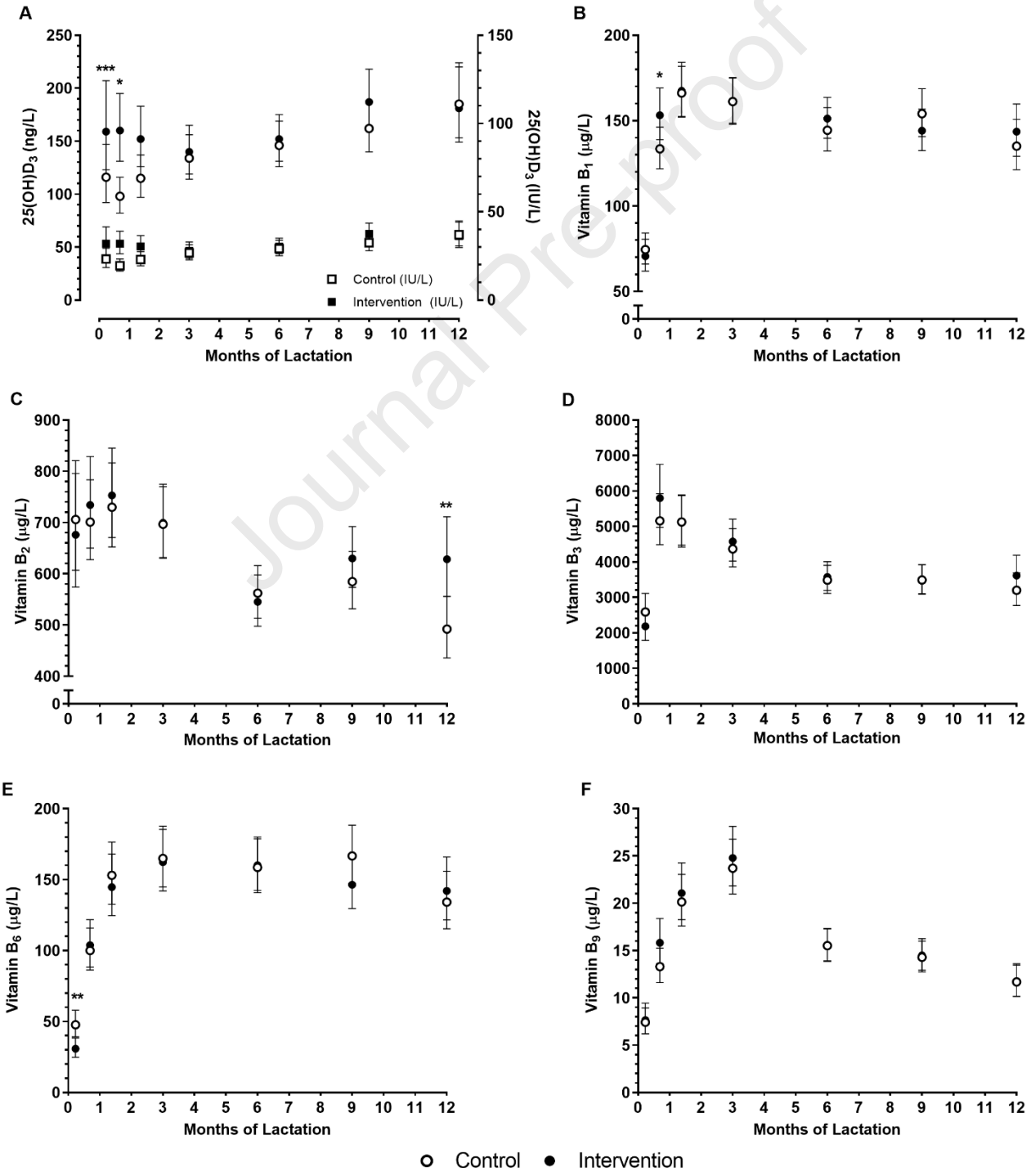
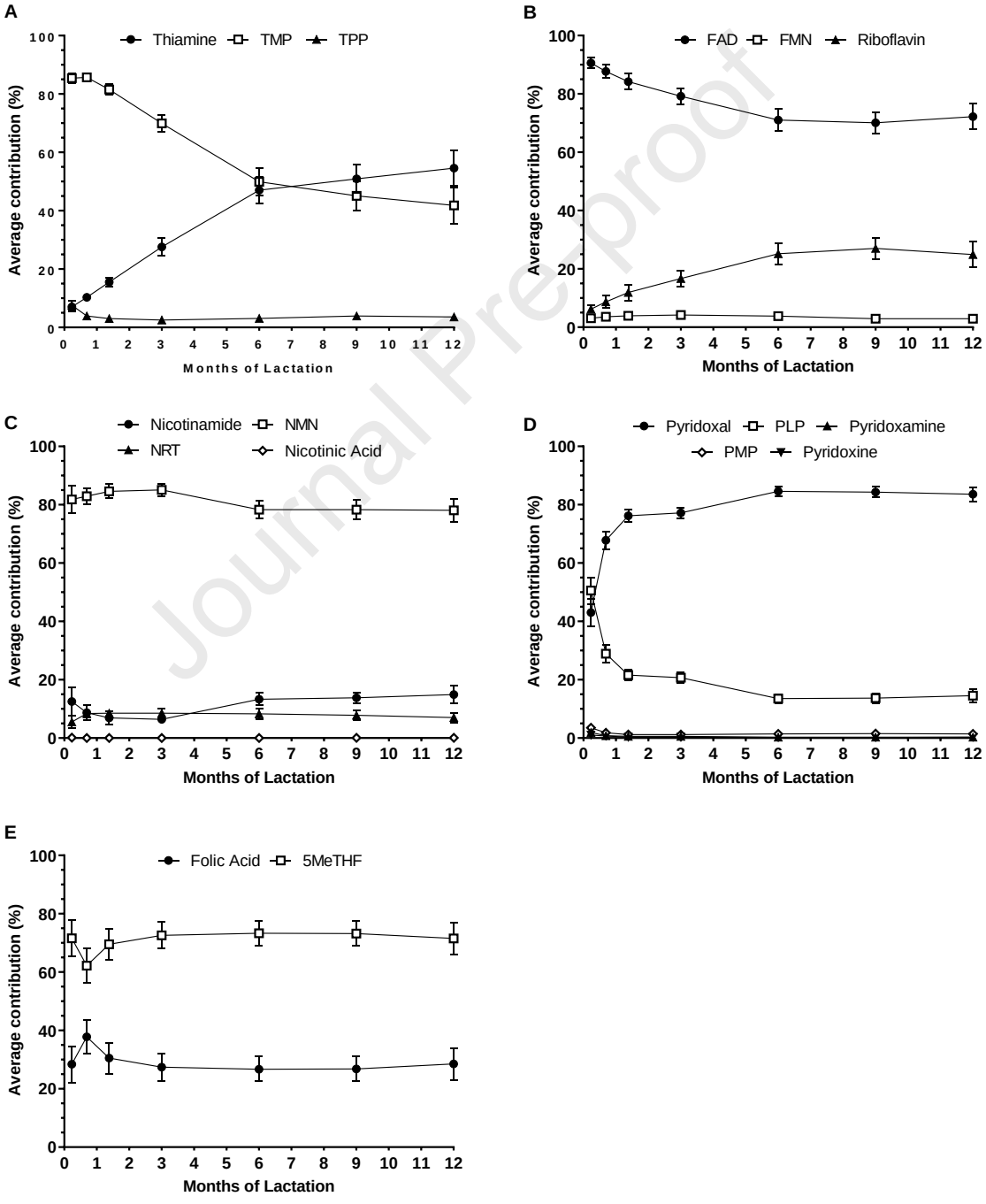


Figure 5. The average contribution (%) of human milk B-vitamers in New Zealand in the NiPPeR study, during the first 12 months of lactation for (A) B_1, (B) B_2, (C) B_3 (D) B_6, and (E) B_9. Data represent the mean contribution of each vitamer at a given visit, and the error bars the respective 95% confidence intervals. Abbreviations: 5MeTHF, 5-methyltetrahydrofolic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine-5'-phosphate; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate.



Discussion

This study showed that an intervention supplement containing 400 IU vitamin D_3 taken before and during pregnancy (but not continued after delivery) increased HM 25(OH)D_3 concentrations over the first 3 months of lactation, particularly in New Zealand. There were no effects of the NiPPeR intervention supplement on HM concentrations of vitamins B_2, B_6, or B_9, and not surprisingly, no effects on the other measured B-vitamins not in the supplement (B_1 and B_3). Maternal supplementation in this study ceased at delivery, a potential reason for the similar HM B vitamin concentrations between the control and intervention groups.

NiPPeR intervention increased HM 25(OH)D_3

HM vitamin D concentrations are highly dependent on maternal vitamin D status (51). Vitamin D transport into the milk is affected by vitamin D binding protein. In maternal circulation, 25(OH)D_3 is tightly bound to vitamin D binding protein, and the transport into the milk is dependent on receptor-mediated endocytosis (52,53). Conversely, the precursor vitamin D_3 is less strongly bound to the vitamin D binding protein, allowing simple diffusion across the cell membranes into the milk (52,53). Previous studies observed a positive association between HM 25(OH)D levels at delivery and maternal serum 25(OH)D levels in second trimester of pregnancy (54). This suggests that maternal vitamin D supplementation during lactation alone, as in some previous studies (30–32), may not ensure adequate HM vitamin D levels in early lactation, especially in mothers with inadequate vitamin D status during pregnancy. However, few studies have investigated the potential influence of vitamin D supplementation during pregnancy on HM vitamin D concentrations. In a double-blind placebo-controlled trial in New Zealand, pregnant women were randomised to receive either placebo, 1,000 IU, or 2,000 IU vitamin D_3 per day from 27 weeks until 36 weeks gestation. At 2 weeks and 2 months, total HM vitamin D_3 concentrations were higher in the 2,000 IU group compared to the 1,000 IU group (55). Similarly, in the current study, HM 25(OH)D_3 concentrations over 3 months of lactation were

higher in the NiPPeR intervention group supplemented from preconception and throughout pregnancy. Notably, this was achieved with a lower daily dose of 400 IU of vitamin D_3, suggesting that cumulative exposure needs to be considered. In the NiPPeR study, accounting for the daily dose (400 IU), the average duration of supplementation (393 days), and average adherence (86.9%), the total cumulative exposure of vitamin D_3 is estimated to have been approximately 136,642 IU in the intervention group. Compared to the study by Wall et al. (55), this is higher than the total of 72,000 IU and close to 140,000 IU exposed to the 1,000 IU and 2,000 IU groups, respectively, over 10 weeks during pregnancy only. In addition, as vitamin D can be stored in adipose tissues (56), vitamin D acquired during the supplemented period could influence maternal vitamin D status over the following months of lactation, contributing to HM vitamin D concentrations. Previous studies focused on short-term effects on HM vitamin D concentrations by maternal supplementation with high doses during lactation only. Our study is distinctive with regards to window of effect, showing that supplementation with a standard (lower) dose over a longer period from preconception and throughout pregnancy could have prolonged effects on HM vitamin D during lactation. Nonetheless, although maternal supplementation during preconception and pregnancy increased HM vitamin D levels by approximately 20%, additional supplementation directly to the infant may be needed. HM alone is an inadequate source of vitamin D (25,51), particularly for infants of mothers with low vitamin D levels, darker skin (27), and/or low sunlight exposure (57).

Of note, the intervention effects on HM vitamin D levels were more marked in New Zealand compared to Singapore. One possible explanation is sunlight exposure; while there is very limited seasonal variation in Singapore (located just above the equator at 1.3° N), in Auckland (≈37° S) the availability of clear-sky ultraviolet radiation for vitamin D synthesis decreases exponentially in winter (47,58). Thus, it is possible that vitamin D supplementation could yield greater benefit to women in Auckland who would be more dependent on vitamin D intake due to reduced vitamin D synthesis over several months of the year. Nonetheless, season was incorporated into the statistical models where 25(OH)D_3

and vitamin D_3 were the outcomes, and, as pointed out in the Results, intervention effects were independent of seasonal variations in vitamin D synthesis at the time of HM sample collection.

NiPPeR intervention did not increase HM B vitamins

No effects of the NiPPeR supplement were observed on HM B vitamins, despite B_2 and B_6 being provided in the intervention but not the control supplement. As B vitamins are water soluble and there are no storage mechanisms in the body (59), the findings are in keeping with our expectations that B vitamins supplementation preconception and during pregnancy would not impact HM concentrations during lactation. In general, B vitamin levels in HM are higher than their levels in maternal plasma (60), suggesting active transport of these vitamins into milk. While various types of B vitamin transporter have been identified (60–63), the regulatory mechanisms in the human mammary gland are still not well understood. Low maternal B_2 and B_6 status are associated with lower concentrations in HM, which are rapidly restored by maternal supplementation, but B_9 concentrations in HM are maintained even when the mother is deficient and are unaffected by maternal folate supplementation (64).

Longitudinal change in HM 25(OH)D_3 and B vitamins

In the New Zealand site, we observed an increasing trend of HM 25(OH)D_3 concentrations from 1 week to 12 months of lactation, with a steady phase between 3 months to 6 months. Previous studies observed an increase in HM vitamin D in mothers supplemented with a large daily dose of 6400 IU of vitamin D over 6 months during lactation (30). Still, they decreased over time in unsupplemented mothers (54,65,66). In the current study, HM vitamin D_3 concentrations increased in both the control and intervention groups, suggesting that such change is a conserved pattern over lactation. We speculate that this may reflect greater fat mobilisation after 3 months of delivery (67,68), leading to the release of vitamin D stored in fat (56). Others have also proposed that outdoor activity increases

as infants get older (30), increasing mothers' sunlight exposure and increasing vitamin D synthesis, which may influence HM vitamin D content.

In the current study, HM B vitamin concentrations increased in earlier stages of lactation: B_1, B_2, and B_3 reaching the highest at 6 weeks, B_6 at 6 months, and B_9 at 3 months, in both control and intervention groups. These observations are comparable to previous studies that reported higher B vitamin concentrations in HM samples collected beyond 15 days postpartum, compared to earlier samples collected within 7 days postpartum (69,70). However, it is not well understood how such increase in HM B vitamins over first 3 months of lactation relates to infant outcomes. It can only be speculated that HM B vitamins increase in early lactation to meet infant demands during this critical phase of development. HM B vitamin concentration decreased from about 3 months to 12 months of lactation, reflecting that HM sources of B vitamins becomes less demanding as infants start eating solid foods from about 6 months of age and breastfeeding becomes complementary. Finally, we observed that contribution of some B-vitamers to their respective B vitamin were not constant but altered with lactation stage. The composition of HM vitamin B_1 was reported to be approximately 30% thiamine and 70% TMP; of B_2 approximately 39% riboflavin and 54% FAD; and of B_6 approximately 75% pyridoxal (64). In our study, we observed that at week 1, thiamine contribution to B_1 was lower at 7% and that of TMP was higher at 85.4% which subsequently increased to 54.6% and decreased to 41.8%, respectively, at 12 months. Contribution of FAD to B_2 ranged from 90.6% to 72.2% and that of pyridoxal to B_6 ranged from 43.0% to 83.6% over the first 12 months of lactation. How these changes in HM B-vitamers relate to the developmental stage of the infant and their implications for infant outcomes requires further investigation.

Strengths and Limitations

Our study had some limitations. Longitudinal samples could not be collected from all participants, but to address this sample size imbalance across lactation, we examined potential treatment effects with

robust linear mixed models based on repeated measures. Accompanying longitudinal measurements of maternal vitamin status during lacation were not available, which precluded examination of their influence on HM vitamin concentrations. Infant blood samples were not collected, and we were, therefore, unable to examine the associations between vitamin concentrations in HM and infant circulation; however, collecting infant blood samples is challenging, requiring strong justification and ethical considerations. Also, while concentrations of many vitamers were below the assay's LLoQ, these values were present at relatively low levels and contributed to a small proportion of the overall vitamin content at a given visit. Thus, even if they could have been more precisely measured, their combined effects on study findings would likely have been negligible. Nonetheless, our study had a number of strengths. Using a gold-standard double-blind randomised controlled trial, we investigated the impact of a nutritional supplement taken from preconception and throughout pregnancy on HM vitamin composition, and its key strengths include: i) adherence to supplementation preconception and pregnancy was high, 87.4% for the control group and 86.9% for the intervention group, ii) HM samples were examined from a large cohort of diverse ethnic groups; iii) standardized methods for HM sample collection, processing, and vitamin quantification; and iv) visit windows that were tightly controlled, each time point representing a distinctive stage of lactation.

Conclusion

A micronutrient supplement including 400 IU of vitamin D daily from preconception through pregnancy until delivery, as recommended in many guidelines, achieved higher levels of HM 25(OH)D_3 and vitamin D_3 concentrations during the first 3 months of lactation. There was no long-term influence of vitamin B_2, B_6, and B_9 supplementation from preconception and pregnancy on levels of these vitamins in HM during lactation. In future studies, ongoing evaluation of infants from this group of supplemented mothers will help to understand both direct and HM vitamin D mediated impacts of

gestational vitamin D supplementation on infant health outcomes such as growth, rickets and bone health, allergic disorders and adiposity during later childhood.

Ethics statement

Ethics approval for the study was obtained at each study site: Southampton, United Kingdom – Health Research Authority National Research Ethics Service Committee South Central Research Ethics Committee (15/SC/0142); Singapore – the National Healthcare Group Domain Specific Review Board (2015/00205); and Auckland, New Zealand – Northern A Health and Disability Ethics Committee (15/NTA/21)]. All participants provided written informed consent.

Data availability statement

The datasets presented in this article are not publicly available because public data sharing was not part of the original participant's informed consent. Requests to access the datasets should be directed to the corresponding author.

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Conflicts of Interest

KMG, S-YC, and WSC are part of an academic consortium that has received grants from Société des Produits Nestlé SA relating to the submitted work, and from Abbott Nutrition, Danone, and BenevolentAI Bio Ltd. outside the submitted work. SMH, FH, JGBD, MHV, SD, KMG, S-YC, SKT, and WSC are co-inventors on patent filings by Société des Produits Nestlé SA relating to the NiPPeR intervention or its components. FH, SD, KR, EC-G, and SKT are employees of Société des Produits Nestlé SA. All other authors report no conflicts of interest.

Author contributions

KMG, S-YC, and WSC led the design of the original study. The present sub-study was developed and undertaken by the SMH, FH, JGBD, MHV, SD, SKT, and WSC. KR and EC-G performed the laboratory analyses. SMH, FH, and JGBD performed the statistical analyses. SMH led the manuscript

writing, and FH and JGBD contributed to sections of the manuscript. SKT and WSC supervised all aspects of the present study. All authors contributed to interpretation, manuscript revision, read, and approved the final version.

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Figure 1. CONSORT diagram with the numbers of human milk (HM) samples analysed for vitamin concentrations in the NiPPeR study.

Reasons for exclusion during the preconception phase have been published previously (42), while reasons for exclusion during pregnancy and birth in Singapore (SGP) and New Zealand (NZL) are provided in Supplementary Table 2. The numbers included in this figure correspond to the number of samples analysed for B vitamins. Of these, 42 samples could not be analysed for vitamin D due to insufficient volume. ‡ There were no HM samples collected in the United Kingdom (UK), so all participants from that site were excluded from this diagram. * Number of participants who provided at least one HM sample during 12 months of lactation. † Number of participants who provided at least one HM sample during the first 3 months of lactation.

Figure 2. 25(OH)D_3 concentrations in human milk of control and intervention groups in the NiPPeR study during the first 3 months of lactation: (A) Overall, (B) Singapore, and (C) New Zealand. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for visit, an interaction term (group*visit), study site, adherence, maternal pre-pregnancy body mass index, gestational age at birth, and season at human milk sample collection; error bars represent the respective 95% confidence intervals. *P<0.05, **P<0.01, and ***P<0.001 for the difference between intervention and control groups at a given time point.

Figure 3. Vitamin B concentrations in human milk of control and intervention groups in the NiPPeR study, during the first 3 months of lactation: (A) B_1, (B) B_2, (C) B_3, (D) B_6, and (E) B_9. Data are the least-squares means (i.e., adjusted means) for each group, adjusted for visit, an interaction term (group*visit), study site, adherence, maternal pre-pregnancy body mass index, and gestational age at birth; error bars represent the respective 95% confidence intervals. *P<0.05 for a difference between intervention and control groups at a given time point.

Figure 4. Vitamin concentrations in human milk from control and intervention groups in New Zealand in the NiPPeR Study, during the first 12 months of lactation: (A) 25(OH)D_3, (B) B_1, (C) B_2, (D) B_3, (E) B_6, and (F) B_9. Data are the least-squares means (i.e., adjusted means) for each group adjusted for visit, a group*visit

interaction term, adherence, maternal pre-pregnancy body mass index, and gestational age at birth, with season at sample collection also included in the model for 25(OH)D_3. Error bars represent the respective 95% confidence intervals. *P<0.05, **P<0.01 for the difference between intervention and control groups at a given time point.

Figure 5. The average contribution (%) of human milk B-vitamers in New Zealand in the NiPPeR study, during the first 12 months of lactation for (A) B_1, (B) B_2, (C) B_3 (D) B_6, and (E) B_9. Data represents the mean contribution of each vitamer at a given visit, and the error bars represent the respective 95% confidence intervals. Abbreviations: 5MeTHF, 5-methyltetrahydrofolic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine-5'-phosphate; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate.