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Macro- and Micronutrients in Milk from Healthy Cambodian Mothers: Status and Interrelations

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ABSTRACT

Background: Except for low thiamin content, little is known about vitamins or macronutrients in milk from Cambodian mothers, and associations among milk nutrients.

Objectives: We measured fat-soluble vitamins (FSVs) and water-soluble vitamins (WSVs), and macronutrients, and explored internutrient associations in milk from Cambodian mothers.

Methods: Milk from women (aged 18–45 y, 3–27 wk postpartum, $n = 68$) who participated in a thiamin-fortification trial were analyzed for vitamins B-2 (riboflavin, FAD), B-3 (nicotinamide), B-5, B-6 (pyridoxal, pyridoxine), B-7, B-12, A, E [α-tocopherol and γ-tocopherol (γ-TPH)], carotenoids, carbohydrate (CHO), fat, and protein. Milk vitamin B-1 [thiamin, thiamin monophosphate (TMP), thiamin pyrophosphate (TPP)] was previously assessed for fortification effects. Milk nutrient concentrations were compared with the Adequate Intake (AI) values for infants aged 0–6 mo. Pearson correlation was used to examine internutrient associations after excluding nutrients affected by fortification.

Results: Fortification increased thiamin and B-1 and decreased γ-TPH. Less than 40% of milk samples met the AIs for all vitamins, and 10 samples did not reach any AI values for the analyzed nutrients. CHO, fat, and energy values were met in 1.5–11.8%, and protein in 48.5%, of the samples. Whereas fat, protein, and energy were related (all $r < 0.5$; $P < 0.001$) and associated with FSVs and WSVs, CHO correlated only with some WSVs. TPP was not correlated with B-1 vitamers, but with other WSVs ($r = 0.28$–0.58; $P < 0.019$). All FSVs, except α-carotene, were correlated with each other ($r = 0.42$–0.98; $P < 0.002$). TPP, FAD, B-2, and B-3 were associated with almost all FSVs ($r = 0.24$–0.63; $P < 0.044$).

Conclusions: Cambodian women might not provide sufficient nutrients to their exclusively breastfeeding infants. Besides thiamin, all other vitamins measured were much lower than the AI. There were many strong correlations among macronutrients and vitamins; the extent to which these are explained by maternal diet, milk volume, maternal physiology, or genetics requires additional exploration. *J Nutr* 2020;150:1461–1469.

Keywords: human milk, micronutrients, macronutrients, Adequate Intake (AI), internutrient relations

Introduction

The WHO recommends exclusive breastfeeding (EBF) for the first 6 mo of life (1), so human milk is the sole source of nutrients for infants during this time. Astonishingly little is known about the nutrient composition of human milk (2, 3), yet milk composition can influence growth, health, and developmental outcomes of infants (4). For instance, infantile beriberi, a potentially fatal disease caused by thiamin deficiency, is an ongoing public health concern in Cambodia and throughout Southeast Asia (5), where lactating women consume little thiamin and therefore produce thiamin-poor milk (6,7). We conducted a trial of thiamin fortification in Cambodia to assess the impact of maternal thiamin intake on milk thiamin (6). Given the availability of milk samples from this study, and the fact that some nutrients have essential and interrelated roles, and often similar sources, we measured additional water-soluble vitamins (WSVs) and fat-soluble vitamins (FSVs) and macronutrients in the milk samples to assess both nutrient concentrations and internutrient associations. For example, B vitamins share many similarities in their dietary sources, absorption, and physiological functions (8), and have dependent interactions, such as involvement in 1-carbon metabolism (8) and the conversion of vitamin B-6 to active derivatives requiring

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riboflavin in the form of both FMN and FAD (9). Furthermore, macro- and micronutrients can have strong associations; high-carbohydrate diets increase thiamin requirements (5), and dietary fats are rich sources of FSVs (10). Given the increased nutrient requirements of lactation (11), reliance on high-carbohydrate dietary staples, and limited access to diverse and/or nutrient-rich foods (e.g., animal source foods) in low-income countries (12), breastfeeding dyads can be at risk of multiple nutrient deficiencies.

Although some milk micronutrients are not affected by maternal status (e.g., iron, zinc, and copper) (13), this is not true for most (7). B vitamins, except for folate, are classified as Group I nutrients in lactation, meaning that milk concentrations are dependent on maternal status and/or intake (7), with low maternal status/intake putting infants at risk of deficiency (3, 11).

When there is insufficient evidence available to establish an estimated average requirement (EAR) and RDA, the Institute of Medicine (IOM) developed an Adequate Intake (AI) that is expected to meet or exceed the needs of individuals in that life-stage group (14). For infants aged 0–6 mo, AIs are calculated using measured nutrient concentrations in milk produced by purportedly healthy EBF mothers, and assuming infant consumption of 780 mL milk/d (14). Many factors including small sample sizes [e.g., $n = 24$ mothers for thiamin (15–17)], potentially outdated and uncertain analytical methodologies (18), and variable milk collection techniques (19), have lead researchers to call AIs for infants into question for a number of micronutrients (2, 6, 20). More recently, the European Food Safety Authority (EFSA) released updated nutrient average requirements (ARs) in 2013, including for infants of the same age (21). Regarding the nutrients analyzed in this study, the ARs were often developed considering the AIs or literature that was used to set the AIs, in conjunction with new evidence where available.

Although we have some information about the nutrient content of milk from women in low-income settings, for example, thiamin (6, 22, 23), choline (20), B vitamins (24–28), and fatty acids (29), to date there has been little investigation of correlations between nutrients in human milk, which could reveal novel information about the mother-milk-infant relation. In this exploratory secondary analysis, using state-of-the art methodological analytical approaches for the complex human milk matrix, we measured the concentrations of FSVs, WSVs, and macronutrients in milk from Cambodian women, compared these values with AIs, and assessed internutrient correlations.

Methods

Participants

This study was a secondary analysis of human milk samples collected from a double-blind, 3-parallel-arm randomized controlled, efficacy trial of thiamin-fortified fish sauce in rural Cambodia between October 2014 (at 23 ± 7 wk gestation) and April 2015 (at infant age 16 ± 8 wk) (clinicaltrials.gov identifier: NCT02221063); details, including a flow diagram, are published elsewhere (6). Briefly, a convenience sample set of 90 pregnant women in Prey Veng province, Cambodia, was recruited. Women needed to be aged 18–45 y, ~3–8 mo pregnant with a singleton fetus with no prior history of pregnancy or delivery complications, and intended to EBF for 6 mo. Participating women could not be taking part in nongovernmental nutrition programs or have consumed thiamin-containing dietary supplements in the previous 4 mo. Women were pregnant at recruitment, and delivered and breastfed their infants during the 6-mo study. All women provided written informed consent to participate. The Cambodian National Ethics Committee for Health Research (0245NECHR) and the University of British Columbia Children's and Women's Health Centre of British Columbia Research Ethics Board (CQ14-0204/H14-01654) approved the study.

Intervention for original trial: thiamin-fortified fish sauce

Upon enrolment to the trial, women were randomly assigned to receive 1 of 3 study fish sauces for ad libitum consumption. The 3 groups were as follows: control (0 g/L thiamin); low-concentration thiamin-fortified [low; 2 g/L thiamin hydrochloride (≥98% purity); Huazhong Pharmaceutical Co]; and high-concentration thiamin-fortified (high; 8 g/L thiamin hydrochloride). All fish sauce was also fortified with iron as ferric sodium ethylenediaminetetraacetate (2.8 g/L Ferrazon; Akzo Nobel Functional Chemicals B.V.) per Cambodian Ministry of Planning guidelines (30). For this secondary analysis, the samples were treated as cross-sectional, and only results unaffected by the intervention were included.

Human milk sample collection

Between April 22 and 29, 2015, when infants were aged 3–27 wk, a full-breast expression was collected using a battery-powered single-breast pump (Swing Breast pump; Medela) from the breast that participants had not most recently fed from. Milk samples were transported on ice to the National Institute of Public Health Laboratory in Phnom Penh within 5 h of collection, mixed well, and transferred into amber cryovials for storage at −80°C. Samples were shipped on dry ice to the University of British Columbia in Vancouver, Canada, and subsequently to the USDA/ARS Western Human Nutrition Research Center in Davis, California, United States, for analysis. Sixty-eight milk samples were available for secondary analysis, 23, 26, and 19 from the control, low, and high groups, respectively.

Biochemical analyses of milk samples

Free thiamin and its phosphorylated forms TMP and thiamin pyrophosphate (TPP) were analyzed by HPLC coupled with fluorescence detection (HPLC-FLD) using an Agilent HPLC 1200 series after precolumn derivatization of the vitamers into their thiochrome esters (23). Results of this analysis in response to fortification have been reported elsewhere (6).

Riboflavin, FAD, nicotinamide (B-3), pyridoxal, pyridoxine, biotin, and pantothenic acid were analyzed using ultra performance liquid chromatography-tandem MS (UPLC-MS/MS) using a Waters ACQUITY UPLC system coupled with a Sciex 4000QTRAP MS system as previously described (31). Vitamin B-12 concentrations were

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Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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Abbreviations used: A, vitamin A; AI, Adequate Intake; AR, average requirement; ASF, animal-source food; B-1, vitamin B-1 (thiamin + TMP + TPP); B-2, vitamin B-2 (riboflavin + FAD); B-3, niacin (nicotinamide); B-5, vitamin B-5 (pantothenic acid); B-6, vitamin B-6 (PL + PN); B-7, vitamin B-7 (biotin); B-12, vitamin B-12; CHO, carbohydrate; E, vitamin E (α-TPH + γ-TPH); EAR, estimated average requirement; EBF, exclusive breastfeeding; EFSA, European Food Safety Authority; FLD, fluorescence detection; FSV, fat-soluble vitamin; lyc, lycopene; IOM, Institute of Medicine; LZ, lutein/zeaxanthin; PL, pyridoxal; PN, pyridoxine; TMP, thiamin monophosphate; TPP, thiamin pyrophosphate; UPLC, ultra-performance liquid chromatography; WSV, water-soluble vitamin; α-C, α-carotene; α-TPH, α-tocopherol; β-C, β-carotene; β-CRP, β-cryptoxanthin; γ-TPH, γ-tocopherol.

TABLE 1 Demographic characteristics of Cambodian women who participated in the thiamin-fortification trial[1]

	Fortification group				
	Combined	Control	Low	High	*P* value
Sample number, *n*	68	23	26	19	
Mother					
Age, y	26.0 ± 4.7	26.7 ± 3.6	26.4 ± 5.4	24.8 ± 4.9	0.37[2]
Highest schooling completed					0.17[3]
None	6 (9)	2 (9)	3 (12)	1 (5)	
Primary	31 (46)	9 (39)	15 (58)	7 (37)	
Lower secondary	26 (38)	11 (48)	5 (19)	10 (53)	
Upper secondary	5 (7)	1 (4)	3 (12)	1 (5)	
Annual household income, US$	1459 ± 1162	1602 ± 1129	1263 ± 902	1553 ± 1500	0.55[2]
Parity, primipara	35 (52)	11 (48)	14 (54)	10 (53)	0.91[3]
Infant					
Sex, girls	30 (44)	11 (48)	11 (42)	8 (42)	0.91[3]
Birth weight, kg	3.1 ± 0.6	3.1 ± 0.4	3.2 ± 0.4	3.1 ± 0.9	0.99[2]
Birth length, cm	49.1 ± 2.3	49.3 ± 1.9	49.1 ± 1.9	48.7 ± 3.3	0.65[2]
Age at milk collection, wk	15.6 ± 7.1	15.0 ± 7.7	17.1 ± 6.8	14.2 ± 6.7	0.38[2]

[1] Results are presented as mean ± SD or *n* (%).
[2] One-factor ANOVA.
[3] Chi-square test.

measured using the IMMULITE/IMMULITE 1000 Vitamin B12 solid-phase, competitive chemiluminescent enzyme immunoassay (Siemens Healthcare Diagnostics) (32). FSVs (retinol, α- and γ-tocopherol, α- and β-carotene, β-cryptoxanthin, lutein/zeaxanthin, lycopene) were analyzed as previously described (33) with a few modifications: 100-μL samples were used for protein precipitation with 0.1% butylated hydroxytoluene (BHT)in ethanol and saponification in the presence of 10% pyrogallol in ethanol and 200 μL aqueous potassium hydroxide (15%). FSVs were extracted with 4 mL hexane, and the organic phase was evaporated to dryness before reconstitution in 50 μL acetonitrile. Twenty microliters of the extract was injected into an Agilent 1260 HPLC system using a multiple wavelength detector (retinol: 325 nm; tocol (internal standard), α- and γ-tocopherols: 295 nm; carotenoids: 450 nm), and equipped with an Agilent Eclipse Plus C18 column (100 × 2.1 mm, 3.5 μm) held at 15°C, protected by a Hypersil BDS C18 Javelin guard (20 × 3 mm, 3 μm; Thermo Scientific). Total carbohydrate (CHO), fat, and protein were measured using the SpectraStar 2600 XT Neonatal Analyzer (Unity Scientific) according to the manufacturer's protocol. Energy, in kilocalories per liter, was calculated based on the results of the macronutrient analysis in percent (%) using the following equation:

$$\text{energy}\left(\frac{\text{kcal}}{\text{L}}\right) = [(\text{CHO} \times 4) + (\text{fat} \times 9) + (\text{protein} \times 4)] \times 10 \quad (1)$$

Statistical analyses

SAS for Windows Release 9.4 (SAS Institute) was used for statistical analyses. The outcomes were concentrations of human milk fat, protein, CHO, energy, thiamin, TMP, TPP, vitamin B-1 (B-1; thiamin + TMP + TPP), free riboflavin, FAD, vitamin B-2 (riboflavin + FAD), vitamin B-3 (nicotinamide), vitamin B-5 (pantothenic acid), pyridoxal (PL), pyridoxine (PN), vitamin B-6 (PL + PN), vitamin B-7 (biotin), vitamin B-12, α-tocopherol (α-TPH), γ-tocopherol (γ-TPH), vitamin E (VE), vitamin A (retinol; VA), α-carotene (α-C), β-carotene (β-C), β-cryptoxanthin (β-Crp), lycopene (Lyc), and lutein/zeaxanthin (L/Z) reported within the 3 defined groups (control, low, high). Log (fat, TPP, thiamin, B-1, FAD, B-2, PL, B-6, B-7, B-12, VA, γ-TPH, α-C, β-C, β-Crp, Lyc, L/Z), square-root (B-1, riboflavin, B-3, B-5, α-TPH, VE), and inverse (1/x; PN, protein, energy) transformations were performed to normalize the distributions of the outcome variables. No transformations were necessary for TMP and CHO. FSVs were also expressed as concentration per gram of fat.

Demographic differences by fortification group were evaluated by 1-factor ANOVA or chi-square tests (**Table 1**). Fortification effects were examined using generalized linear models adjusted for time of sample collection (infant age) and multiple comparisons with Tukey–Kramer test (**Table 2**). Treatment groups were pooled and only outcome variables unaffected by fortification were included in the Pearson correlation analyses. The correlation plot is presented in **Figure 1**, and correlation coefficients and *P* values in **Supplemental Table 1**. The correlation strength was defined as follows: weak ($\rho \leq 0.3$), moderate ($\rho = 0.3$–0.5), good ($\rho = 0.5$–0.7), or strong ($\rho = 0.7$–1.0). Measured milk nutrient concentrations were also compared with the AIs for infants aged 0–6 mo recommended by the IOM (32–35). The obtained dichotomous results were examined by chi-square tests for differences in percentage of samples meeting the requirements due to fortification. Fortification groups were pooled when no significant differences were found (**Table 3**). *P* values <0.05 were considered significant for all statistical tests.

R Studio for Windows, Version 1.1453 (RStudio Inc) was used to create the correlation plot (corrplot) in Figure 1.

Results

Maternal and infant characteristics

Demographic characteristics were comparable among study participants ($P > 0.05$; Table 1). On average, women were 26 y old, half were first-time mothers, and nearly half of the infants were female. The participant flow and follow-up is published elsewhere (6). At the time of milk sample collection 88.1% (59/67) of the participants were EBF.

Vitamin and macronutrient concentrations and comparison with AI values for infants aged 0–6 mo

The effects of thiamin fortification on human milk B-1 concentrations are reported elsewhere (6). As shown by Whitfield et al. (6), thiamin and B-1 were increased by thiamin fortification; however, we found that γ-TPH, and fat-adjusted γ-TPH, α-C, and β-Crp also responded to the fortification (Table 2). All other results were pooled for further analyses. As expected, TMP, FAD, and PL represented the main vitamers of their respective vitamins (B-1, B-2, and B-6).

TABLE 2 Concentrations of nutrients in milk from Cambodian women 3–27 wk postpartum[1]

Nutrient	Fortification group			
	Combined	Control	Low	High
Number of samples, *n*	68	23	26	19
Energy, kcal/L	542 (568, 619)	527 (486, 576)	586 (545, 632)	512 (474, 555)
Carbohydrates, g/L	69.5 (68.7, 70.3)	69.4 (68.1, 70.6)	70.1 (68.6, 71.5)	69.2 (67.4, 70.9)
Fat, g/L	23.7 (21.2, 26.5)	22.0 (17.6, 27.4)	28.4 (24.1, 33.5)	20.6 (16.3, 25.3)
Protein, g/L	11.8 (11.3, 12.3)	11.6 (10.8, 12.4)	12.3 (11.5, 11.4)	11.4 (10.5, 12.5)
Thiamin pyrophosphate,[2] μg/L	3.88 (3.34, 4.51)	3.21 (2.51, 4.11)	4.29 (3.39, 5.44)	4.37 (3.33, 5.72)
Thiamin monophosphate,[2] μg/L	148 (135, 160)	133 (112, 153)	166 (146, 186)	140 (111, 169)
Thiamin,[2] μg/L	—	12.1[a] (8.88, 16.5)	49.1[b] (36.5, 66.1)	39.4[b] (28.0, 55.4)
Vitamin B-1,[2] μg/L	—	131[a] (110, 154)	203[b] (178, 231)	172[ab] (146, 202)
Riboflavin, μg/L	11.6 (8.87, 14.7)	16.5 (11.4, 22.6)	8.90 (5.33, 13.4)	10.4 (6.04, 15.8)
FAD, μg/L	103 (84.6, 125)	99.3 (71.7, 138)	108 (78.9, 148)	102 (71.5, 147)
Vitamin B-2, μg/L	60.2 (49.7, 73.0)	62.2 (45.0, 85.9)	60.2 (44.2, 82.1)	59.4 (41.6, 84.7)
Vitamin B-3, μg/L	442 (370, 519)	421 (312, 552)	454 (340, 583)	468 (340, 617)
Vitamin B-5, mg/L	1.81 (1.62, 2.00)	1.93 (1.61, 2.26)	1.70 (1.42, 2.01)	1.79 (1.46, 2.15)
Pyridoxal, μg/L	82.9 (72.2, 95.0)	86.9 (69.0, 110)	81.9 (65.6, 102)	80.1 (62.1, 103)
Pyridoxine, μg/L	0.24 (0.23, 0.26)	0.26 (0.23, 0.30)	0.24 (0.21, 0.27)	0.23 (0.21, 0.27)
Vitamin B-6, μg/L	83.7 (73.0, 95.8)	87.8 (69.8, 110)	83.1 (66.7, 104)	80.4 (62.4, 103)
Vitamin B-7, μg/L	5.44 (4.82, 6.13)	6.29 (5.18, 7.64)	5.14 (4.26, 6.19)	4.84 (3.89, 6.04)
Vitamin B-12, pmol/L	248 (205, 299)	245 (180, 335)	215 (159, 289)	316 (224, 445)
Vitamin A, mg/L	0.41 (0.35, 0.48)	0.42 (0.31, 0.57)	0.46 (0.38, 0.55)	0.37 (0.25, 0.54)
α-Tocopherol, mg/L	2.45 (2.07, 2.86)	2.74 (1.88, 3.76)	2.63 (2.12, 3.18)	1.91 (1.35, 2.57)
γ-Tocopherol, mg/L	—	0.18[a] (0.13, 0.25)	0.20[a] (0.15, 0.26)	0.09[b] (0.07, 0.13)
Vitamin E, mg/L	2.67 (2.29, 3.09)	2.97 (2.07, 4.05)	2.94 (2.49, 3.43)	2.03 (1.45, 2.70)
α-Carotene, μg/L	9.63 (8.39, 11.0)	11.4 (8.59, 15.2)	9.19 (7.53, 11.2)	8.37 (6.44, 10.9)
β-Carotene, μg/L	48.6 (39.1, 60.4)	63.4 (41.4, 97.1)	47.9 (35.1, 65.4)	36.4 (23.2, 57.2)
β-Cryptoxanthin, μg/L	8.70 (7.42, 10.2)	10.2 (7.36, 13.9)	8.11 (6.42, 10.2)	7.47 (5.53, 10.1)
Lutein/zeaxanthin, μg/L	51.4 (43.6, 60.6)	56.1 (40.7, 77.3)	60.8 (47.8, 77.4)	37.4 (27.2, 51.4)
Lycopene, μg/L	8.33 (7.36, 9.43)	8.87 (7.22, 10.9)	9.33 (7.46, 11.7)	6.66 (5.30, 8.37)
FSVs adjusted by milk-fat				
Vitamin A mg/g fat	0.18 (0.16, 0.19)	0.19 (0.17, 0.23)	0.16 (0.14, 0.19)	0.18 (0.14, 0.22)
α-Tocopherol, mg/g fat	0.96 (0.86, 1.07)	1.11 (0.91, 1.33)	0.90 (0.76, 1.06)	0.87 (0.67, 1.09)
γ-Tocopherol, μg/g fat	—	81.9[a] (63.0, 107)	70.6[a] (53.0, 94.1)	46.7[b] (34.8, 62.5)
Vitamin E, mg/g fat	1.06 (0.96, 1.17)	1.21 (1.00, 1.44)	1.02 (0.89, 1.16)	0.92 (0.72, 1.15)
α-Carotene, μg/g fat	—	5.76[a] (4.67, 7.11)	3.95[b] (3.30, 4.72)	4.16[ab] (3.22, 5.36)
β-Carotene, μg/g fat	21.2 (17.7, 25.3)	29.3 (22.0, 39.1)	17.7 (13.1, 24.1)	18.2 (12.7, 26.0)
β-Cryptoxanthin, μg/g fat	—	5.13[a] (4.14, 6.34)	3.56[b] (3.01, 4.22)	4.16[ab] (3.32, 5.21)
Lutein/zeaxanthin, μg/g fat	22.3 (19.9, 24.9)	26.0 (21.6, 31.3)	22.1 (18.3, 26.8)	18.7 (15.0, 23.2)
Lycopene, μg/g fat	4.12 (3.78, 4.48)	4.57 (4.07, 5.13)	3.98 (3.37, 4.71)	3.77 (3.17, 4.48)

[1]Results are geometric means (95% CIs). Fortification effects were examined using generalized linear models adjusted for time of sample collection (infant age) and multiple comparisons (Tukey–Kramer). Different letters indicate significant differences (all $P < 0.023$), all adjusted by infant age. Vitamin B-1: thiamin + (thiamin monophosphate × 0.871) + (thiamin pyrophosphate × 0.707); vitamin B-2: riboflavin + (FAD × 0.479); vitamin B6: pyridoxal + (pyridoxine × 1.012). FSV, fast-soluble vitamin.
[2]Published in Whitfield et al. (6).

When comparing the milk vitamin concentrations with the values used for setting the AI for infants aged 0–6 mo (32–37), none of the samples met the accepted value for vitamin B-2 or B-3 (Table 3). Vitamins B-5, B-6, B-7, B-12, and VA revealed somewhat higher concentrations, with 17.7–38.2% of the milk samples meeting the AI recommendations, whereas VE was only adequate in 8.8% of the analyzed samples. As previously shown (6), none of the samples in the control group met the AI value for B-1, whereas only 24.0% and 10.5% of the samples in the low and high groups, respectively, met the AI value despite maternal consumption of thiamin-fortified fish sauce. CHO, fat, and energy values met the AI in 1.49–11.8% of the samples, whereas almost half of all samples were sufficient in protein. Although no AI is provided for carotenoids, the infant's β-carotene intake has been estimated to be 8–163 μg/d (10.3–209 μg/L); 89.7% of the samples analyzed were within this range, whereas 4.4% were below and 5.9% were above.

Using the more recently reviewed EFSA ARs (21), the same percentage of samples reached the AR for vitamins B-1, B-2, B-3, and B-12 as when using the IOM's AIs, whereas the biggest differences in reaching recommendation values were found for vitamins B-5, B-7, and E (AI compared with AR: 33.8% compared with 19.1%; 37.3% compared with 56.7%; 8.8% compared with 22.1%).

Correlations among milk nutrients

All intervention groups were pooled for this analysis to increase sample size. Correlations were not evaluated for nutrient affected by the intervention (B-1, thiamine, and γ-TPH; Table 2). Fat-adjusted concentrations for FSVs were not considered because milk fat was included in this correlation matrix.

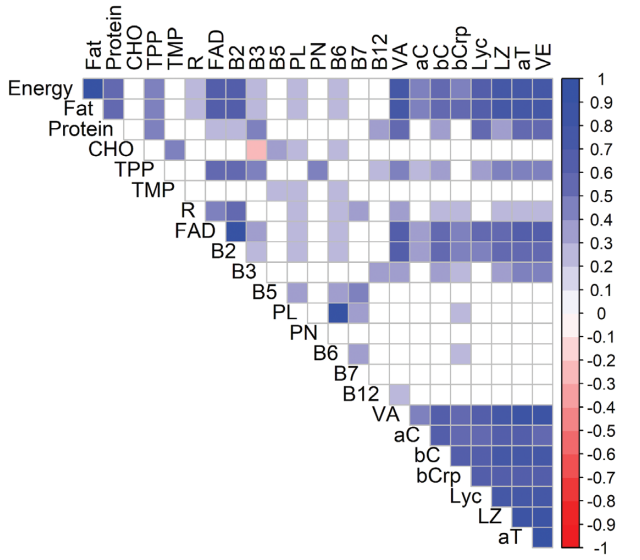

FIGURE 1 Correlation plot of B vitamins and vitamers in milk from Cambodian women 3–27 wk postpartum ($n = 68$). Pearson correlation analysis was used to examine nutrient associations. Fortification-affected thiamin, vitamin B-1, and γ-tocopherol were not included. Significant correlations are indicated by blue and red colors ($P < 0.05$). Correlation coefficients are provided for all significant correlations and are shown in Supplemental Table 1. aC, α-carotene; aT, α-tocopherol; bC, β-carotene; bCrp, β-cryptoxanthin; B2, vitamin B-2; B3, vitamin B-3; B5, vitamin B-5; B6, vitamin B-6; B7, vitamin B-7; B12, vitamin B-12; CHO, carbohydrates; Lyc, lycopene; LZ, lutein/zeaxanthin; PL, pyridoxal; PN, pyridoxine; R, free riboflavin; TPP, thiamin pyrophosphate; VA, vitamin A; VE, vitamin E.

TABLE 3 Percentage of milk samples from Cambodian women 3–27 wk postpartum that met the recommended requirements for infants aged 0–6 mo by the Institute of Medicine[1]

Nutrient	*n*	AI[2] (IOM)	Percentage of samples reaching AI
Energy, kcal/L	68	711.7[4]	10.3
Carbohydrate, g/L	68	76.9	1.49
Fat, g/L	68	39.7	11.8
Protein, g/L	68	11.7	48.5
Vitamin B-1[3]		256	
Control, μg/L	23		0
Low, μg/L	26		24.0
High, μg/L	19		10.5
Vitamin B-2, μg/L	68	385	0
Vitamin B-3, mg/L	68	2.56	0
Vitamin B-5, mg/L	68	2.18	33.8
Vitamin B-6, μg/L	68	128	17.7
Vitamin B-7, μg/L	68	6.41	37.3
Vitamin B-12, pmol/L	68	376	26.5
Vitamin A, mg RAE/L	68	0.51[5]	38.2
Vitamin E, mg α-TPH/L	68	5.13[6]	8.82

[1] Treatment groups from the thiamin fortification trial were combined when chi-square test did not reveal significant differences between groups. Vitamin B-1: thiamin + (thiamin monophosphate × 0.871) + (thiamin pyrophosphate × 0.707); vitamin B-2: riboflavin + (FAD × 0.479); vitamin B-6: pyridoxal + (pyridoxine × 1.012). AI, Adequate Intake; IOM, Institute of Medicine; RAE; retinol activity equivalent; TPH, tocopherol.
[2] Adequate Intake as recommended by the Institute of Medicine expressed as concentrations per liter based on a milk consumption of 780 mL/d (34–37).
[3] Published in Whitfield et al. (6).
[4] AI for energy is calculated based on AI for macronutrients.
[5] The IOM does not consider carotenoids in the infant AI for vitamin A.
[6] The IOM only uses α-tocopherol for the infant AI for vitamin E.

Macronutrient associations.

Good to strong correlations ($r = 0.51$–0.99; $P < 0.0001$; Figure 1, Supplemental Table 1) were found between fat, protein, and energy, whereas CHO revealed no associations with any of these macronutrients or energy. About half of the correlations of the macronutrients with WSVs in milk were weak ($r < 0.30$); only vitamin B-2 and FAD had strong correlations with fat and energy ($r = 0.61$–0.66; $P < 0.0001$). CHO had moderate correlations with TMP, B-5, B-6, and PL ($r = 0.29$–0.49; $P < 0.017$). TPP was moderately correlated with all macronutrients ($r = 0.42$–0.45; $P < 0.0004$) except CHO. CHO had a weak inverse association with B-3 ($r = -0.26$; $P = 0.031$), the only inverse correlation in the dataset.

Almost all correlations (>95%) found between macronutrients and energy with FSVs were at least moderately strong ($r > 0.3$). Unsurprisingly, all FSVs had moderate to good associations with milk fat ($r = 0.42$–0.79; $P < 0.0004$) and energy ($r = 0.41$–0.78; $P < 0.0007$). Of the FSVs, only α-C and β-Crp showed no relation to protein, and none of the FSVs correlated with CHO ($P > 0.05$).

Micronutrient correlations.

The observed significant correlations among WSVs and their vitamers in human milk were all positive but mostly weak or moderate ($r < 0.5$; $P < 0.05$; Figure 1, Supplemental Table 1). Good to strong correlations were observed between vitamers of each vitamin (B-1, B-2, B-6; $r > 0.6$; $P < 0.001$). Whereas TPP was not correlated with TMP, the only other B-1 vitamer unaffected by fortification, it revealed moderate intervitamin associations with B-2, FAD, B-3, PN, B-7 ($r = 0.36$–0.57; $P < 0.003$). B-5 was moderately correlated with PL, B-6, and B-7 ($r = 0.38$–0.47; $P < 0.001$).

Almost all correlations between FSVs were good or strong ($r = 0.53$–0.98; $P < 0.0001$; Figure 1, Supplemental Table 1). α-TPH, the main vitamer of VE, was strongly correlated with VE ($r = 0.98$), and both were good to strongly associated with VA and all carotenoids ($r = 0.61$–0.83). VA was associated with all measured carotenoids, with the weakest relation to α-C ($r = 0.44$) and the strongest with L/Z ($r = 0.71$). All 5 measured carotenoids were correlated with each other ($r = 0.56$–0.78).

Significant correlations between WSVs and FSVs were all positive ($P < 0.044$; Figure 1, Supplemental Table 1). Only ~29% were weak correlations ($r < 0.28$) and none were strong ($r > 0.7$). Of all WSVs analyzed, only B-2 and its main vitamer FAD correlated with all FSVs ($r = 0.34$–0.62). TPP was the only B-1 vitamer with significant associations with FSVs, with the weakest association to α-C ($r = 0.24$); the remaining FSVs had good to strong correlations with TPP ($r = 0.35$–0.49). Whereas B-3 was correlated with all FSVs except α-C and Lyc, and more strongly with VE and α-TPH ($r = 0.46$–0.47) than with VA and carotenoids ($r = 0.28$–0.4), PL, B-6, and B-12 were only weakly correlated with β-Crp and α-C ($r < 0.28$). The remaining WSVs were not significantly correlated with any FSV ($P > 0.05$).

Discussion

Comparison of human milk composition with other studies

The nutrient composition from our samples is comparable with values from women in other low-income countries. Our Cambodian milk was lower in fat and VE but higher in B-2, B-3, and B-12 compared with Bangladeshi women 2–4 mo postpartum (19), possibly due to the known low B-2 and B-12 intakes (38) and low meat consumption in Bangladesh. Conversely, people in Southeast Asia have high intakes of nuts and seeds, good VE sources (39). Another Bangladeshi study on milk FSVs (1.5–26 mo postpartum) was in agreement with our Cambodian results (40). Japanese women had similar concentrations of most milk WSVs, except lower B-6 and higher B-2, B-5, and B-12 (41). The typical higher intake of animal-source foods (ASFs; e.g., meat, eggs, dairy) in Japan could be reflected in the higher ASF-derived B-2, B-5, and B-12 in the milk (8). A recent study in Indonesia found lower milk B-1, B-3, B-5, B-6, and B-12, whereas B-2, B-7, and the FSVs were higher (42). Low daily micronutrient intakes of these Indonesian mothers could contribute to the low milk micronutrient content; in fact intakes of vitamins A, niacin, and riboflavin were positively correlated with their milk concentrations (42).

Comparing milk macronutrients with available data from Finland, Spain, Australia, and the United States obtained with similar analytical techniques (43–46), our Cambodian sample set contained comparable amounts of CHO (Finland, United States) and protein (Finland, Australia), whereas the fat content was up to 60% lower (29). The Australian and Spanish samples were lower in CHO, and the Spanish and US samples were lower in protein. However, the use of different analytical approaches, instruments, and lactation stages in these studies, plus the naturally occurring macronutrient-specific diurnal and other variations in concentrations (47, 48) could contribute to these observed differences.

Vitamin and macronutrient concentrations and comparison with AIs for infants aged 0–6 mo

Our milk samples were low in all measured WSVs and FSVs. No sample met the AI values for B-2 or B-3. Given that the measured B-2 vitamers represent >90% of total milk B-2, the low concentrations could indicate that EBF infants in Cambodia might be at risk of B-2 deficiency (18, 49). This is not surprising given that thiamin, a known public health concern in Cambodia, and riboflavin are found in similar foods, and it aligns with previous work reporting that 78% of urban and 82% of rural Cambodian women of reproductive age are riboflavin deficient (50). B-3 in this study was solely represented by nicotinamide. However, nicotinamide mononucleotide, NAD, and nicotinamide riboside have been reported in human milk (51), but were not analyzed here. Thus, the total B-3 concentration in this sample is unknown because possible significant contributions of other B-3 vitamers were not included.

As previously reported (6), no sample of the control group met the AI for B-1, but the geometric mean (131 μg/L) was comparable with milk B-1 from US women (22). Even when thiamin fortification was most effective, only 24% of the samples reached the AI value (Table 3) (34). The highest proportion of samples reaching AI was found for VA, yet this number was still <40%. VA supplementation for lactating mothers ceased in Cambodia in 2012 and thus was not a contributing factor to our findings. Milk macronutrients were also low: 12% reached the AI for fat, 10% for energy, and <2% for CHO. The geometric mean of CHO, fat, and energy, however, reflected 90%, 60%, and 76% of the respective AI; thus, the macronutrient concentrations are not as alarmingly lower than the AI as the dichotomous comparison might suggest. Nevertheless, 10 samples (14.7%) did not meet any AI value for any nutrient evaluated.

The similar results using IOM's AIs and EFSA's ARs for most of the milk nutrients are not unexpected given the similarities between the AI and AR values; the ARs are often based on the AIs. The notable differences between AIs and ARs for vitamins B-5, B-7, and E reflect the new evidence available to EFSA that warranted an adjustment of the recommendations as set by the IOM.

As noted, AIs are set when there are insufficient data available to establish an EAR and RDA. The AI is not a requirement, but the recommended intake expected to meet or exceed the needs of individuals of specific life-stage groups (14). Although AIs are the currently accepted estimates of milk concentrations adequate for infant growth and development, these values are based on few reports and outdated data with missing information regarding validation for the methods applied, which could be unsuitable for the complex milk matrix (18, 49). Furthermore, the IOM does not subdivide AIs during the first 6 mo of life despite the known physiological alterations in nutrient concentrations (13, 52). Therefore, AI has very limited use in assessment and is used here with the understanding that nutrient values below those used to set AIs do not signify inadequacy. The EFSA's ARs, although including more recent literature in their recommendations, should still be viewed in a similar fashion, because the EFSA continues to use available reports in the literature, none of which were conducted for the purpose of reviewing intake recommendations.

Possible reasons for correlations among milk nutrients

Human milk is the sole food matrix for EBF infants, and therefore the single source of all nutrients required for growth and development (1). Consequently, the milk matrix is thought to supply these nutrients in distinct concentrations throughout lactation. Thus, examining the internutrient relations in human milk can help gain further insight into milk nutrient secretion and nutrient availability to the EBF infant.

There has been relatively little investigation of associations among milk macronutrients, and especially micronutrients. The new analytical methods pioneered in our laboratory for adequate and reliable analysis of vitamins and vitamers in the complex human milk matrix enable us to produce data that can explore these correlations. The strong associations found in this study could result from multiple factors, possibly including similar maternal diets. Unfortunately, dietary intake was not measured, but intake of the measured vitamins would affect concentrations of the milk nutrients. This is likely not the case for the macronutrients and does not explain the observed correlations between some milk macro- and micronutrients. Also, milk volume could affect milk nutrients, with higher concentrations possibly coinciding with lower milk production. This hypothesis has not been adequately explored but is being investigated in our ongoing studies. However, it does not explain the variability in correlation strength among the nutrients. Finally, correlations might be affected by maternal physiology and nutrient transport mechanisms in the mammary gland.

Macronutrient associations.
The concentrations of milk macronutrients vary throughout lactation and even within a feed. Fat has been reported to be the most variable, whereas lactose, the principal CHO, is the most stable (44), as we also observed here. Whereas fat and protein were strongly correlated, neither was associated with CHO. The calculated energy only correlated with fat and protein. Because fat is one of the main energy sources in human milk (47), its strong relation with energy is expected, and has been previously reported (47, 53).

Nommsen et al. (53) showed that milk fat, but not protein, is significantly correlated with maternal protein intake. Others reported significant relations between dietary and milk protein (54), and higher milk protein in colostrum of well-nourished mothers compared with undernourished women (55). Thus, the evidence base remains inconsistent. Mucins, a family of glycosylated proteins surrounding the lipid globules in milk (56), might contribute to the relation between milk fat and protein, but given they are only a minor protein fraction (57), mucins are unlikely to drive this assocation.

The protein fraction in milk consists of proteins synthesized in the mammary gland, which decrease during early lactation (e.g., lactoferrin, caseins, α-lactalbumin), and proteins derived from the maternal bloodstream (e.g., serum albumin), which are mainly synthesized in the liver and remain constant (48, 58). Thus, associations with dietary protein could be driven by proteins derived from the mother's blood, and depending on the ratio of milk-specific and blood-derived proteins, a potential relation with dietary protein could be masked.

CHO was the sole macronutrient that showed no correlation with any other macronutrient or energy, even though CHO is a major contributor to energy. Stable lactose concentrations are needed to regulate milk osmolality and volume, and our sample set revealed only very limited variation (CV <5%). Thus, CHO should be viewed as a constant and therefore would not correlate with milk nutrients with varying concentrations throughout lactation.

FSVs are associated with the milk-fat globules (59–61), thus the observed relations between milk fat/energy and FSVs were expected. Little information is available regarding WSVs and macronutrient relations. Particularly, the strong relation between FAD (and B-2) and fat/energy remains to be elucidated. Coenzymatic forms of vitamins such as FAD or TPP could be involved in enzymatic processes, such as lactose synthesis, which requires a coupled reaction of α-lactalbumin and galactosyltransferase (62). TPP has been shown to possess a close topological association to this transferase in *trans*-Golgi cisternae (63). TMP, the main B-1 vitamer in milk, had the strongest correlation to CHO, but has no known role in metabolic processes (64). Its secretion into human milk might occur via transporters closely related to thiamin transporters (23, 64) yet it does not represent the main B-1 vitamer in food items (65). Thus, its function and relations with other milk nutrients are not yet well understood. However, dietary CHO intake has been shown to affect thiamin status of adults but not enzyme activities (66), indicating the existence of such interrelation in human metabolism.

The only inverse association in this dataset was between nicotinamide and CHO ($r = -0.26$). Vitamin B-3's coenzymatic form NAD and its reduced form NAD(H) are required in many redox reactions including their function as electron carriers in the Krebs cycle (9), which would suggest a positive relation. However, this weak association was exclusively based on free nicotinamide; thus, we cannot examine the true nature of this relation.

Micronutrient correlations.
All WSV vitamers contributing to total WSV content were strongly correlated with each other. TPP was linked to FAD (and therefore to B-2), B-3, and the minor B-6 contributor PN. All these vitamers are coenzymes or coenzyme precursors (18) and interact in energy metabolism, including the Krebs cycle, respiratory chain complexes I and II, or mitochondrial 1-carbon transfer (67), and therefore essential for the EBF infant. B-5 and B-7 are involved in heme biosynthesis (68), which could be a contributing factor to their connected milk concentrations.

Besides their importance as antioxidants and in immune response (68), carotenoids function as provitamin A and are therefore a VA source for the infant. All FSVs, including carotenoids, decrease during lactation (62, 68), and are associated with milk fat, which all could contribute to the correlations identified with FSVs in our samples.

Relations between WSVs and FSVs were mainly observed for FAD, B-2, and B-3, thus involving coenzymatic or pre-coenzymatic forms. Many metabolic processes in the human body require complex interactions between WSVs and FSVs. FAD, FMN, and NAD(P)H, are involved in the production of oxygen radicals, whereas these cofactors and VE are part of the scavenging system against reactive oxygen species (69, 70). Retinal oxidase, a metalloflavoenzyme involved in retinoic acid synthesis, contains 2 FAD molecules (71), and animal studies have reported riboflavin-dependent photoreceptors involved in the dark adaptation process by stimulating the neuronal signal for pupillary response. Thus, it has been speculated that B-2 deficiency could contribute to increased risk of night blindness, a condition mainly caused by VA deficiency (72).

These relations between macro- and micronutrients could indicate just how precisely human milk is produced for optimal and efficient provision of nutrients to the EBF infant to ensure sufficient energy and availability of enzymes/coenzymes for healthy growth and development.

Limitations

Although our data are, to our knowledge, the first to examine human milk macro- and micronutrient status in Cambodia and novel aspects of internutrient relations in human milk, the small sample size did not permit examining the effects of thiamin fortification on associations among the affected nutrients. Because maternal dietary data were not collected, the nutritional status of the participants remains unknown, as well as the extent to which maternal diet, or other factors such as milk volume, maternal physiology, or genetics, contribute to observed relations among milk macronutrients and vitamins.

The samples were not collected at a fixed time point but between 3 and 27 wk of lactation; consequently, the milk nutrients were subject to the naturally occurring changes in concentrations over time. However, all samples were collected using a standard protocol with the same pump as a full-breast expression in the morning, limiting effects introduced by collecting convenience sample aliquots and interparticipant diurnal variations (19).

Conclusion

Milk from Cambodian mothers revealed low concentrations of macro- and micronutrients, indicating a potential for higher risk of deficiency of the EBF infant, particularly for vitamin B-2,

because none of the samples met the AI value. Multiple observed internutrient associations could be due to the intertwined relations of the nutrients in metabolic processes needed for healthy growth and development, as well as shared maternal food sources of these nutrients. More research is needed to gain better insight into these interconnections, in which maternal diet could also be an important contributor.

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