Total folate and unmetabolized folic acid in the breast milk of a cross-section of Canadian women[1,2]

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ABSTRACT

Background: Folate requirements increase during pregnancy and lactation. It is recommended that women who could become pregnant, are pregnant, or are lactating consume a folic acid (FA)–containing supplement.

Objectives: We sought to determine breast-milk total folate and unmetabolized folic acid (UMFA) contents and their relation with FA-supplement use and doses in a cohort of Canadian mothers who were enrolled in the MIREC (Maternal-Infant Research on Environmental Chemicals) study.

Design: Breast-milk tetrahydrofolate (THF), 5-methyl-THF, 5-formyl-THF, 5,10-methenyl-THF, and UMFA were measured with the use of liquid chromatography–tandem mass spectrometry ($n = 561$). Total daily supplemental FA intake was based on self-reported FA-supplement use.

Results: UMFA was detectable in the milk of 96.1% of the women. Total daily FA intake from supplements was associated with breast folate concentration and species. Breast-milk total folate was 18% higher ($P < 0.001$) in supplement users ($n = 401$) than in nonusers ($n = 160$), a difference driven by women consuming >400 μg FA/d ($P \leq 0.004$). 5-Methyl-THF was 19% lower ($P < 0.001$) and UMFA was 126% higher ($P < 0.001$) in supplement users than in nonusers. Women who consumed >400 μg FA/d had proportionally lower 5-methyl-THF and higher UMFA than did women who consumed ≤400 μg FA/d.

Conclusions: FA-supplement use was associated with modestly higher breast-milk total folate. Detectable breast-milk UMFA was nearly ubiquitous, including in women who did not consume an FA supplement. Breast-milk UMFA was proportionally higher than 5-methyl-THF in women who consumed >400 μg FA/d, thereby suggesting that higher doses exceed the physiologic capacity to metabolize FA and result in the preferential uptake of FA in breast milk. Therefore, FA-supplement doses >400 μg may not be warranted, especially in populations for whom FA fortification is mandatory. *Am J Clin Nutr* 2017;105:1101–9.

Keywords: breast milk, folate, folic acid, supplements, unmetabolized folic acid

INTRODUCTION

The demand for folate increases during pregnancy and lactation to support fetal and neonatal growth and development (1). Folates are actively transported across the mammary epithelium, thereby allowing breast-milk folate to be maintained at the expense of maternal folate status in the absence of supplementation (2). Because exclusive breastfeeding is recommended in the first 6 mo of life, it is of interest to assess factors that may affect the breast-milk folate content.

Randomized controlled trials have clearly shown that supplementation with folic acid (FA),[8] which is the synthetic form of folate, in the periconceptional period reduces risk of having a baby with a neural tube defect (3–5). As such, several countries, including the United States and Canada, have implemented the mandatory FA fortification of white flour (6). In addition, it is recommended that women of child bearing age who could become pregnant and women who are pregnant or lactating should consume a multivitamin that contains 400 μg FA/d (1). However, lactating Canadian women who consume FA from both FA-enriched foods and vitamin supplements are likely to exceed the Tolerable Upper Intake Level of 1000 μg FA/d (7).

The presence of unmetabolized folic acid (UMFA) in the circulation is nearly ubiquitous in areas where FA fortification is permitted (8–10). Breast-milk folate species are predominantly in the reduced polyglutamate form with 5-methyl-tetrahydrofolate (THF) making up the majority (11, 12). However, evidence has been accumulating that UMFA is also detected in the breast milk of women who are exposed to FA (9, 13). Although it is not clear

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[2] Supplemental Methods, Supplemental Methods Tables 1–10, and Supplemental Table 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[8] Abbreviations used: DFE, dietary folate equivalent; FA, folic acid; FBP, folate binding protein; FFQ, food-frequency questionnaire; LOD, limit of detection; MeFox, 4α-hydroxy-5-methyl-tetrahydrofolate; MIREC, Maternal-Infant Research on Environmental Chemicals; THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

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whether UMFA has any health benefits or consequences for the mother or child, it can be hypothesized that the appearance of UMFA in the breast milk is an indicator of supraphysiologic FA intake. The aims of this study were to determine the total folate content of breast milk, investigate the prevalence of detectable UMFA in breast milk, and examine the relation of breast-milk folate and FA-supplement use and doses in a cohort of mothers who were enrolled in the MIREC (Maternal-Infant Research on Environmental Chemicals) study. The MIREC cohort was established from 2008 to 2011 to examine the potential health effects on maternal and fetal health that may be associated with environmental and nutritional exposures in pregnant Canadian women (14).

METHODS

Ethics

The MIREC study was approved by the Research Ethics Board of Health Canada, the Research Ethics Committee of the Coordinating Center of Sainte-Justine Hospital in Montreal, and the academic and hospital ethics committees of the 10 study sites across Canada (14). All participants provided informed consent on enrollment.

Subjects

The MIREC study is a national-level, multiyear cohort study that recruited women between February 2008 and March 2011 from 10 sites located in Vancouver, Edmonton, Winnipeg, Sudbury, Ottawa, Kingston, Hamilton, Toronto, Montreal, and Halifax (14, 15). Eligibility criteria were as follows: ability to consent and communicate in English or French, age ≥18 y; <14 wk of gestation, and willing to provide a sample of cord blood and planning on delivering at a local hospital. Women with a specific medical history, as described in Arbuckle et al. (14), were excluded from the study. Of 1983 women who consented and completed visit 1 (6–13 wk of pregnancy), 1385 women completed visit 6 (2–10 wk postpartum), and 1017 women provided a milk sample at that time (**Figure 1**). Of the milk samples, 561 samples were allocated proportionally for a folate analysis on the basis of maternal age (<30 and ≥30 y), parity (primiparous and multiparous), and region (Maritimes, Quebec, Ontario, Prairies, and British Columbia) (14). The sample of women who had milk samples allocated for the folate analysis included 401 self-identified FA-supplement users and 160 nonusers.

Identification of FA-supplement users and determination of FA doses

Study participants were queried about their supplement and medication intakes in the past 30 d at the time of milk sampling. Participants were asked to provide the name and description of the product, the drug identification number on the bottle (drug identification number or natural product number), the amount taken each time (number of, e.g., pills, tablets, capsules, or teaspoons), and the frequency of intake. A supplement user was defined as someone who consumed FA in the form of a multivitamin or single-vitamin supplement. A total of 78 unique vitamin supplements that contained FA were identified by the participants. The FA content and recommended daily intake for each product were obtained from the Health Canada Licensed Natural Health Products Database (16). For products that were not present in the database, the FA content and recommended daily intake were identified from the manufacturer's website. If the participant indicated a brand but did not specify the product, the mean FA content for all prenatal supplements from that manufacturer was used (n = 5 participants). For vitamin supplements that were not present in the Licensed Natural Health Products Database, or if the manufacturer's website could not be found, the mean FA content for all supplement products that were identified in the sample were used to estimate total daily FA intake (784 μg FA; n = 8 participants). When a participant did not indicate the frequency or number of pills consumed, daily intake recommended by the manufacturer was assumed.

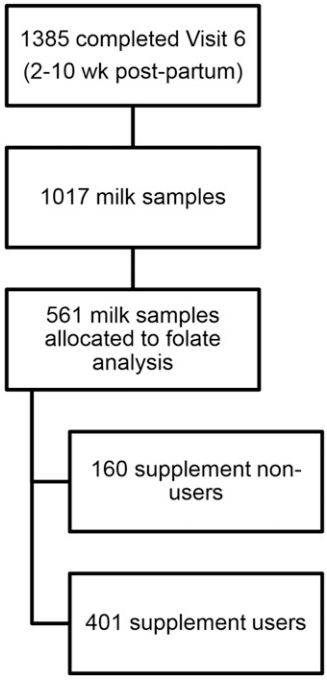

FIGURE 1 Participant flowchart indicating the number of Maternal-Infant Research on Environmental Chemicals participants who completed visit 6 and provided a breast-milk sample for analyses. A subset of milk samples were allocated proportionally for folate analysis on the basis of maternal age (<30 or ≥30 y), parity (primiparous or multiparous), and region (Maritimes, Quebec, Ontario, Prairies, or British Columbia).

To calculate total daily FA intake from supplements for each participant, the FA content from all vitamin supplements was summed. The doses of total daily FA intake from supplements were divided into 4 groups as follows: 0 μg (n = 160), >0 to ≤400 μg (n = 36), >400–1000 μg (n = 319), and >1000 μg (n = 46). Recommended FA intake from supplements for women who could become pregnant or were pregnant or lactating was 400 μg/d (17). However, over-the-counter vitamin supplements on the Canadian market can have up to the Tolerable Upper Intake Level of 1000 μg, which is a dose that is commonly found in prenatal supplements (18). FA supplements with higher doses are available in Canada by prescription (18).

Variables of interest

Maternal characteristics of interest were age, the time from parturition to milk sampling, education, household income, FA-supplement use and dose, milk type (foremilk, hindmilk, or

both), time of day of collection (morning, afternoon, evening, overnight, or combined), duration of sample storage at −20°C to −38°C, duration of sample storage at −80°C, and breast-milk folates. Maternal education brackets were defined as less than a postsecondary education; a college, trade-school diploma, or undergraduate degree; and a graduate degree. Brackets of annual household income were defined as lower income (<$60,000), which comprised the bottom quartile, and a middle-high income (≥$60,000), which comprised the upper 3 quartiles. The milk type was defined as foremilk, hindmilk, or both when the mother combined them. The time of day that the milk was collected was defined as morning, afternoon, evening, overnight, or combined when more than one period was indicated by the mother.

Milk collection, handling, and storage

Mothers were asked to collect breast milk between the third and eighth weeks after delivery. Mothers were asked to express both left and right breasts by hand, if possible, into provided sterile glass jars; however, they could provide milk expressed from either breast with the use of a sterilized breast pump if necessary. Mothers were advised to follow their normal routines of feeding their babies or pumping milk for their babies first and to collect the last amount of milk for the study. Milk collection was repeated until 200 mL was achieved. Milk was stored in a refrigerator until 200 mL was achieved or for ≤3 d, at which point the jar was placed in the freezer. If the collection took >3 consecutive days, the sample was frozen at the end of the third day even if the milk had not reached 200 mL. Mothers were asked to record the date, time of day (morning, 0600–1159; afternoon, 1200–1759; evening, 1800–2359; or overnight, 2400–0559), and whether the milk was foremilk, hindmilk, or both.

A research nurse collected the frozen milk sample from the participant's home. Samples were stored in a domestic freezer until they were shipped frozen on dry ice to the Food Research Division of Health Canada in Ottawa, Ontario. Samples were stored at −38°C until aliquots were made, after which they were stored at −80°C.

All sample handling was performed under yellow light. For aliquots, milk samples were thawed at room temperature in the dark for ∼1 h. Jars were heated to 38°C while shaken for 30 min and divided into amber glass jars and stored at −80°C. Aliquots were shipped overnight and frozen on dry ice to the Health Canada Quebec Regional Laboratory in Longueuil, Quebec, and stored at −80°C until folate analysis.

Measurement of breast-milk folate via liquid chromatography–tandem mass spectrometry

Breast-milk folates were measured with the use of liquid chromatography–tandem mass spectrometry (17, 19, 20). A detailed protocol is shown in **Supplemental Methods**, which includes **Supplemental Methods Tables 1–10**. In brief, folates were extracted from breast milk with an acidic buffer, which was followed by a multistep enzymatic digestion that was performed with the use of amylase, protease, and folate deconjugase from rat serum. Proteins were precipitated, and the extracts were purified, via solid-phase extraction. Quantitation was achieved with the use of internal standards and an external standard calibration curve. Because of the differences in stability for each folate, matching internal standards were included to correct for losses during sample workups as well as the interconversion between folate forms. The limit of detection (LOD), limit of quantification, and method performance data are presented in Supplemental Methods Tables 6–9. Samples were analyzed with the use of liquid chromatography–tandem mass spectrometry in positive electrospray mode, and FA, THF, 5-methyl-THF, 5-formyl-THF, and 5,10-methenyl-THF were quantified. The sum of these folates was reported as total folate. FA and 5-methyl-THF were reported separately; interconversions of other folate forms precluded them from being reported individually with certainty. Reduced folates represented the sum of THF, 5-formyl-THF, 5,10-methenyl-THF, and 5-methyl-THF.

Dietary folate intake

After completion of the milk collection, mothers were asked to complete the visit-6 questionnaire, which included the food-frequency questionnaire (FFQ). A 1-mo semiquantitative FFQ was used to assess the self-reported frequencies of intakes of 46 food items that were listed in 6 subgroups (vegetables; fruits; meat, poultry, fish, and alternatives; milk products; grain products; and other foods). The FFQ was validated to obtain rankings of iron, calcium, and vitamin D intakes but not of folate intake (17). However, of the 46 food items, 24 items were categorized as moderate-to-excellent sources of folate on the basis of the total dietary folate equivalent (DFE) content for that food in the Canadian Nutrient File (21). Foods were categorized on the basis of Wilson et al. (18) as follows: an excellent folate source (≥80 μg DFE/usual serving size); a good folate source (40–79 μg DFE/usual serving size); and a moderate folate source (20–39 μg DFE/usual serving size). Foods that were considered to be poor folate sources (<20 μg DFE/usual serving size) were not included in our analysis. Participants were asked whether they had consumed the food item or group of food items in the past month, the frequency with which the food was consumed (daily, weekly, or monthly), and the usual serving size (small, medium, or large) with a guide that was provided describing the usual serving size (e.g., 1 medium potato, 125 mL, or 1 cup). Responses were converted to servings per day with a 33% reduction factor for small servings and a 33% inflation factor for large servings. Of 561 women, FFQ data were incomplete for n = 7–9 women depending on the food item. Missing data were replaced with values that represented the median usual frequency of consumption for each food item or category.

Statistics

An unpaired Student's t test and chi-square test were used to determine differences between supplement nonusers and users in participant descriptive variables. Missing data were excluded from the χ^2 analysis. The Mann-Whitney rank-sum test was used to compare the frequency of daily consumption of folate food sources between supplement nonusers and users. Pearson's product-moment correlation was used to analyze the relation between concentrations of breast-milk folate species and between breast-milk folate species and descriptive variables.

Because the breast-milk folate data were skewed, all data were log transformed for the statistical analysis. A backward stepwise regression analysis was performed to identify variables that added

significantly to the ability of a regression equation to predict the dependent variable. Variables included were maternal age, maternal education, time from parturition (days), time of day of milk collection (morning, afternoon, evening, overnight or combined), type of milk (foremilk, hindmilk, or combined), frequency of intake of dietary sources of folate, duration of sample storage at −20°C to −38°C, and duration of sample storage at −80°C. Total daily FA intake from supplements was forced into the equation. Because total folate and reduced folates are composite variables that include UMFA, 5-methyl-THF, or both, independent variables that were shown to be significant predictors of any of the dependent variables were included in the multiple linear regression analysis. These variables included maternal age (UMFA), maternal education (5-methyl-THF), time from parturition (total folate, reduced folates, and 5-methyl-THF), duration of sample storage at −20°C to −38°C (UMFA), duration of sample storage at −80°C (5-methyl-THF), and total daily FA intake from supplements (reduced folates, 5-methyl-THF, and UMFA).

A 1-factor ANCOVA with a Holm-Sidak pairwise post hoc analysis was used to assess differences in FA dose groups. Dose-group means were adjusted for maternal education, time from parturition, duration of sample storage at −20°C to −38°C, and duration of sample storage at −80°C. Maternal age was not included in the ANCOVA analyses because it showed a collinear relation with total daily FA intake from supplements.

Data are presented as means ± SEMs. For calculating the mean UMFA of a group, UMFA samples that were less than the LOD (<0.9 nmol/L; n = 22) were assigned a value of one-half the LOD (0.45 nmol/L). For calculating total folate and the prevalence of detectable UMFA, UMFA was treated as 0. Statistical analyses were performed with the use of SigmaPlot 13 software (Systat Software Inc.). $P < 0.05$ was considered statistically significant.

RESULTS

Subject characteristics

A total of 561 women were included in the study (**Table 1**). The mean maternal age was 33.0 ± 0.0 y. The mean time from parturition to milk sampling was 34 ± 1.0 d. For annual family income, the majority of women had a middle-high income (≥$60,000) and a postsecondary education. Mean total folate, reduced folates, 5-methyl-THF, and UMFA were 119.0 ± 1.9, 72.0 ± 1.4, 49.7 ± 1.0, and 47.0 ± 1.6 nmol/L, respectively (**Figure 2**A). UMFA was detectable in the breast milk of almost

TABLE 1
Study participant characteristics

	Supplement use			
Variable	All	Nonuser	User	*P*
Participants, *n* (%)	561 (100)	160 (28.5)	401 (71.5)	—
Age,[1] y	33.0 ± 0.0	31.0 ± 0.0	33.0 ± 0.0	<0.001
Time from parturition to milk sampling,[1,2] d	34.0 ± 1.0	33.0 ± 1.0	34.0 ± 1.0	0.16
Annual family income,[3] %				0.02
Lower income (<$60,000)	16.9	23.8	14.2	
Middle-high income (≥$60,000)	78.6	71.3	81.5	
Missing	4.5	5.0	4.2	
Maternal education,[3] %				0.01
Less than postsecondary	9.1	14.4	7.0	
College or trade-school diploma or undergraduate degree	63.5	63.1	63.6	
Graduate degree	27.3	22.5	29.2	
Missing	0.2	0	0.2	
Time of collection,[3] %				0.79
Morning	15.7	13.1	16.7	
Afternoon	7.8	6.9	8.2	
Evening	8.6	9.4	8.2	
Overnight	2.3	1.9	2.5	
Combined	57.4	50.0	60.3	
Missing	8.2	18.8	4.0	
Foremilk or hindmilk,[3] %				0.03
Foremilk	13.2	15.0	12.5	
Hindmilk	8.9	11.3	8.0	
Combined	68.4	53.1	74.6	
Missing	9.4	20.6	5.0	
Prevalence of detectable UMFA[4] in breast milk,[3,5] %	96.1	95.1	96.5	0.70

[1] Values are means ± SEs. Differences between supplement nonusers and users were assessed with the use of Student's unpaired *t* test; $P < 0.05$ was considered significant.

[2] Because of missing data, the sample size was n = 533 (n = 138 supplement nonusers; n = 395 supplement users).

[3] Differences in proportions between supplement nonusers and users were assessed with the use of a χ^2 analysis; $P < 0.05$ was considered significant. Missing data were not included in the χ^2 analysis.

[4] UMFA, unmetabolized folic acid.

[5] The limit of detection for UMFA concentrations was <0.9 nmol/L.

all participants (Table 1). The majority of study participants provided milk samples that were collected across multiple days (data not shown) or across multiple times of day or samples that consisted of both foremilk and hindmilk.

Dietary folate intake

At the time of milk collection, participants were asked to complete an FFQ. However, the FFQ was not validated for folate intake (22). Nevertheless, 24 of 46 food items were classified as being at least a moderate source of folate. A comparison to 2 studies that conducted weighed food records in a comparable cohort of pregnant and lactating Canadian women showed that these 24 food items could represent 50–60% of total dietary folate intake (19, 20). With these limitations in mind, we showed that supplement nonusers had a lower frequency of daily consumption of foods that were considered to be excellent ($P = 0.006$) or moderate ($P < 0.001$) sources of natural folate only compared with that of supplement users (**Supplemental Table 1**). There was no difference in the frequency of intake of good sources of natural folate. There were also no differences in the frequency of intake of foods that contained natural folates and FA. When included in our multiple regression analysis, we showed no association between the frequency of intake of dietary folate sources with or without FA or combined and breast-milk folates (data not shown).

Supplement users compared with nonusers

Supplement users were defined as women who consumed FA as either a multivitamin or a single FA supplement. Of 561 study participants, 28.5% of subjects were supplement nonusers, and 71.5% of subjects were supplement users (Table 1). Median total daily intake of FA from supplements was 1000 μg/d, and the mean was 979 ± 39 μg/d. Compared with supplement nonusers, supplement users were older and had a higher education and annual family income. The mean age of supplement users was 2 y older than that of nonusers ($P < 0.001$). The proportion of women who consumed an FA supplement was ~40% lower in the lower income group (<$60,000) and ~15% higher in the middle-high income group (≥$60,000) ($P = 0.02$). The proportion of women with less than a postsecondary education was 50% lower, and the proportion of women with a postgraduate education was 33% higher, in FA-supplement users ($P = 0.03$) than in nonusers. There were no differences between supplement nonusers and users for the time from parturition to milk sampling. There was no difference in the time of collection between supplement users and nonusers; however, the majority of samples were combined across a number of collections. There was a significant difference in the prevalence of foremilk and hindmilk samples between supplement nonusers and users, but, as with the time of collection, the majority of samples were combined foremilk and hindmilk.

Breast-milk total folate, reduced folates, 5-methyl-THF, and UMFA concentrations were significantly different between supplement nonusers and users (P-all comparisons < 0.001) (Figure 2B). Total folate was 18% higher in supplement users than in nonusers. Reduced folates and 5-methyl-THF were 15% and 19% lower, respectively, in supplement users than in nonusers. In contrast, UMFA was 126% higher in supplement users than in nonusers. UMFA was observed in >95% of participants regardless of supplement use (Table 1).

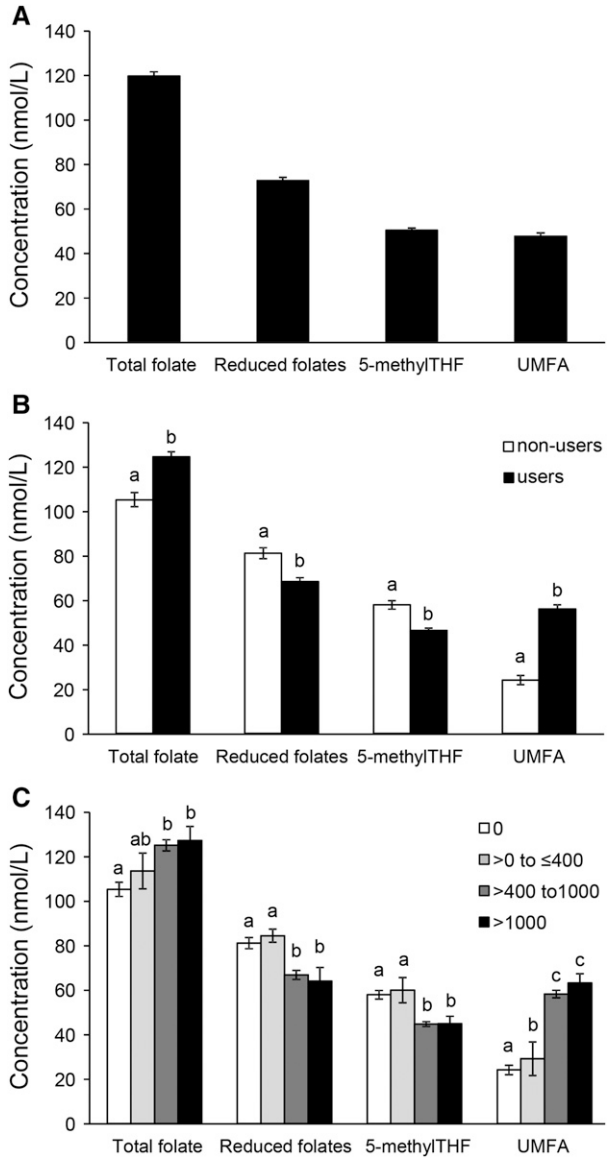

FIGURE 2 Mean ± SE total folate, reduced folates, 5-methylTHF, and UMFA contents in the breast milk of Canadian women (n = 561) (A), in women who were folic acid–supplement nonusers (n = 160) and users (n = 401) (B), and in women who consumed 0 μg folic acid/d (n = 106), >0–400 μg folic acid/d (n = 43), >400–1000 μg folic acid/d (n = 312), and >1000 μg folic acid/d (n = 46) from supplements (C). Total folate represents the sum of UMFA, THF, 5-methylTHF, 5,10-methenyl-THF, and 5-formyl-THF. Reduced folates represent the sum of THF, 5-methylTHF, 5,10-methenyl-THF, and 5-formyl-THF. Values that do not share a lowercase letter are significantly different. Differences in dose groups were assessed with the use of a 1-factor ANCOVA that was adjusted for maternal education, time from parturition, duration of sample storage at −20°C to −38°C, and duration of sample storage at −80°C followed by a Holm-Sidak pairwise post hoc analysis. $P < 0.05$ was considered significant. THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

The differences in the concentrations of folate species that were observed between supplement nonusers and users resulted in a shift in the relative proportions of folate species (**Figure 3**). The minor reduced folates (THF, 5-formyl-THF and 5,10-methenyl-THF) represented 18–20% of total folate regardless of

supplement use. However, 5-methyl-THF represented 55% of total folate in nonusers compared with 37% in users; conversely, UMFA represented 23% of total folate in nonusers and 45% in users ($P = 0.004$).

Breast-milk folate in relation to FA-supplement dose

The total daily FA-supplement dose ($P = 0.028$) and time from parturition ($P < 0.001$) were positively associated with total folate. When dose groups were compared, only doses >400 μg FA/d were significantly higher than those of nonusers ($P \leq 0.004$) (Figure 2C).

In contrast, total daily FA intake from supplements ($P < 0.001$) was negatively associated with reduced folates and 5-methyl-THF. The time from parturition was positively associated ($P < 0.001$), and education was negatively associated ($P = 0.046$–0.072), with reduced folates and 5-methyl-THF. Reduced folate was higher in women who consumed 0 or ≤400 μg FA/d than in with women who consumed >400 μg FA/d ($P < 0.001$–0.038). Similarly, women who consumed 0 or ≤400 μg FA/d had higher 5-methyl-THF than that of women who consumed >400 μg FA/d ($P < 0.001$–0.040).

Total FA intake from supplements ($P < 0.001$), maternal age ($P = 0.026$), and the duration of storage at −20 to −38°C (0.007) were positively associated with UMFA. When dose groups were compared, UMFA was significantly lower in supplement nonusers than in all supplement dose groups ($P < 0.001$–0.044). However, there was >2 times the amount of UMFA in women who consumed FA supplements that contained >400 μg FA/d ($P \leq 0.001$) than in women who consumed 0–400 μg/d. The prevalence of detectable UMFA was not significantly different in the dose groups (range: 93.0–97.9%; $P = 0.29$).

Differences in folate concentrations that were related to the FA dose resulted in a significant shift in the proportion of total folate that consisted of 5-methyl-THF and UMFA in women who consumed >400 μg FA/d (**Figure 4**). In women who consumed 0–400 μg/d, 5-methyl-THF comprised 53–55% of the total folate compared with 35% of the total folate in women who consumed >400 μg/d. For women who consumed 0–400 μg FA/d, UMFA comprised 23–26% of the total folate compared with 47–50% of the total folate when intakes were >400 μg FA/d. Furthermore, 5-methyl-THF and UMFA were negatively correlated ($r = -0.25$, $P < 0.001$). The proportion of total folate that was comprised of the other reduced folates THF, 5-formyl-THF, and 5, 10-methenyl-THF was not significantly different in the supplemental FA–dose groups.

DISCUSSION

Folate is especially important during times of growth and development, which highlights its importance during pregnancy and lactation because both the mother and fetus or neonate are undergoing rapid tissue growth (1). In the current study, we showed that the total folate in breast milk was modestly but significantly higher in women who consumed FA supplements. However, the higher total milk folate was due to higher UMFA concentrations but not to reduced folates. 5-Methyl-THF was significantly lower in milk from women who consumed supplements of >400 μg FA/d, which suggested that FA doses that are higher than recommended (>400 μg FA/d) result in

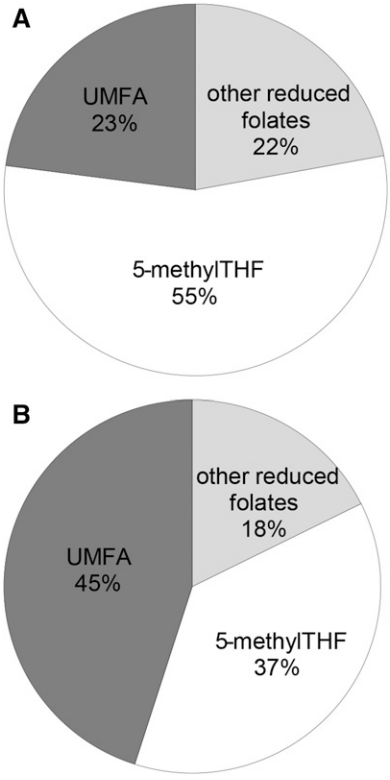

FIGURE 3 Proportions of total folate consisting of 5-methylTHF, other reduced folates, and UMFA in the breast milk of Canadian women who were folic acid–supplement nonusers ($n = 160$) (A) and users ($n = 401$) (B). THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

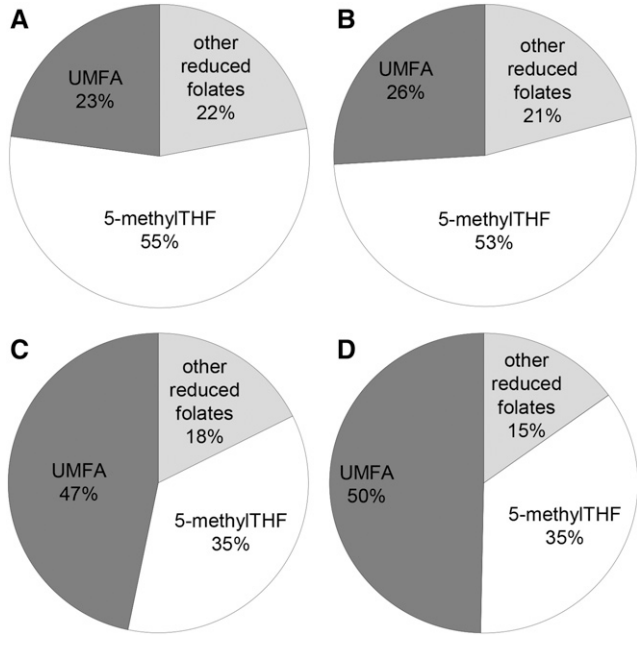

FIGURE 4 Proportions of total folate consisting of 5-methylTHF, other reduced folates, and UMFA in the breast milk of Canadian women who had daily folic acid supplement intake of 0 ($n = 160$) (A), >0–400 μg ($n = 43$) (B), >400–1000 μg ($n = 312$) (C), or >1000 μg ($n = 46$) (D). THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

disproportionately higher UMFA in breast milk at the expense of 5-methyl-THF.

Other than in overt deficiency, the concentration of milk folate is independent of a mother's folate status during lactation because the active transport of folate across the mammary epithelium (23–25). FA supplementation with 400–1000 μg FA/d has been shown to maintain maternal folate status but does not result in higher total milk folate, thereby suggesting that milk folate secretion reaches a maximum threshold in women with (at least) an adequate folate status (2, 9, 13, 24–26). In contrast, we observed significantly higher total milk folate in FA-supplement users and a modest positive association between the FA-supplement dose and total milk folate. The categorical analysis indicated that the difference in total milk folate was significant in women who consumed >400 μg FA/d. The differences between our observations and those of other authors were likely due to our large sample size, our method for analyzing the folate species and total folate, and the broad range of supplemental FA doses that was represented in this group of women.

Although the relation between maternal folate status and intake and total milk folate is reasonably understood, the impact of FA-supplement use on milk folate species remains unclear. We showed that UMFA represents ~25% of the total milk folate in Canadian women who were exposed to FA fortification and consumed 0–400 μg supplemental FA/d. Similarly, UMFA was shown to represent 8–18% of the total milk folate in women who consumed no supplemental FA (9, 13) or a 400 μg FA supplement/d for 16 wk (13). We also showed that UMFA was the major folate species in breast milk in women who consumed FA supplements in doses >400 μg FA/d, which represented ~50% of the total folate. Our results build on the observations by West et al. (9) who showed that UMFA represented 40% of the total milk folate in women who consumed a 750 μg FA supplement/d for 10 wk. We also showed that the 5-methyl-THF concentration was lower in milk from women who consumed higher doses of supplemental FA both in absolute and proportional terms. Together, our data indicate that there is a fundamental shift in milk folate species when FA supplements are consumed at doses that are higher than recommended (400 μg FA/d) with UMFA becoming the predominant folate species at the expense of 5-methyl-THF.

FA is more bioavailable than natural reduced folates and is rapidly absorbed across the intestine (27). Oral doses >260–280 μg FA/d saturate the hepatic metabolic capacity, which results in the appearance of UMFA in the circulation (28). In populations who are exposed to grain fortification, the prevalence of detectable UMFA in the blood is nearly ubiquitous (8, 10), albeit at low concentrations, and makes up 4% of the total folate, whereas 5-methyl-THF makes up >85% of the total folate (29). In contrast, the breast-milk UMFA concentration is 1 order of magnitude higher than that present in the circulation and represents 25–50% of the total folate. The mammary epithelial folate receptor α, which transports folate across the epithelium, has a higher affinity for UMFA than for 5-methyl-THF, and thus, UMFA can outcompete 5-methyl-THF for folate receptor α or inhibit its transport into breast milk (30). Milk folate binding protein also has a greater affinity for UMFA than for reduced folates, which can allow accumulation of UMFA in milk (31). Because milk folate secretion has an apparent maximum threshold, the shift in breast-milk folate species at higher FA-supplement doses may be explained by the preferential uptake and accumulation of UMFA in milk.

The potential health effects of UMFA on the mother and child remain unclear. UMFA has been hypothesized to disturb folate homeostatic regulation (13). FA must be sequentially reduced to dihydrofolate and THF by the enzyme dihydrofolate reductase (32, 33). The activity of human hepatic dihydrofolate reductase is relatively low, and the presence of UMFA could lead to an accumulation of dihydrofolate. In vitro, dihydrofolate can inhibit the activities of methylene-THF reductase and thymidylate synthase, which are 2 key enzymes that are required for one-carbon metabolism, theoretically leading to functional folate deficiency. Perhaps a more pertinent concern to infant nutrition is related to the bioavailability of breast-milk UMFA compared with reduced folates. Because the folate binding protein (FBP) has a higher affinity for FA than for 5-methyl-THF, FA may not be as readily released from FBP and absorbed in the infant gastrointestinal tract (34–36), which could affect infant folate status.

Strengths of our study include our quantitative method for measuring the various milk folate species (liquid chromatography–tandem mass spectrometry) and our large sample of women (n = 561) with a broad range of FA-supplement intakes. These strengths gave us the power to examine the dose-response relation between FA-supplement intake and breast-milk folate across a broad range of intakes (95% of the sample consumed doses that ranged from 0 to 2200 μg FA). In addition, the strengths may explain our ability to identify a significant association, albeit weak, between supplement use and dose and breast-milk total folate in contrast with the results of smaller intervention studies (9, 13).

Our study also has limitations. Although the MIREC study is a prospective cohort, the milk sampling was cross-sectional. Ideally, our findings will be verified in an intervention study with FA-supplement doses that span the range that was observed in our sample. The milk folate content can change according to the milk type (i.e., foremilk or hindmilk) and the time of day of collection; however, we did not observe an association between the milk folate content and these factors. This result was likely due to the majority of our samples being a combination of milk types that were collected across multiple times of day. Because the samples represent an infant's overall breast-milk folate exposure, we believe our findings hold. Our data suggest that the frequency of intake of FA-fortified foods (e.g., white bread and ready-to-eat cereal) does not differ between supplement nonusers and users. We also showed no association between dietary folate intake and the breast-milk folate content even in supplement nonusers only (data not shown) as was previously reported. However, the FFQ was not validated for folate intake, and thus, we cannot conclude that dietary folate does not influence breast-milk folates. However, FA-supplement use is the main determinant of folate status and nearly doubles total folate intake in lactating women (37, 38). We showed that total supplemental FA intake was a determinant of breast-milk UMFA and 5-methyl-THF regardless of the variables included in our regression model (not all data shown), which suggests that our finding is robust. Our study population was biased toward more older women with a higher socioeconomic status; these women are more likely to breastfeed and consume FA supplements (39, 40). However, these characteristics would not necessarily influence the FA metabolism. We did not measure the milk FBP concentration or maternal folate status,

which could have improved the interpretation of the data. We also did not measure the 5-methyl-THF oxidation product 4α-hydroxy-5-methyl-THF (MeFox), which, since the completion of our study, was shown to contribute $\sim$5% to the total serum folate (29). Our method separated MeFox chromatographically from the other folate analytes, and thus, it would not have contributed to our reported total folate. It remains to be seen whether MeFox is naturally present in breast milk or how sample handling and preparation could contribute to its formation.

In conclusion, our findings suggest that FA supplementation during lactation can result in significantly but modestly higher breast-milk total folate than in supplement nonusers. A significant inverse relation is observed between 5-methyl-THF and UMFA concentrations in breast milk, specifically at supplemental FA doses >400 μg/d. Our data suggest that intakes exceeding the recommended FA intake of 400 μg/d are supraphysiologic with no clear benefit. Therefore, higher-dose FA-supplement use may not be warranted, especially in populations for whom FA fortification is mandatory.

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