Associations between folic acid supplement use and folate status biomarkers in the first and third trimesters of pregnancy in the Maternal–Infant Research on Environmental Chemicals (MIREC) Pregnancy Cohort Study

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ABSTRACT

Background: Achieving optimal folate status during early gestation reduces the risk of neural tube defects (NTDs). While inadequate folate intake remains a concern, it is becoming increasingly common for individuals to consume higher than recommended doses of folic acid (FA) with minimal additional benefit.

Objective: Here, we sought to investigate the determinants, including FA supplement dose and use, of plasma total and individual folate vitamer concentrations in the first and third trimesters of pregnancy.

Methods: Using data from the Maternal–Infant Research on Environmental Chemicals (MIREC) Study, a cohort exposed to mandatory FA fortification, we measured plasma total folate and individual folate vitamer [5-methyltetrahydrofolate (5-methylTHF), unmetabolized FA (UMFA), and non-methyl folates (sum of THF, 5-formylTHF, 5,10-methenyl-THF)] concentrations in the first and third trimesters (n = 1,893). Using linear mixed models, we estimated associations between plasma folate concentrations, total daily supplemental FA intake, plasma vitamin B-12 concentrations, and multiple demographic, maternal, and reproductive factors.

Results: Almost 95% of MIREC study participants met or exceeded the recommended daily supplemental FA intake from supplements (≥400 μg/d), with approximately 25% consuming more than the Tolerable Upper Intake Level (>1000 μg/d). Over 99% of MIREC participants had a plasma total folate status indicative of maximal NTD risk reduction (25.5 nmol/L) regardless of FA supplement dose. UMFA was detected in almost all participants, with higher concentrations associated with higher FA doses. Determinants of adequate FA supplement intake and folate status associated with reduced NTD risk included indicators of higher socioeconomic position, higher maternal age, nulliparity, and lower prepregnancy BMI.

Conclusions: In the context of mandatory FA fortification, our data indicate that higher-than-recommended FA doses are unwarranted, with the exception of individuals at higher risk for NTDs. Ideally, prenatal supplements would contain 400 rather than 1000 μg FA, thereby enabling the consumption of optimal and safe FA doses. *Am J Clin Nutr* ;116:1852–1863.

Keywords: folate, folic acid, pregnancy, supplements, folate status, unmetabolized folic acid

Introduction

Folate status during early pregnancy is associated with the risk of neural tube defects (NTDs), including spina bifida, anencephaly, and encephalocele (1–3). Mandatory folic acid (FA) fortification of white wheat flour and other cereal products was implemented in the United States and Canada (4) to increase

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Supplemental Figures 1–3 and Supplemental Tables 1–8 are 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/ajcn/.

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Abbreviations used: FA, folic acid; ICC, intraclass correlation coefficient; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; MeFox, 4α-hydroxy-5-methylTHF; MIREC, Maternal–Infant Research on Environmental Chemicals; NTD, neural tube defect; THF, tetrahydrofolate; UL, Tolerable Upper Intake Level; UMFA, unmetabolized folic acid; UPLC, ultra-performance liquid chromatography.

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folate intake. Individuals who could become pregnant, or are pregnant, are recommended to consume a folate-rich diet and take an FA supplement; however, optimal folate status for reducing NTD risk may not be achieved through diet alone (5), even in the context of fortification (6). For instance, in Canada where white wheat flour and cereal products are fortified, ~25% of individuals of childbearing age who do not consume a supplement have nonoptimal folate status for NTD risk reduction (7). As such, Canadians who could become pregnant are recommended to consume an FA–containing supplement. Those with no known NTD risk factors are recommended to consume a daily multivitamin containing 0.4 mg FA, while those with NTD risk factors, such as a previous NTD-affected pregnancy, may require a daily FA dose of 4 mg (4). The goal is to ensure folate intakes that achieve an RBC total folate cutoff of 906 nmol/L, which is associated with maximal NTD risk reduction. The WHO (1, 8, 9) did not set a serum/plasma cutoff, but recent modeling suggests that a plasma total folate cutoff of 25.5 nmol/L approximates the RBC total folate threshold among individuals with adequate vitamin B-12 status (10).

While it is essential for a population to achieve an optimal folate status, individuals who consume FA-fortified foods and supplements often demonstrate FA intakes above the Tolerable Upper Intake Level (UL; 1 mg FA/d) (6, 11). Concerns have been voiced related to potential adverse health effects associated with high FA intake, including growth promotion of pre-existing cancer, reduced NK cell cytotoxicity (12), and asthma in offspring exposed to high FA intake in utero (13). However, other than the UL, there is no consensus on a definition of "high" or "excessive" FA intake or status, or FA-associated adverse effects (14, 15).

With these uncertainties, it is important to investigate the patterns and determinants of circulating folate concentrations and to identify the FA doses required to achieve optimal folate status while limiting unnecessarily high intakes. Here, we examined the relations between plasma total folate and individual folate vitamers, including unmetabolized FA (UMFA), FA supplement dose, and plasma vitamin B-12 concentrations in the first and third trimesters of pregnancy in a cohort of Canadian participants with adequate vitamin B-12 status.

Methods

Subjects

We used data and biospecimens from the Maternal–Infant Research on Environmental Chemicals (MIREC) Study. The MIREC study is a pan-Canadian prospective cohort designed to assess the impact of environmental chemical and nutritional exposures on maternal, infant, and child health (16). Briefly, pregnant participants in their first trimester were recruited from 11 sites located in Vancouver, Edmonton, Winnipeg, Sudbury, Ottawa, Kingston, Hamilton, Toronto, Montreal, and Halifax across Canada from February 2008 to March 2011. Eligibility criteria were as follows: ability to consent and to communicate in English or French, ≥18 y of age, <14 wk of gestation, willing to provide a sample of cord blood, planning on delivering at a local hospital, and no history of known fetal abnormalities (including NTD) or serious medical complications. Participants with specific medical histories were excluded, as previously described (16). Participants provided biospecimens and completed questionnaires on sociodemographic, lifestyle, and nutritional factors during pregnancy. Of 8716 potential participants invited to join MIREC, 5108 were eligible and 2001 consented to participate; 73 dropped out during pregnancy. Our analysis included 1893 participants who consented to contribute to the MIREC Biobank, and excluding those with no plasma folate values during the first or third trimester ($n = 33$) and those considered to have implausibly high self-reported supplemental FA intake ($n = 2$; >10,000 μg/d) (**Supplemental Figure 1**). While all participants had at least 1 plasma folate value from the first or third trimester, FA intake from supplements was available for a subset of participants ($n = 1476$).

Ethics

The MIREC study was approved by the Research Ethics Board of Health Canada, the Research Ethics Committee of the coordinating center of Ste-Justine's Hospital in Montreal, and the academic and hospital ethics committees of the study sites (16). In addition, this MIREC biobank project to analyze plasma folate and vitamin B-12 in biobanked samples was approved separately by the Research Ethics Board of Health Canada. All participants provided informed consent upon enrollment.

FA supplement dose

After the first-trimester study visit, study participants were surveyed about their supplement and medication intake in the last 30 d (17). Participants provided the name and description of the product, the product identification number (Drug Identification Number or Natural Product Number), the amount taken each time (number of pills, tablets, capsules, teaspoons, etc.), and the frequency of intake. A supplement user was defined as someone who consumed FA in the form of a multi- and/or single-vitamin supplement. We identified 72 unique vitamin supplements containing FA reported by MIREC participants. The FA content and recommended daily intake for each product was obtained from the Health Canada Licensed Natural Health Products Database (18). For those products not found in the database, the FA content and recommended daily intake were identified on the manufacturer's website. If the participant indicated a brand but not the specific product, the mean FA content for all prenatal supplements from that manufacturer was assigned. For vitamin supplements that were not found in the Licensed Natural Health Products Database, or the manufacturer's website could not be found, the median FA contents for all supplement products identified in the sample were assigned to estimate the total daily FA intake (800 μg FA; $n = 39$). When the participant did not indicate the frequency or number of pills consumed, the daily intake recommended by the manufacturer was assumed.

To calculate total daily FA intake from supplements for each participant, the FA content from all vitamin supplements was summed. We categorized total daily FA intake from supplements into 3 groups: not meeting recommended FA intake (0 to <400 μg/d), meeting recommended FA intake (≥400 to ≤1000 μg/d), and exceeding recommended FA intake (>1000 μg/d). The recommended FA intake from supplements for individuals who could become pregnant or are pregnant

or lactating in Canada is 400 μg/d (19). However, over-the-counter vitamin supplements on the Canadian market can have up to the UL of 1000 μg, the dose found most commonly in prenatal supplements (18). FA supplements with higher doses are available in Canada by prescription only (18).

Sociodemographic, perinatal, and lifestyle characteristics

We estimated the associations of sociodemographic, maternal, and reproductive factors with folate biomarkers based on biologic plausibility and a priori knowledge using a directed acyclic graph (**Supplemental Figures 2** and **3**). Household income, maternal age, maternal birth country, educational attainment, parity, use of fertility treatments, time to pregnancy, prepregnancy BMI, and self-reported tobacco use during pregnancy were collected via participant self-report on questionnaires completed during the baseline visit (first trimester). We also considered gestational week of collection as a predictor.

Blood sampling

Nonfasting peripheral blood samples were collected by venipuncture at scheduled clinic visits in the first and third trimester visits. Most participants provided a blood sample in the first trimester (n = 1844; 97%) with fewer providing blood in the third trimester (n = 1602; 85%). The majority of participants (n = 1553; 82%) provided blood samples in both trimesters. Blood was collected into Vacutainer tubes (BD-Canada, Mississauga, ON) containing EDTA. EDTA whole blood for plasma was centrifuged at 1500 × g for 15 minutes at 4°C, and plasma immediately placed into aliquots and stored at −80°C. Samples from the MIREC biobank were shipped to Health Canada Food Laboratory in Toronto, Ontario, in May 2018 for analysis of folate and stored at –80°C until analysis.

Plasma folate

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to determine the contribution of individual folate vitamers to plasma total folate following the US CDC method adapted for manual wet chemistry as opposed to automated processing (20), as previously described (20–22). Folate vitamers included the following: tetrahydrofolate (THF), 5,10-methenylTHF, 5-formylTHF, 5-methylTHF, and UMFA. The 5-methylTHF oxidation product 4α-hydroxy-5-methylTHF (MeFox) was also measured. In brief, plasma samples were spiked with an internal standard mixture followed by addition of the extraction buffer (at pH 5) and incubated at 4°C for 30 min. Sample extraction and cleanup were performed by solid-phase extraction using sodium acetate elution buffer and the final extracts were filtered through 0.2-μm syringe filters prior to LC-MS/MS analysis. Folate peaks were separated by ultra-performance LC (UPLC) gradient mobile phase and detected by MS/MS in multiple reaction monitoring (MRM) mode. Quantitation was performed using external calibration standards based on peak area ratio using internal standards. Quality-control metrics for LC-MS/MS measurements are provided in **Supplemental Table 1**. Samples were analyzed using the Waters Xevo TQ-XS mass spectrometer coupled to the Waters Acquity UPLC system (Waters Limited). Samples were maintained at room temperature for no longer than 50 min during processing and returned to –80°C. Data collection and reduction were achieved using MassLynx software (version 4.2; Waters Corporation, Milford MA). Nonmethylated folate was calculated by summing the concentrations of THF, 5-formylTHF, and 5,10-methenylTHF. Total folate was calculated by summing the concentrations of 5-methylTHF, FA, THF, 5-formylTHF, and 5,10-methenylTHF. We used the proposed cutoff for optimal folate status for NTD prevention defined as a plasma total folate value of 25.5 nmol/L (10).

Plasma vitamin B-12

Plasma samples subjected to 1 thaw cycle were shipped to the Nutrition Research Canadian Health Measures Survey Reference Laboratory in Ottawa, Ontario. Plasma total vitamin B-12 was measured according to the manufacturer's instructions (Siemens ADVIA Centaur XP). Policies, procedures, and processes within the laboratory meet licensing and accreditation standards of clinical laboratories in Canada and the Institute for Quality Management in Healthcare. Assay performance targets (intra- and interassay) were set at a CV ≤10%. All assays were verified to meet targets prior to sample analysis utilizing third-party quality-control materials (BioRad). The plasma vitamin B-12 cutoff value for deficiency was defined as <148 pmol/L (10, 23).

Statistical analyses

Values below the limit of detection (LOD) were assigned a value of the LOD/$\sqrt{2}$. Plasma folate concentrations were right skewed, so we $\log_{10}$-transformed the data to reduce the influence of extreme observations and satisfy normality assumptions of our models. We calculated univariate statistics for maternal, sociodemographic, and reproductive factors, as well as maternal plasma folate and vitamin B-12 concentrations and daily supplemental FA intake. We assessed distributions of plasma folate concentrations across categories of daily supplemental FA intake. We calculated the mean relative contribution of folate vitamers to total plasma folate concentration. We calculated Spearman rank correlation coefficients among $\log_{10}$-transformed folate concentrations and folate concentrations and daily supplemental folic acid intake. We assessed reproducibility of repeated plasma folate concentrations using intraclass correlation coefficients (ICCs) using a 2-way random-effects model (24).

Our primary analysis used linear mixed models with random effects to estimate associations between covariates and repeated plasma folate or vitamin B-12 concentrations, where separate models were developed for folate and vitamin B-12 biomarkers. We calculated % differences in $\log_{10}$-transformed folate biomarker (UMFA, 5-methylTHF, non-methyl folate, and total folate) and vitamin B-12 concentrations with increasing levels of predictors, or across categories of predictors relative to the reference group. We examined categories of daily supplemental FA intake, maternal age, educational attainment, birth country, household income, parity, and gestational week of collection.

We conducted sensitivity analyses to assess the robustness of our results to various adjustments. First, in addition to previously specified covariates used in our primary analyses, we also adjusted for prepregnancy BMI and maternal tobacco use during pregnancy. We also considered reproductive factors by additionally adjusting for fertility treatment use and time

TABLE 1 First- and third-trimester maternal plasma total folate by maternal factors among MIREC study participants[1]

		Total folate,[2] nmol/L			
		First trimester		Third trimester	
Variable	Full sample,[3] *n* (%)	*n* (%)	Median (first quartile, third quartile)	*n* (%)	Median (first quartile, third quartile)
Participants	1893 (100)	1844 (100)	96 (78, 118)	1602 (100)	98 (74, 133)
Maternal age					
<25 y	130 (7)	128 (7)	87 (62, 106)	98 (6)	74 (39, 107)
25 to <35 y	1123 (59)	1088 (59)	95 (77, 116)	961 (60)	98 (73, 131)
≥35 y	640 (34)	628 (34)	99 (81, 122)	543 (34)	103 (79, 147)
Missing	0 (—)				
Education					
<High school	166 (9)	164 (9)	88 (66, 115)	130 (8)	82 (48, 116)
Some college	99 (5)	95 (5)	90 (71, 106)	84 (5)	88 (73, 113)
College/trade school	448 (24)	436 (24)	94 (75, 120)	369 (23)	93 (66, 126)
University	1178 (62)	1147 (62)	98 (81, 118)	1017 (63)	103 (79, 141)
Missing	2 (0.1)				
Mother born in Canada					
Yes	1544 (82)	1500 (81)	95 (78, 117)	1298 (81)	98 (74, 132)
No	349 (18)	344 (19)	97 (77, 119)	304 (19)	101 (76, 139)
Missing	0 (—)				
Household income (Canadian dollars)					
<30,000	141 (8)	138 (7)	89 (60, 111)	113 (7)	83 (45, 131)
30,000–80,000	571 (32)	549 (30)	95 (76, 115)	487 (30)	96 (68, 130)
>80,000	1099 (61)	1076 (58)	97 (80, 119)	939 (59)	102 (78, 136)
Missing	82 (4)				
Marital status					
Not married	532 (28)	524 (28)	94 (75, 118)	439 (27)	96 (65, 132)
Married	1360 (72)	1319 (72)	97 (79, 117)	1162 (73)	100 (76, 135)
Missing	1 (0.1)				
Prepregnancy BMI (kg/m^2)					
<25	1108 (63)	1081 (59)	95 (78, 117)	946 (59)	100 (77, 136)
25–29.9	387 (22)	377 (20)	99 (79, 121)	315 (20)	98 (67, 139)
≥30	260 (15)	253 (14)	96 (78, 117)	222 (14)	91 (65, 126)
Missing	138 (7)				
Tobacco use[4]					
Nonsmoker	1148 (61)	1116 (61)	97 (79, 118)	990 (62)	102 (77, 134)
Any smoking	743 (39)	726 (39)	94 (76, 117)	610 (38)	95 (68, 133)
Missing	2 (0.1)				
Parity					
0	840 (44)	818 (44)	98 (81, 122)	716 (45)	108 (85, 149)
1	764 (40)	741 (40)	95 (77, 114)	642 (40)	93 (68, 124)
≥2	289 (15)	285 (15)	92 (72, 112)	244 (15)	85 (57, 124)
Missing	0 (—)				
Folic acid supplementation[5]					
0–400 μg/d	79 (4)	75 (4)	80 (69, 109)	75 (5)	83 (53, 125)
≥400–1000 μg/d	1030 (54)	1001 (54)	96 (79, 117)	934 (58)	101 (78, 134)
>1000 μg/d	367 (19)	358 (19)	105 (84, 129)	320 (20)	105 (83, 146)
Missing	417 (22)				
First-trimester vitamin B-12					
<148 pmol/L	1 (0.1)	NR	NR	NR	NR
148–220 pmol/L	55 (0.3)	55 (3)	78 (61, 97)	48 (3)	73 (47, 106)
>220 pmol/L	1774 (94)	1772 (96)	96 (78, 118)	1492 (93)	100 (75, 135)
Missing	63 (3)				
Third-trimester vitamin B-12					
<148 pmol/L	20 (1)	19 (1)	85 (66, 93)	20 (1)	69 (48, 86)
148–220 pmol/L	278 (15)	267 (14)	88 (71, 108)	278 (17)	80 (56,112)
>220 pmol/L	1296 (68)	1259 (68)	98 (79,121)	1296 (81)	102 (78,140)
Missing	299 (16)				

[1]The sample included $n = 1553$ repeated measures from a total of $n = 1893$ participants. MIREC, Maternal–Infant Research on Environmental Chemicals; NR, not reported; THF, tetrahydrofolate.

[2]Total folate: sum of the 5 folate vitamers: 5-methylTHF, folic acid, tetrahydrofolate (THF), 5-formylTHF, and 5,10-methenylTHF.

[3]Note: *n* and % values may not round to 100 or sum to the full sample size since there may be missingness.

[4]Assessed at baseline visit (first trimester)

[5]Daily folic acid intake from supplements was based on maternal self-report on standardized questionnaires collected once after the first-trimester baseline visit. Participants who indicated that they did not consume a folic acid supplement were assigned "0".

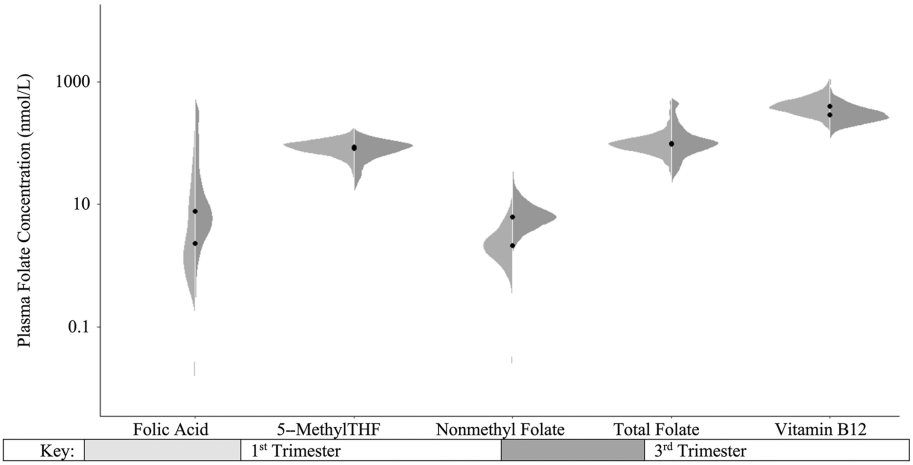

FIGURE 1 Violin plot of the distribution of plasma folate vitamer concentrations comparing the first and third trimesters among MIREC study participants. The sample included a total of $n = 1893$ participants, with $n = 1553$ repeated measures. The *y*-axis is presented on the log scale. Non-methyl folate values are the sum of THF, 5-formylTHF, and 5,10-methenylTHF. Total folate values are the sum of the 5 folate vitamers: 5-methylTHF, folic acid, THF, 5-formylTHF, and 5,10-methenylTHF. Black dots represent the median values. MIREC, Maternal–Infant Research on Environmental Chemicals; THF, tetrahydrofolate.

to pregnancy. Finally, we adjusted for recruitment year and recruitment center.

We completed statistical analyses using R Studio (version 3.6.2; R Foundation for Statistical Computing) (25).

Results

Study participant characteristics

MIREC study participants were predominantly married (72%), Canadian born (82%), and nonsmokers (61%) and had high levels of educational attainment (62% had university degrees) and household incomes (61% >$80,000 annually; **Table 1**). The majority were older than 25 y, multiparous (55%), and had a BMI (kg/m^2) <25 (63%). Within the full analytic sample, FA intake from supplements was reported for 1476 (78%) participants. Over 99% of participant samples had a plasma vitamin B-12 concentration >148 pmol/L; only 1 participant in the first and 20 participants in the third trimester had values <148 pmol/L. Vitamin B-12 status was lower in the third trimester, with 94% of participants in the first trimester and 68% in the third trimester having adequate vitamin B-12 values (>220 pmol/L).

First- and third-trimester plasma folate concentrations

Nearly all participant samples had a plasma total folate concentration above the proposed 25.5-nmol/L cutoff for NTD prevention; only 8 participants in the first and 12 participants in the third trimester had values that were lower. In both trimesters, higher plasma total folate was associated with higher maternal age, maternal educational attainment, annual household income, FA intake from supplements, and lower parity (Table 1). In the third trimester, lower plasma total folate was associated with higher prepregnancy BMI.

The distributions of plasma total folate, 5-methylTHF, and MeFox concentrations were similar when comparing the first and third trimesters (**Figure 1**, **Supplemental Table 2**). However, the distributions of plasma UMFA and non-methyl folate concentrations were higher in the third trimester compared with the first trimester, and distributions of plasma vitamin B-12 concentrations were higher in the first trimester compared with the third trimester.

In the first trimester, the mean relative contribution of 5-methylTHF to plasma total folate was 83%, 15% for UMFA, and 3% for non-methyl folates (**Figure 2**, top panel). In contrast, 5-methylTHF had a lower mean relative contribution of 64% in the third trimester, whereas UMFA and non-methyl folates had higher relative contributions of 30% and 6%, respectively.

Pearson correlation coefficients among the plasma folate vitamer concentrations within the first or third trimesters were positive, and stronger in the third trimester (range: 0.26–0.85) relative to the first trimester (range: 0.04–0.84) (**Supplemental Table 3**). The strongest correlations were between plasma total folate and 5-methylTHF in both the first (0.84) and third (0.85) trimesters followed by correlations between plasma total folate and UMFA (first trimester: 0.61; third trimester: 0.79). Correlations between plasma folate vitamers and vitamin B-12 concentrations were weakly positive but similar between the first (range: 0.07–0.17) and third (0.05–0.21) trimesters.

Repeated $\log_{10}$-transformed plasma folate biomarker concentrations had poor reproducibility (ICCs <0.40) between the first and third trimesters (**Supplemental Table 4**). 5-MethylTHF had the highest (ICC: 0.39) and nonmethyl folate concentrations had the lowest (ICC: 0.06) reproducibility. Repeated $\log_{10}$-transformed plasma vitamin B-12 concentrations had fair reproducibility (ICC: 0.51) between the first and third trimesters.

Supplemental FA intake

Of participants who provided information on FA supplement use ($n = 1476$), the majority (95%) of participants reported FA intake from supplements that met or exceeded the daily recommended dose of 400 μg (**Table 2**, **Supplemental Table 5**). Participants who did not meet daily recommendations

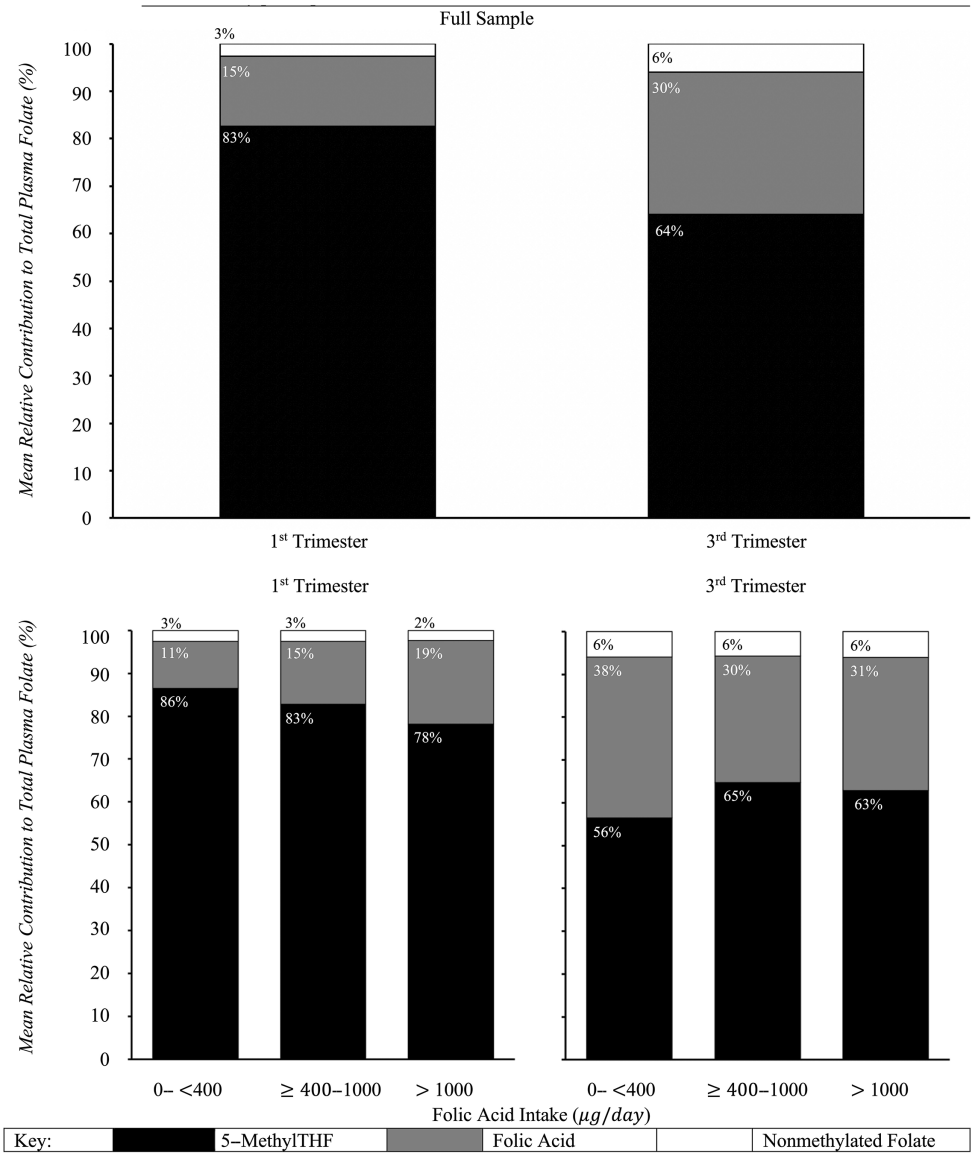

FIGURE 2 Mean relative contributions of folate vitamers to plasma total folate concentrations within the full sample are presented on the top row, and by categories of folic acid intake from supplements on the bottom row. The sample included $n = 1553$ repeated measures from a total of $n = 1893$ participants. Non-methyl folates values are the sum of THF, 5-formylTHF, and 5,10-methenylTHF. Note, percent values for individual folate vitamers may not add to 100% due to rounding. THF, tetrahydrofolate.

for supplemental FA intake were more likely to have lower educational attainment and lower annual household income and were not nulliparous. Of note, 25% of participants reported intakes that exceeded the UL. They were older (>35 y), with higher educational attainment, higher household income, and nulliparous.

Association between supplemental FA intake with plasma folate and vitamin B-12 concentrations

Spearman rank correlation coefficients between estimated FA intake with plasma folate and vitamin B-12 biomarker concentrations were weakly positive (range: 0.04–0.18), with similar correlation patterns in the first and third trimesters of pregnancy (**Supplemental Table 6**). However, these results should be interpreted with caution given that FA supplement use was only assessed once and also that "ties" in the rankings of FA intake might bias these correlations because a large number of participants reported the same FA intake (i.e., 1000 μg/d; Table 2).

Concentrations of the folate biomarkers plasma 5-methylTHF, non-methyl folate, and total folate increased with increasing supplemental FA intake in both trimesters, with the exception of UMFA, which increased across dose groups in the first trimester but not in the third trimester (**Figure 3**). The largest increase was observed when comparing the folate vitamer concentrations of participants with supplemental FA intake of 0 to <400 μg/d compared with those consuming ≥400 to 1000 μg/d. Concentrations of plasma vitamin B-12 were similar across categories of supplemental FA intake during the first trimester.

TABLE 2 Folic acid supplement intake by maternal factors among MIREC study participants[1]

		Folic acid supplementation,[2] μg/d					
		Below recommendations (0–<400)		Meets recommendations (≥400–1000)		Exceeds recommendations (>1000)	
Variable	Full sample, *n* (%)[3]	*n* (%)	Median (first, third quartile)	*n* (%)	Median (first, third quartile)	*n* (%)	Median (first, third quartile)
Participants	1476 (100)	79 (100)	20 (0, 200)	1030 (100)	1000 (1000, 1000)	367 (100)	2000 (1200, 5000)
Maternal age							
<25 y	68 (5)	2 (3)	0 (0, 0)	57 (6)	1000 (1000, 1000)	9 (2)	2000 (1700, 2500)
25 to <35 y	897 (61)	49 (62)	20 (0, 143)	642 (62)	1000 (1000, 1000)	206 (56)	2000 (1176, 5000)
≥35 y	511 (35)	28 (35)	63 (0, 213)	331 (32)	1000 (1000, 1000)	152 (41)	2000 (1200, 3550)
Education							
<High school	166 (9)	6 (8)	67 (0, 133)	80 (8)	1000 (1000, 1000)	19 (5)	1700 (1100, 2250)
Some college	99 (5)	6 (8)	72 (0, 186)	48 (5)	1000 (1000, 1000)	16 (4)	1850 (1175, 5000)
College/trade school	448 (24)	20 (25)	0 (0, 143)	232 (23)	1000 (1000, 1000)	77 (21)	2000 (1200, 5000)
University	1178 (62)	47 (59)	27 (0, 214)	670 (65)	1000 (1000, 1000)	253 (69)	2000 (1229, 4100)
Mother born in Canada							
Yes	1218 (83)	64 (81)	23 (0, 200)	852 (83)	1000 (1000, 1000)	302 (82)	2000 (1289, 5000)
No	254 (17)	15 (19)	0 (0, 171)	178 (17)	1000 (1000, 1000)	61 (18)	1700 (1100, 2500)
Household income (Canadian dollars)							
<30,000	89 (6)	5 (6)	0 (0, 0)	68 (7)	1000 (1000, 1000)	16 (4)	1900 (1550, 5000)
30,000–80,000	432 (29)	30 (38)	0 (0, 186)	312 (30)	1000 (1000, 1000)	90 (25)	1900 (1100, 4082)
>80,000	902 (61)	41 (52)	86 (0, 250)	616 (60)	1000 (1000, 1000)	245 (67)	2000 (1200, 5000)
Marital status							
Not married	382 (26)	22 (28)	10 (0, 143)	267 (26)	1000 (1000, 1000)	93 (25)	2000 (1200, 5000)
Married	1093 (74)	57 (72)	108 (0, 229)	762 (74)	1000 (1000, 1000)	274 (75)	2000 (1200, 4082)
Prepregnancy BMI (kg/m^2)							
<25	882 (60)	49 (62)	0 (0, 200)	612 (59)	1000 (1000, 1000)	221 (60)	2000 (1171, 2500)
25–29.9	296 (22)	12 (15)	114 (0, 217)	215 (21)	1000 (1000, 1000)	69 (19)	2000 (1429, 5000)
≥30	198 (13)	12 (15)	71 (0, 234)	126 (12)	1000 (1000, 1000)	60 (16)	2000 (1200, 5000)
Tobacco use[4]							
Nonsmoker	886 (60)	49 (62)	100 (0, 229)	599 (58)	1000 (1000, 1000)	238 (65)	2000 (1249, 5000)
Any smoking	514 (35)	28 (35)	0 (0, 157)	382 (37)	1000 (1000, 1000)	104 (28)	1879 (1138, 3100)
Parity							
0	692 (47)	28 (35)	0 (0, 108)	476 (46)	1000 (1000, 1000)	188 (51)	2000 (1300, 5000)
1	587 (40)	39 (49)	143 (0, 225)	404 (39)	1000 (1000, 1000)	144 (39)	2000 (1197, 5000)
≥2	197 (13)	12 (15)	138 (0, 207)	150 (15)	1000 (1000, 1000)	35 (10)	1600 (1014, 2000)
First-trimester vitamin B-12							
<148 pmol/L	1 (0.1)	0 (—)	—	0 (—)	—	1 (0.3)	5000 (—)
148–220 pmol/L	55 (0.3)	2 (3)	286 (286, 286)	28 (3)	1000 (1000, 1000)	4 (1)	1643 (1239, 3000)
>220 pmol/L	1774 (94)	74 (94)	23 (0, 200)	965 (94)	1000 (1000, 1000)	348 (95)	2000 (1200, 5000)

[1]The sample included a total of $n = 1476$ participants with complete folic acid supplement intake data (78% of the full analytic sample). MIREC, Maternal–Infant Research on Environmental Chemicals.

[2]Daily folic acid intake from supplements was based on maternal self-report on standardized questionnaires collected once after the first-trimester baseline visit. Participants who did not consume a folic acid supplement were assigned "0."

[3]Note: *n* and % values may not round to 100 or sum to the full sample size since there may be missingness.

[4]Assessed at baseline visit (first trimester).

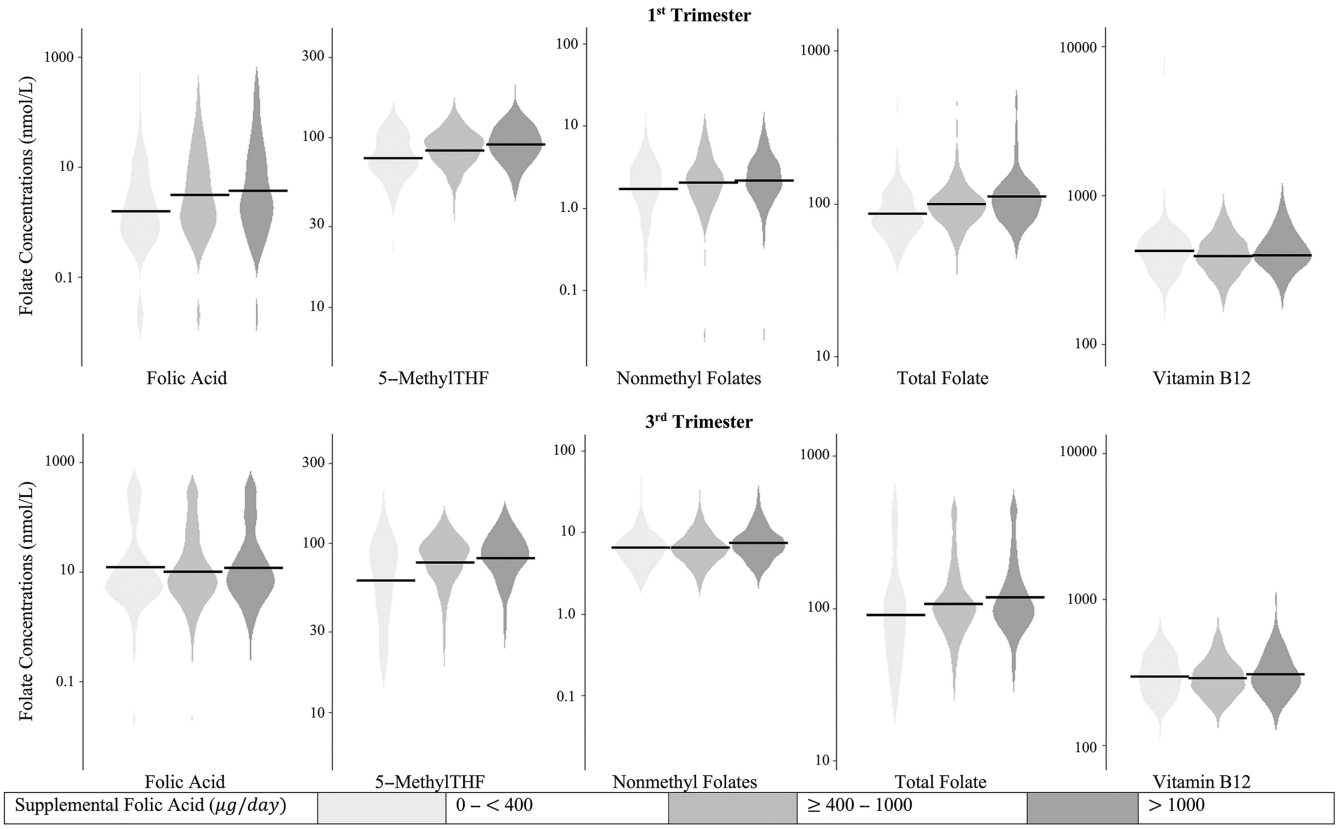

FIGURE 3 Distributions of plasma folate vitamers and vitamin B-12 by supplemental folic acid doses are presented on the top row from the first trimester and on the bottom row from the third trimester. The sample included $n = 1553$ repeated measures from a total of $n = 1893$ participants. The *y*-axis is presented on the log scale. Non-methyl folate values are the sum of THF, 5-formylTHF, and 5,10-methenylTHF. Total folate values are the sum of the 5 folate vitamers: 5-methylTHF, folic acid, THF, 5-formylTHF, and 5,10-methenylTHF. Horizonal lines represent the median values. THF, tetrahydrofolate.

In the first trimester, 5-methylTHF made up the largest proportion of mean relative contribution to total folate regardless of FA supplemental dose (Figure 2, lower panel). However, it was lower with higher FA doses (86% vs. 83% vs. 78%). Conversely, plasma UMFA as a relative contribution to total folate was higher with higher FA doses (11% vs. 15% vs. 19%). In the third trimester, 5-methylTHF was the largest contributor to total folate but a smaller proportion than observed in the first trimester (64% vs. 83%). Differences in relative contributions of folate vitamers to total folate by FA dose reflected higher relative contributions of UMFA and lower relative contributions of 5-methylTHF with higher FA dose; note that absolute UMFA concentrations did not differ by FA dose in the third trimester. The mean relative contribution to total folate by non-methyl folates remained similar across FA doses within each trimester.

After adjusting for maternal age, educational attainment, birth country, household income, and parity, we observed a monotonic increase in plasma folate biomarkers across categories of FA intake from supplements (**Figure 4**, **Supplemental Table 7**). Plasma UMFA concentrations were 38% (95% CI: 4%, 89%) and 54% (95% CI: 12%, 111%) higher among those meeting or exceeding daily supplemental FA intake recommendations, respectively, compared with those who did not meet daily FA intake recommendations. Moreover, 5-methylTHF concentrations were 17% (95% CI: 11%, 24%) and 23% (95% CI: 16%, 31%) higher among those consuming ≥400–1000 μg/d and >1000 μg/d of FA from supplements, respectively, relative to those not meeting daily recommendations of FA (0 to <400 μg/d) (Figure 4, Supplemental Table 7). Plasma vitamin B-12 concentrations did not substantially differ among those meeting (–5%; 95% CI: –10%, 1%) or exceeding (0%; 95% CI: –5%, 7%) daily FA intake recommendations, compared with those who did not meet daily recommendations.

Associations of sociodemographic, perinatal, and lifestyle characteristics with plasma folate concentrations

After adjustment for covariates, concentrations of FA, 5-methylTHF, non-methyl folate, and total folate were positively associated with maternal age and negatively associated with parity (**Table 3**). Higher household income was associated with higher 5-methylTHF, total folate, and vitamin B-12. We did not observe associations between gestational age, maternal education, or country of origin and plasma folate or vitamin B-12.

In sensitivity analyses, we examined prepregnancy BMI category, tobacco use, fertility treatment use, and time to pregnancy in relation to total folate concentrations. Compared with those who had a prepregnancy BMI <25, those with a prepregnancy BMI >30 tended to have lower total folate

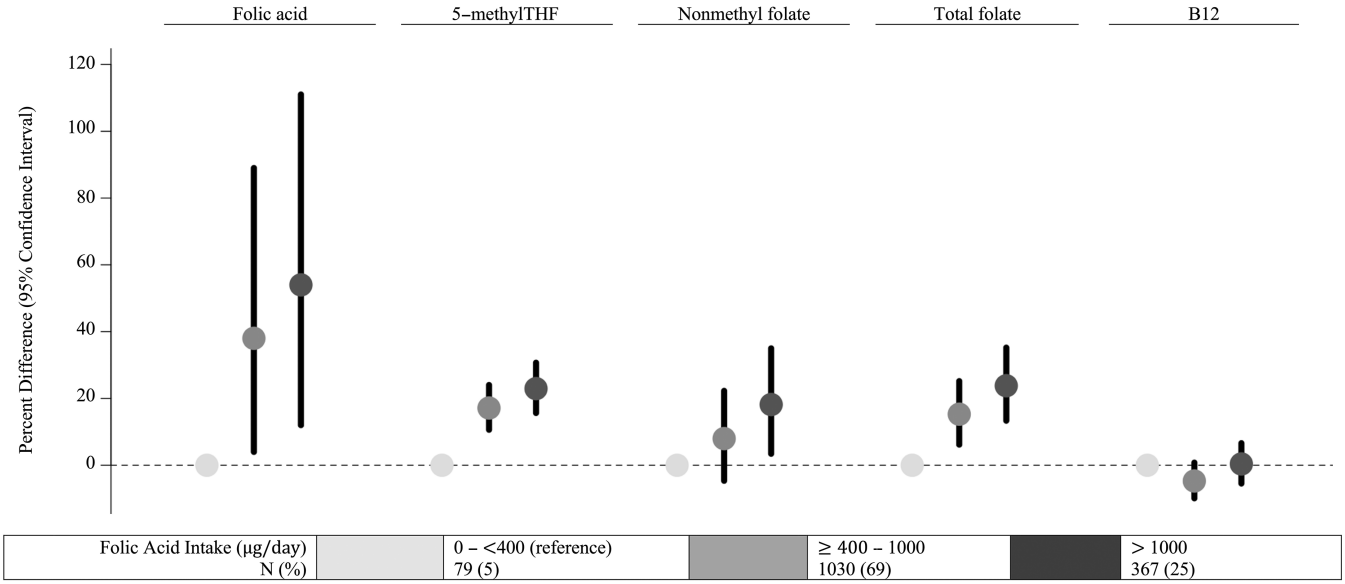

FIGURE 4 Adjusted percentage difference in plasma folate biomarkers (nmol/L) and vitamin B-12 (pmol/L) by categories of folic acid intake from supplements (μg/d) during pregnancy among MIREC study Participants. Models adjusted for maternal age in years (continuous), maternal education (categorical), maternal birth country (Canada vs. other), household income (category), parity (categorical), folic acid intake from supplements (categorical), gestational age (continuous), and visit. The sample included $n = 1553$ repeated measures from a total of $n = 1893$ participants, and folic acid intake from supplements was available among $n = 1476$. Non-methyl folate values are the sum of THF, 5-formylTHF, and 5,10-methenylTHF. Total folate values are the sum of the 5 folate vitamers: 5-methylTHF, folic acid, THF, 5-formylTHF, and 5,10-methenylTHF. Folic acid intake from supplements <400 μg/d serves as the reference group. Daily folic acid intake from supplements was based on maternal self-report on standardized questionnaires collected once after the first-trimester baseline visit. Participants who did not consume a folic acid supplement were assigned "0." Note, percentage difference values were calculated using linear mixed models with random effects to estimate associations between covariates and repeated plasma folate concentrations, where separate models were developed for each folate biomarker. B12, vitamin B-12; MIREC, Maternal–Infant Research on Environmental Chemicals; THF, tetrahydrofolate.

concentrations (–5%; 95% CI: –11%, 1%) (**Supplemental Table 8**). Compared with those not receiving fertility treatments, those receiving fertility treatments also tended to have lower total folate concentrations (–7%; 95% CI: –13%, 0%). We did not observe evidence that smoking status or time to pregnancy were associated with concentrations of plasma folate.

Discussion

The goal of mandatory FA fortification is to increase folate intake and reduce the risk of NTDs; the success of this policy is clear with the prevalence of NTDs in the United States and Canada dropping by 28% (anencephaly and spina bifida) and 46% (anencephaly, spina bifida, and encepahlocele), respectively (26–28). While most individuals in the general population who could become pregnant in Canada achieve a folate status associated with maximal NTD risk reduction, approximately 25% do not, primarily non–FA-supplement users (7, 29). As such, it remains prudent to recommend FA supplements in the preconceptional period. The recommended supplemental FA dose in Canada for individuals at low risk of an NTD-affected pregnancy is 0.4 mg/d (30), but the majority of daily prenatal supplements contain 1 mg FA (18), and many individuals without indications for high NTD risk are nevertheless consuming high-dose FA (6). Here we show that almost 100% of individuals consuming FA supplements, regardless of dose, achieved a folate status in the first trimester associated with maximal NTD risk reduction.

Determinants of FA supplement intake and folate status were similar in this cohort, as observed in previous studies (7, 29, 31). Generally, participants with higher indicators of socioeconomic status were more likely to consume higher-dose supplemental FA and had higher plasma total folate concentrations in both the first and third trimesters. Older age was associated with higher plasma total folate. Nulliparous participants were also more likely to consume FA supplements and had higher plasma total folate than those who were multiparous. Finally, participants with a higher prepregnancy BMI had lower plasma total folate in the third trimester. However, it must be emphasized that, in the context of mandatory fortification, essentially everyone in this cohort had a folate status associated with lower NTD risk, even with lower FA supplement doses.

The critical period for attaining an optimal folate status is in the periconceptional period (3 mo before conception and first 3 mo of pregnancy) because the NTD closes in the first few weeks of pregnancy. In the first trimester of pregnancy, >99% of MIREC participants met or exceeded the folate status cut-off of 25.5 nmol/L associated with maximal NTD risk reduction, as measured by plasma total folate (10); this was consistent across supplemental FA-dose groups. Our findings are similar to other studies of folate status biomarkers in pregnancy among Canadians. In an ancillary study of the Folic Acid Clinical Trial, a randomized controlled trial estimating the effect of high-dose FA supplements on pre-eclampsia, all participants regardless of dose group (≤1.1 mg vs. 4.0–5.1 mg/d FA) exceeded the WHO RBC folate cutoff for maximal NTD risk reduction (22). This suggests that, in the context of FA fortification, higher FA doses (e.g., above over-the-counter levels) are unnecessary unless an individual meets the definition of being at higher risk of having an NTD-affected pregnancy (19).

TABLE 3 Adjusted percent difference in plasma folate biomarkers (nmol/L) and vitamin B-12 (pmol/L) during pregnancy by covariates among MIREC study participants[1]

		Percent difference (95% CI)				
Variable	*n* (%)	Folic acid	5-MethylTHF	Non-methyl folate[2]	Total folate[3]	Vitamin B-12
Maternal age (y)[4]		12 (4, 20)	5 (3, 6)	8 (5, 11)	7 (4, 9)	0 (−2, 1)
Education						
<High school	166 (9)	(ref)	(ref)	(ref)	(ref)	(ref)
Some college	99 (5)	− 37 (−58, −6)	1 (−6, 9)	− 10 (−24, 7)	− 5 (−15, 6)	− 5 (−12, 3)
College/trade school	448 (24)	− 8 (−32, 25)	0 (−6, 6)	1 (−11, 15)	− 2 (−10, 7)	− 3 (−9, 2)
University degree	1178 (62)	− 2 (−27, 32)	3 (−3, 9)	− 5 (−16, 8)	0 (−8, 9)	− 2 (−7, 4)
Mother born in Canada						
Yes	1544 (82)	(ref)	(ref)	(ref)	(ref)	(ref)
No	349 (18)	− 13 (−27, 5)	2 (−2, 5)	− 6 (−13, 1)	1 (−4, 6)	3 (−1, 6)
Household income (Canadian dollars)						
<30,000	141 (8)	(ref)	(ref)	(ref)	(ref)	(ref)
30–80,000	571 (32)	9 (−20, 48)	10 (4, 17)	− 4 (−15, 10)	8 (0, 18)	7 (1, 14)
>80,000	1099 (61)	5 (−23, 43)	12 (5, 19)	− 7 (−18, 6)	9 (0, 19)	13 (7, 20)
Parity						
Nulliparous (0)	840 (44)	(ref)	(ref)	(ref)	(ref)	(ref)
1	764 (40)	− 30 (−39, −18)	− 10 (−13, −7)	− 6 (−12, 0)	− 12 (−16, −9)	− 2 (−5, 0)
≥2	289 (15)	− 28 (−42, −11)	− 13 (−17, −10)	− 16 (−23, −8)	− 15 (−20, −10)	− 2 (−6, 2)
Gestational age (wk)[5]		− 3 (−13, 8)	0 (−1, 1)	2 (−3, 7)	0 (−3, 2)	− 1 (−3, 2)

[1]Models adjusted for maternal age in years (continuous), maternal education (categorical), maternal birth country (Canada vs. other), household income (category), parity (categorical), folic acid intake from supplements (categorical), gestational age (continuous), and visit. The sample included $n = 1553$ repeated measures from a total of $n = 1893$ participants. Note: results for folic acid intake from supplements are displayed in Figure 4. Daily folic acid intake from supplements was based on maternal self-report on standardized questionnaires collected once after the first-trimester baseline visit. Participants who reported no folic acid supplement use were assigned "0." Note: percent difference values were calculated using linear mixed models with random effects to estimate associations between covariates and repeated plasma folate concentrations, where separate models were developed for each folate biomarker. MIREC, Maternal–Infant Research on Environmental Chemicals; ref, reference; THF, tetrahydrofolate.

[2]Non-methyl folates: sum of THF, 5-formylTHF, and 5,10-methenylTHF.

[3]Total folate—sum of the 5 folate vitamers: 5-methylTHF, folic acid, THF, 5-formylTHF, and 5,10-methenylTHF.

[4]Maternal age was assessed continuously and scaled to the SD (5.1 y).

[5]Gestational age at the time when each plasma sample was mean centered and was modeled continuously in weeks as a linear and time-varying covariate.

While there is a focus on NTD risk reduction and FA supplementation in the periconceptional period, folate requirements remain higher throughout pregnancy to support fetal growth and development beyond neural tube closure (4, 19, 30). As such, Health Canada recommends the consumption of a multivitamin that contains 400 μg FA throughout pregnancy (and lactation) (19, 30). We observed that MIREC participants had a folate status in the third trimester similar to that observed in the first trimester. There is no cutoff for folate status associated with optimal fetal growth beyond neural tube closure, but our data suggest that the status of these pregnant Canadians was likely sufficient throughout pregnancy given how few participants had plasma total folate concentrations below the cutoff for NTD risk reduction.

Ideally, an optimal folate intake and status can be achieved that reduces NTD risk without increasing FA intake beyond the UL (1000 μg FA/d). In the absence of FA supplement consumption, most Canadians do not consume the recommended amount of folate from diet alone, even with fortification (6, 32–34). As such, it remains prudent to maintain the recommendation for those who could become pregnant to consume an FA supplement. However, the vast majority of prenatal supplements available over-the-counter in Canada contain 1000 μg FA (18), as was evident among the MIREC participants who mostly consumed 1000 μg supplemental FA. In addition, approximately 25% of participants who reported consuming supplemental FA consumed >1000 μg. Given that Canada has mandatory FA fortification, all of these participants (assuming consumption of some fortified food products) would have a combined FA intake above the UL, consistent with other studies (6, 32–34).

Gaps exist regarding potential adverse effects of high FA intake and there is no definition for "excessive" FA intake (14, 35). Nevertheless, the appearance of UMFA in the circulation suggests supraphysiological FA intake (36). Plasma total folate represents the combination of multiple forms of circulating folate (i.e., 5-methylTHF, non-methyl folates, and UMFA). 5-MethylTHF made up the largest proportion of circulating first-trimester total folate in MIREC participants (83%), which is comparable to the general US population (87%) (37). Among MIREC participants, UMFA represented a larger relative contribution to plasma total folate than expected (15% and 30% in the first and third trimesters, respectively), higher than estimates from the general US population (∼10%) (37). We speculate that these differences reflect the relatively high prevalence of high-dose supplemental FA consumption within MIREC and suggest FA supplement consumption among these relatively healthy participants was higher than required.

In addition to folate, vitamin B-12 plays an important role in fetal and neonatal development. Vitamin B-12 inadequacy has been associated with increased NTD risk (38). In addition,

maternal vitamin B-12 status in pregnancy determines early postnatal status in the neonate, with implications for vitamin B-12 deficiency–associated anemia (39, 40). We noted a decrease in status in the third trimester, similar to previous observations that vitamin B-12 status indicators are lower as pregnancy proceeds (41). However, MIREC participants generally had adequate vitamin B-12 status (>220 pmol/L) in the first trimester, suggesting that risk of a vitamin B-12–dependent NTD was likely low.

Our study has a number of limitations and strengths. The MIREC participants provided only 1 measure of self-reported FA intake from supplements. Supplement intake ascertainment occurred shortly after the first-trimester visit and long before the third-trimester blood collection. It is possible that maternal FA intake from supplements changed over the course of the pregnancy, thus resulting in misclassification. The food-frequency questionnaire used for MIREC was not validated for folate, limiting our ability to take into consideration dietary folate intake; based on a previous analysis of a subset of MIREC participants, we assume that baseline folate intakes were likely similar among the FA-dose groups (17). There were also very few participants who reported consuming no supplements, and of those who did, the majority consumed 1 mg/d, limiting comparisons among FA-dose groups. Maternal plasma folate samples were nonfasting samples that could contribute to higher within-participant variability in folate vitamer concentrations. In addition, plasma samples were stored at –80°C for 8–11 y before analysis for the current study; therefore, some degree of folate degradation would be expected. A previous study showed that the major folate forms, 5-methylTHF and folic acid, as well as MeFox, in serum pools decreased ≤11% when stored up to 11 y at –70°C (42), whereas 5-formylTHF and 5,10-methenylTHF decreased <12% and THF decreased 20% in serum stored up to 6 y. The folate concentrations in our participants were much higher than that for the pooled samples in the CDC study (42), which might impact the rate of degradation, but this is not known. Despite potential degradation, this would not necessarily impact the rank order of folate concentrations among the MIREC participants, nor the range (difference between minimum–maximum) (43). If anything, our data would underestimate the number of participants meeting the cutoff for NTD risk reduction (10). Finally, as has been previously reported (17), the MIREC cohort was biased toward older females with higher socioeconomic status, which may make our findings less generalizable to populations who might be at higher risk of lower folate status.

There are also strengths to this study. The MIREC study includes a large and well-characterized sample of participants with a broad range of FA intakes and status biomarker concentrations, allowing for an in-depth assessment of the determinants of folate vitamer concentrations in both the first and third trimesters of pregnancy. The use of the LC-MS/MS method allowed for an examination of the relations among individual folate vitamers and supplemental FA dose, which may be useful in defining supraphysiological intakes of FA. The use of this method also facilitates the comparison of findings from other studies, such as the NHANES, which have used the same method of folate analysis.

In conclusion, our findings provide insight into the association between supplemental FA intake and folate status in pregnancy. Over 99% of MIREC participants had a plasma total folate status indicative of maximal NTD risk reduction, regardless of FA supplement dose. UMFA was detected in all participants with higher concentrations among those consuming high FA doses. In the context of a population with mandatory FA fortification, our data indicate that higher-than-recommended FA doses are unwarranted, with the exception of individuals at higher risk of NTDs. Ideally, prenatal supplements would contain 400 rather than 1000 μg FA, thereby enabling the consumption of optimal and safe FA doses.

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The authors' responsibilities were as follows—AJM and JMB: conceptualized the project; TEA: contributed to study design and oversight; AJM: was responsible for the folate and vitamin B-12 analyses; MAP: analyzed the data and performed the statistical analyses and drafted the manuscript; AJM: had primary responsibility for final content; and all authors: contributed to the final version and read and approved the final manuscript.

Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request in a de-identified form pending review and approval of a formal request to MIREC (https://www.mirec-canada.ca/en/).

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