Folate-status response to a controlled folate intake in nonpregnant, pregnant, and lactating women[1–4]

Allyson A West, Jian Yan, Cydne A Perry, Xinyin Jiang, Olga V Malysheva, and Marie A Caudill

ABSTRACT

Background: Folate dose-response studies in women of childbearing age who consumed a folic acid (FA)–containing multivitamin in the era of FA fortification are lacking.

Objective: We sought to investigate folate-status response to a known folate dose comprising an FA-containing prenatal supplement (750 μg/d) plus natural food folate (400 μg/d) in third-trimester pregnant women, lactating women 5–15 wk postpartum, and nonpregnant women.

Design: Pregnant ($n = 26$), lactating ($n = 28$), and nonpregnant ($n = 21$) women consumed the study folate dose under controlled intake conditions for 10–12 wk. Blood, urine, and breast milk were collected at baseline, study midpoint, and study end.

Results: Study-end serum total folate concentrations averaged ∼30 ng/mL and did not differ by physiologic group ($P = 0.876$). Study-end urinary folate excretion represented ∼9–43% of total folate intake and ranged from 100 to 500 μg/d. Third-trimester pregnant women excreted less urinary folate than did lactating ($P = 0.075$) and nonpregnant ($P < 0.001$) women. Lactating women excreted less ($P < 0.001$) urinary FA than did nonpregnant women. Breast-milk total folate concentrations remained constant ($P = 0.244$; 61.8 ng/mL at study end), whereas breast-milk FA concentrations increased ($P = 0.003$) to 24.1 ng/mL at study end.

Conclusions: The consumption of the study folate dose yielded a supranutritional folate status regardless of the physiologic state. Based on urinary folate excretion, folate use was greatest to least: pregnant > lactating > nonpregnant women. Breast-milk folate species were responsive to maternal folate intake, and FA made up ∼40% of breast-milk total folate at study end. These findings warrant revisiting prenatal supplement FA formulation in populations exposed to FA-fortification programs. This trial was registered at clinicaltrials.gov as NCT01127022. *Am J Clin Nutr* 2012;96:789–800.

INTRODUCTION

Folate refers to a group of related molecules with essential roles in cellular methylation, nucleotide biosynthesis, and amino acid metabolism. Folic acid (FA)[5] is a synthetic oxidized form of folate added to fortified foods and used in dietary supplements. In contrast, the majority of natural food folate is reduced, has a polyglutamate tail, and is ∼40% less bioavailable than FA. In the United States, folate requirements are met through natural food folate, FA-fortified foods, and folate or FA-containing vitamin supplements (1). To account for differences in bioavailability, folate recommended intakes are given in dietary

folate equivalents (DFEs) for which 1 μg natural food folate and 1 μg FA are equivalent to 1 DFE and 1.7 DFEs, respectively (2).

It is well established that pregnancy and lactation increase the demand for folate; current Recommended Dietary Allowances (RDAs) for nonpregnant, pregnant, and lactating women are 400, 600, and 500 DFEs/d, respectively (2). The Institute of Medicine and CDC also recommend that women of childbearing age consume 400 μg FA/d to decrease the incidence of neural tube birth defects (2, 3). In 1996 the FDA mandated that enriched grain products be fortified with FA to facilitate the achievement of this recommended intake (4). As a result of FA fortification, folate status in women of childbearing age has dramatically improved (5), and the incidence of neural tube birth defects has decreased 19–50% (6, 7).

Multivitamins are the most common dietary supplement consumed in the United States (8). Most multivitamins contain 400 μg FA; however, prenatal multivitamin supplements often contain 800–1000 μg FA (9). Women of childbearing age are advised to consume 400 μg FA/d through a combination of FA-fortified food and supplements (2); however, no explicit public health recommendation for healthy pregnant or lactating women to consume FA-containing supplements currently exists (10).

NHANES 2003–2006 data and Gallup polling for the March of Dimes indicated that 30–38% of women of childbearing age

consume FA-containing supplements or multivitamins (8, 11). Research has shown that 77–93% of pregnant (12–14) and 73% of lactating (14) women use multivitamins; however, the accurate prevalence is unknown in lactating women because this estimate does not come from a nationally representative study (10).

Folate dose-response studies are lacking in third-trimester pregnant and lactating women. In addition, there has not been a comprehensive longitudinal assessment of folate status achieved by women of childbearing age who consume an over-the-counter FA-containing prenatal supplement in the era of FA fortification. This study sought to quantify the folate-status response to a known dose of folate comprising an FA-containing prenatal supplement plus natural food folate ($\sim$400 μg DFEs) in third-trimester pregnant women, lactating women 5–15 wk postpartum, and nonpregnant women of childbearing age.

SUBJECT AND METHODS

Study participants

Nonpregnant, third-trimester pregnant (27 wk gestation), and lactating women 5 wk postpartum aged $\geq$21 y were recruited from the Ithaca, New York, area from January 2009 to October 2010 as described by Yan et al (15). During the screening stage, interested women provided a blood sample for blood chemistry profiling and a complete blood count. In addition, these women completed a health history and demographics questionnaire. Important inclusion criteria for all participants were as follows: general healthiness as determined by the questionnaire, blood chemistry profile, and complete blood count; no drug or alcohol use; normal kidney and liver function; and willingness to comply with the study protocol (ie, eating a certain number of meals at the on-site location and not consuming food or beverages outside what was provided in the study). Additional inclusion criteria for pregnant women included a singleton pregnancy and no pregnancy-associated complications (eg, preeclampsia or gestational diabetes). An additional criterion for lactating women was the intention to exclusively breastfeed for the duration of the study. Exclusion criteria included the inability to comply with study protocols and use of prescription medications known to affect liver function. Eligible pregnant women were admitted to the study on a rolling basis at 26–29 wk gestation, eligible lactating women were admitted to the study on a rolling basis at the start of 5 wk postpartum, and eligible nonpregnant women were added as scheduling and space constraints allowed until the desired number of participants completed the study.

The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and the Cayuga Medical Center (the hospital where pregnant participants delivered their babies). Informed consent was obtained from all participants before their entry into the study.

Study design, diet, and supplements

Design

This study was an extension of a controlled feeding study conducted in pregnant, lactating, and nonpregnant women who were randomly assigned to consume 480 or 930 mg choline/d for 10–12 wk (15). Throughout the controlled-consumption period (10–12 wk), all participants consumed a daily over-the-counter prenatal multivitamin supplement (Pregnancy Plus; Fairhaven Health LLC) that contained 750 μg FA, plus natural food folate ($\sim$400 μg/d) from a mixed diet. Nonpregnant and pregnant women participated in the controlled-consumption study for 12 wk, whereas lactating women participated in the study for 10 wk. Biological samples, including blood, urine, and breast milk, were collected at baseline, study midpoint (week 6), and study end (week 10 or 12).

Diet

The study diet, as previously described (15), provided $\sim$2000 kcal/d; however, participants could individualize their caloric intake by choosing to consume more or less of menu items and foods that contained <5 μg folate/serving, such as unenriched rice, selected juices and sodas, chips, and pudding. Nonpregnant and pregnant women consumed meals of their choosing on $\geq$5 d/wk in the Francis A Johnston and Charlotte M Young Human Metabolic Research Unit (HMRU) at Cornell University. Lactating women consumed meals of their choosing on $\geq$3 d/wk in the HMRU. All other food and beverages were provided as take-aways.

To control FA intake, commercial items manufactured without FA (eg, whole-wheat bread and organic specialty pasta) were used, and menu items prepared in the HMRU (eg, pizza dough and muffins) used unenriched wheat flour. Thus, dietary folate intake was restricted to naturally occurring forms with the only source of FA being the study prenatal supplement.

Supplements

Each day, study participants consumed an over-the-counter prenatal supplement (Pregnancy Plus; Fairhaven Health LLC). Although this prenatal supplement was labeled to contain 600 μg FA per tablet, the measured amount was 750 μg FA (see Analytic measurements for details on methodology) and was the value used throughout this article. To meet recommendations (16), study participants also consumed a 200-mg docosahexaenoic supplement/d (Neuromins; Nature's Way Products) and a thrice-weekly potassium and magnesium supplement (General Nutrition Corp). When eating on site, participants consumed supplements under the supervision of study personnel. For days when study participants did not eat on site, supplements were provided in baggies along with the take-away meals of subjects. Participants were instructed to consume the supplements with a meal of their choice.

Compliance

The study protocol was well tolerated, and 91% of enrolled participants completed the study (21 of 22 nonpregnant, 26 of 29 pregnant, and 28 of 31 lactating women). Reasons for stopping the study included nausea, early delivery, personal challenges, and food dislikes (15).

Study participants completed daily checklists that indicated that they had received and consumed all menu items and supplements. For meals consumed on site, study personnel were able to directly monitor compliance. For meals consumed off site, participants were asked to return all empty baggies and take-away

food containers to study personnel during their next visit to the HMRU. In addition, study personnel had daily contact with participants throughout the study to maintain positive rapport and enhance compliance.

Sample collection and processing

Blood

Fasting venous blood was drawn at baseline (all participants) and study weeks 6 (all participants), 10 (all participants), and 12 (nonpregnant and pregnant participants) in the HMRU ward by a trained phlebotomist. Blood samples were collected in EDTA-containing and serum separator tubes, processed within 2 h, and stored in cryostat tubes at $-80°$C until analysis as previously described (15). Ascorbic acid (10 g/L) was added to whole blood and serum samples during processing to preserve labile folates.

Urine

Participants completed 24-h urine collections at baseline (all participants) and study weeks 6 (all participants), 9 (nonpregnant and pregnant), 10 (lactating), and 12 (nonpregnant and pregnant) in acid-washed opaque 2-L bottles as previously described (15). To preserve labile urinary folates, 5 g sodium ascorbate was added to each bottle ahead of collection.

Breast milk

Breast-milk samples were collected at baseline and study weeks 6, 9, and 10 with a Medela electric breast pump (Medela Inc) in the HMRU. Lactating women were fasted for breast-milk sample collection that occurred on the same morning as the corresponding week's blood collection. Breast-milk samples consisted of the full expression of one breast 2 h after the first feed of the day. Women expressed the same breast throughout the study. On collection, breast-milk samples were immediately placed on ice, dispensed within 30 min of collection into 4.5-mL cryostat vials (Cryo; NUNC), and stored at $-80°$C until analysis.

Analytic measurements

Food and breast-milk folate extracts

Natural food folate provided by the study menu (dietary folate) and breast-milk folate concentrations were quantified from extracts prepared by using the trienzyme method. Blank extracts were prepared from extraction buffer subjected to trienzyme digestion to quantify folate in enzyme preparations. All incubations were performed at $37°$C.

For dietary folate extraction, prepared study meals were homogenized with cold 0.1 mol/L potassium phosphate buffer with 57 mmol/L ascorbic acid and stored at $-80°$C. On thawing, meal samples (2 g) were mixed with extraction buffer [0.05 mol/L 2-(cyclohexylamino)ethanesulfonic acid, 0.05 mol/L HEPES, 0.1 mol/L sodium ascorbate, and 0.2 mol/L 2-mercaptoethanol] and subjected to trienzyme digestion as described by Tamura et al (17). After digestion, sample extracts were filtered through Whatman paper (Fisher Scientific) and stored at $-80°$C until quantification.

Breast-milk sample extractions were prepared by using the method of Lim et al (18) with the following modifications. First,

samples were mixed with 10 mg/mL sodium ascorbate and extraction buffer on thawing. Second, after protease inactivation, pH was adjusted to 7.2, and samples were incubated for 5 h with rat plasma conjugase (500 μL/mL breast milk). Finally, after conjugase incubation, samples were centrifuged at $4°$C, filtered with 0.45-μm syringe filters (MillexHV; Millipore Corp), and stored at $-80°$C. Breast-milk extracts for liquid chromatography–tandem mass spectrometry (LC-MS/MS) and microbiological assay (MA) quantification were prepared separately. A stable-isotope internal standard (IS) mix was added to breast milk before enzyme digestion for samples destined for LC-MS/MS quantification. Extracts for LC-MS/MS and MA methods were prepared from 4 and 0.1 mL breast milk, respectively.

MA measurements

The MA method (19), with *Lactobacillus casei* (ATCC 7469) as the test organism and 10 ng FA/mL (Sigma) as the standard calibrator, was used to determine total folate concentrations in serum, whole blood, urine, breast milk, and dietary folate. CVs were calculated by using a positive quality-control sample of each analyte (ie, repeated measurement of control samples prepared for each biological material in our laboratory). The purpose of the positive quality-control sample was to ensure that the assay and calibrators were working correctly and to determine the precision of the assay. MA intraassay CVs were <10%. MA interassay CVs were as follows: <10% for serum, whole blood, and breast milk and <13% for urine and dietary folate.

Red blood cell (RBC) folate values for nonpregnant and pregnant women were calculated from whole blood folate concentrations, serum total folate concentrations, and hematocrit values. Lactating serum folate was not measured via MA; 5-methyltetrahydrofolate (5-methyl-THF) + FA measured via LC-MS/MS was used a proxy for serum total folate.

Dietary folate was determined twice, the first time by using meal extracts prepared before the start of the study and the second time by using meal extracts prepared during the study. Dietary and breast-milk folate values were corrected for exogenous folate (ie, folate from amylase and conjugase preparations added during trienzyme digestion) by subtracting blank-extract MA values.

LC-MS/MS measurements

5-Methyl-THF and FA were quantified in serum, urine, and breast milk by using LC-MS/MS stable-isotope dilution methods. Serum, urinary, and breast-milk extracts were prepared by using solid-phase extraction (SPE) clean-up methods on a 12-port vacuum manifold (JT Baker Inc). Extract eluents were concentrated with a centrifuge (SpeedVac Concentrator; Savant) under vacuum with no heat before injection.

Standard curves were prepared from 5-methyl-THF (Sigma) and FA (Sigma) stock solutions as described in Pfeiffer et al (20). National Institute of Standards and Technology (NIST) standard reference materials SRM 3280 (multivitamin tablets) and SRM 1955 (homocysteine and folate in frozen human serum) were used to validate the accuracy of FA and 5-methyl-THF calibrator curves, respectively. IS solutions were prepared by using ^{13}C-5-methyl-THF and ^{13}C-FA (Merck Eprova) as described by Pfeiffer et al (20). CVs were calculated by using positive quality-control

samples included with each extraction batch. Extraction batches included all time points (ie, baseline, week 6, and study end) for 4–10 participants. Details specific to the extraction method for each biological material are detailed below.

Serum

5-Methyl-THF and FA were extracted from 400 μL serum by using the SPE clean-up method of Pfeiffer et al (20). Serum 5-methyl-THF and FA were quantified via LC-MS/MS (20) with modifications that were based on our instrumentation. The LC-MS/MS system used to quantify serum folates was a TSQ Quantum mass spectrometer (Thermo) equipped with a refrigerated Accela autosampler (Thermo) and Accela pump with degasser (Thermo). 5-Methyl-THF and FA were separated with a Luna C18 column (250 × 4.6 mm, 5-μm bead size; Phenomenex) and matching guard column (4 × 3 mm) by using a gradient mobile phase (500 μL/min) that consisted of solution A (10 mL acetic acid/L, 400 mL methanol/L, and 100 mL acetonitrile/L) and solution B (0.15% formic acid). During the first 7 min of the run, a linear gradient brought the mobile phase from 50% to 100% solution A. This was followed by a linear gradient that returned the mobile phase to 50% solution A at minute 12. From minutes 12 to 15, the column was re-equilibrated under isocratic conditions with 50% solution A. Retention times for 5-methyl-THF and FA were 5 and 9 min, respectively. The mass spectrometer was operated with electrospray ionization in positive-ion mode with a multiple-reaction monitoring mode of the following transitions: for 5-methyl-THF, m/z 460 → m/z 313; for ^{13}C-5-methyl-THF, m/z 465 → m/z 313; for FA, m/z 442 → m/z 295; and for ^{13}C-FA, m/z 447 → m/z 295. The total run time was 15 min, the injection volume was 15 μL, and the autosampler and column temperatures were set at 10°C and 25°C, respectively. Intraassay and interassay CVs were 3.3% and 3.9%, respectively, for serum 5-methyl-THF and 8.5% and 13.7% for serum FA.

Because of the limited availability of ascorbic acid–preserved serum (400–500 μL) and the small amount of FA present, a limit of detection (LOD) and a limit of quantification (LOQ) were defined. With the use of serially diluted serum samples and a noise-to-signal ratio of 15, the serum FA LOD was set at 0.075 ng/mL. The intraassay CV for samples <0.11 ng/mL was 19.2%, whereas the CV for samples with concentrations >0.11 ng/mL was 8.5%; thus, the serum FA LOQ was set at 0.11 ng/mL.

Urine

5-Methyl-THF and FA were extracted from 3 mL urine by using an adaptation of the phenyl SPE method of Pfeiffer et al (20). Briefly, urine samples were spiked with a ^{13}C-5-methyl-THF and ^{13}C-FA IS preparation and mixed with 3 mL sample buffer (10 g ammonium formate/mL and 1 g ascorbic acid/L). Each sample was brought to pH 2.9 and allowed to equilibrate for 20 min at 20°C. Urine samples and calibrator points were cleaned up with 1-mL phenyl cartridges (100 mg, Bond Elut C18; Agilent) conditioned with 2 mL each of acetonitrile, methanol, and sample buffer (pH 2.9). After samples were loaded, cartridges were washed with 3 mL wash buffer (0.5 g ammonium formate/L and 0.05 g ascorbic acid/L; pH 3.4), and folates were eluted with 500 μL elution buffer (400 mL methanol/L, 100 mL acetonitrile/L, 10 mL acetic acid/L, and 1 g ascorbic acid/L).

The LC-MS/MS ion-trap system used to quantify urinary folates consisted of an LCQ Advantage Max mass spectrometer (Thermo) equipped with a refrigerated Surveyor autosampler (Thermo) and Surveyor pump with degasser (Thermo). Urinary 5-methyl-THF and FA were separated by using the same column and gradient system as for serum folates previously described. Retention times for 5-methyl-THF and FA were 5 and 8 min, respectively. The mass spectrometer was operated with electrospray ionization in negative-ion mode with full-scan parameters that monitored the following transitions: for 5-methyl-THF, m/z 458 → m/z 329; for ^{13}C-5-methyl-THF, m/z 463 → m/z 329; for FA, m/z 440 → m/z 311; and for ^{13}C-FA, m/z 445 → m/z 311. The total run time was 15 min, the injection volume was 10 μL, and the autosampler and column temperatures were set at 10°C and 25°C, respectively. Intraassay and interassay CVs were 7.3% and 10.1%, respectively, for urinary 5-methyl-THF and 10.4 and 11.6% for urinary FA.

Breast milk

5-Methyl-THF and FA were extracted from 4 mL breast milk by using a strong anion exchange (SAX) SPE cleanup method that was based on the methods of Vishnumohan et al (21) and Friesleben et al (22). Briefly, trienzyme digested breast-milk extracts with ^{13}C-5-methyl-THF and ^{13}C-FA IS as described previously were adjusted to pH 7.5 and cleaned up with 3 mL SAX cartridges (500 mg; Bond Elut SAX; Agilent) conditioned with 3 mL each of hexane, methanol, and purified water and 10 mL conditioning solution (0.01 mol/L potassium phosphate, 0.1% ascorbic acid, and 0.01% 2-mercaptoethanol; pH 7.5). After samples were loaded, cartridges were washed with 3 mL conditioning solution, and folates were eluted with 1 mL elution buffer (5% sodium chloride, 1% sodium ascorbate, and 0.1 mol/L sodium acetate; pH 5.4).

Breast-milk folates were separated and quantified by using the same Luna column and LC-MS/MS system as for serum folates as previously described. However, the mobile phase had to be modified to wash SAX elution-buffer salts from breast-milk extracts (22). After 10 min of isocratic 7% solution A, a 3-min linear gradient brought the mobile phase to 13% solution A. Then, a 4-min linear gradient brought the mobile phase to 30% solution A followed by a second 4-min linear gradient that brought the mobile phase to 90% solution A. A third 4-min linear gradient brought the mobile phase back to 7% solution A. Finally, the column was reequilibrated under isocratic conditions with 7% solution A for 10 min. The retention times for 5-methyl-THF and FA were 22 and 25 min, respectively, and the total run time was 35 min. Breast-milk folate concentrations were corrected for exogenous folate added during trienzyme digestion by subtracting blank-extract LC-MS/MS values. Intraassay and interassay CVs were 10.0% and 12.5%, respectively, for breast-milk 5-methyl-THF and 5.0% and 3.5%, respectively, for breast-milk FA.

Prenatal supplement FA

The FA content of the prenatal supplement was determined by using the LC-MS/MS negative-ion mode method described by Nelson et al (23). FA in the study prenatal supplement and NIST

SRM 3280 multivitamin were extracted by using the same protocol and quantified in the same LC-MS/MS run. The measured NIST 3280 FA concentration (412 mg FA/kg supplement) was consistent with the reference value published by Nelson et al (413 mg/kg) (23).

MTHFR genotyping

The MTHFR C677>T genotype was determined after purifying polymerase chain reaction products (QIAquick PCR Purification kit; Qiagen) (24) and sequencing the double-stranded DNA templates with an Applied Biosystems Automated 3730 DNA analyzer (Applied Biosystems).

Statistical methods

Normality was not achievable in several dependent variable baseline concentrations, and thus, Kruskal-Wallis H and Mann-Whitney U tests were used to test baseline differences in folate status marker concentrations by physiologic group. Kruskal-Wallis H tests were also used to test for differences in ethnicity/race, MTHFR C677>T genotype, choline-intake random assignment, and supplement use before study-enrollment distribution in physiologic groups.

Linear mixed models (LMMs) were used to investigate folate-status response over time when dependent variables or transformed dependent variables conformed to model assumptions. Log-transformed serum 5-methyl-THF, log-transformed serum 5-methyl-THF + FA, square-root transformed urinary 5-methyl-THF, square-root transformed urinary total folate, and log-transformed RBC folate were assessed by using LMMs. Physiologic state (nonpregnant, pregnant, or lactating), time (study week), and choline intake (480 or 930 mg/d) were entered as fixed factors and subject identification was entered as random factor. Because of varying supplement use within and among physiologic groups before study entry, supplement use before study enrollment was entered as fixed factor. In addition, because the MTHFR C677>T genotype can have robust effects on folate requirements (25, 26), and ethnicity may affect folate status (27), both variables were included as fixed factors in the LMMs. In the initial model, all 2-way interactions between fixed factors were included. Nonsignificant interactions were progressively removed until final models were derived. Bonferroni corrections were made for multiple comparisons where applicable. All breast-milk folate variables were square-root transformed and analyzed with LMMs as previously described without the physiologic group fixed factor.

To compare physiologic groups and assess the response over time in variables that did not conform to LMM assumptions, nonparametric analyses were used. Kruskal-Wallis H and Mann-Whitney U tests were performed with baseline, week 6, and study-end concentrations as the test variable and physiologic state as the grouping variable. Wilcoxon rank-sum tests stratified by physiologic state were used to assess the change over time within and among physiologic groups. Serum FA and urinary FA were analyzed with nonparametric techniques. A portion of the serum FA values fell between the LOD and LOQ; concentrations for these samples were set equal to the LOQ (0.11 ng/mL) for statistical analyses. All statistics were performed with IBM SPSS software (version 19; SPSS Inc).

RESULTS

Participant characteristics and baseline measurements

Seventy-five women were included in the final analyses. Twenty-one nonpregnant women completed 12 wk of the study. Twenty-three pregnant women completed 12 wk of the study, and 3 women completed 10 wk of the study. Twenty-five lactating participants completed 10 wk of the study, 2 participants completed 9 wk of the study, and one participant completed 8 wk of the study. The study-end time point was used in all statistical analyses and reflected the last sample collection for each study participant. The ethnicity/race of study participants and MTHFR C677>T genotype and choline supplement random assignment were balanced by physiologic group (**Table 1**).

At baseline, 85%, 75%, and 33% of pregnant, lactating, and nonpregnant participants, respectively, reported the consumption of FA-containing supplements ($P = 0.001$; Table 1). The differing rates of supplement use before study enrollment corresponded to differences in baseline folate status marker concentrations with pregnant and lactating participants having, on average, higher serum folate, greater urinary folate excretion, and higher RBC folate than did nonpregnant participants (Table 1).

Folate dose

The average intake of natural food folate was 404 μg/d ($\sim$400 DFEs). The FA content of the prenatal multivitamin supplement was 750 μg FA, which yielded a total folate intake of 1150 μg/d (400 + 750 μg) or 1675 DFEs after adjustment for the 1.7$\times$ greater bioavailability of FA.

Serum folate

Serum folate is a sensitive indicator of recent folate intake and can be a valid indicator of folate status (2). 5-Methyl-THF is the primary form of folate found in circulation, whereas the presence and quantity of FA in fasted serum is of interest because of concerns regarding excess FA exposure (28).

Serum 5-methyl-THF

The consumption of the study folate dose (FA-containing prenatal supplement plus 400 μg natural food folate) yielded a small but significant increase (time: $P < 0.001$) in serum 5-methyl-THF over the course of the study that did not differ by physiologic group (physiologic group $\times$ time interaction: $P = 0.536$) after ethnicity, MTHFR C677>T genotype, choline intake, and supplement use before study enrollment were controlled for (**Figure 1**A). At study end, serum 5-methyl-THF concentrations did not vary by physiologic group ($P = 0.855$; Figure 1A).

Serum FA

The consumption of the study folate dose did not alter serum FA concentrations within physiologic groups ($P = 0.122–0.302$) or in the cohort of participants as a whole ($P = 0.398$) over the course of the study (Figure 1B). At study end, serum FA concentrations did not vary by physiologic group ($P = 0.251$; Figure 1B). Unmetabolized FA was present in fasted serum among

TABLE 1
Participant characteristics and baseline folate status marker concentrations in pregnant women at ∼27 wk gestation, lactating women ∼5 wk postpartum, and nonpregnant women of childbearing age[1]

	Pregnant ($n = 26$)	Lactating ($n = 28$)	Nonpregnant ($n = 21$)	P
Ethnicity (n)				0.804
White	16	20	14	
African American	1	1	2	
Hispanic	4	3	2	
Asian	4	1	1	
Other	1	3	2	
MTHFR genotype (n)				0.974
CC	11	11	9	
CT	13	15	9	
TT	2	2	3	
Choline intake (n)				0.916
480 mg/d	13	15	10	
930 mg/d	13	13	11	
Percentage of subjects who used an FA-containing supplement before study enrollment	85[a]	75[a]	33[b]	0.001
Serum folate (ng/mL)				
5-Methyl-THF	30.0 (24.4–34.7)[2,a]	31.4 (25.5–37.1)[a]	18.6 (14.7–23.7)[b]	<0.001
FA	0.41 (0.1–0.65)[a,b]	0.54 (0.42–0.69)[b,*]	0.32 (0.0–0.45)[a]	0.008
Percentage of subjects with detectable FA	73[a,b]	93[b,*]	67[a]	0.062
5-Methyl-THF + FA[3]	30.3 (24.9–35.6)[a]	33.7 (25.5–40.5)[a]	18.7 (15.2–23.7)[b]	<0.001
FA percentage of 5-methyl-THF + FA	1.5 (0.3–2.1)	1.9 (1.3–3.2)	1.5 (0.0–3.0)	0.222
Urinary folate excretion (μg/24 h)				
5-Methyl-THF	144.0 (39.7–411.9)[a]	53.5 (14.2–165.6)[b]	5.3 (3.2–32.5)[c]	<0.001
FA[4]	5.6 (0.0–89.0)[a]	5.0 (0.0–18.4)[a]	0.0 (0.0–0.0)[b]	0.012
Percentage of subjects with detectable FA	62[a]	57[a]	19[b]	0.008
Total folate	178.3 (84.0–475.9)[a]	113.4 (32.1–425.6)[a]	22.4 (10.0–65.8)[b]	<0.001
RBC folate (ng/mL)				
Total folate	916.1 (735.7–1019.5)[a]	1295.8 (1095.6–1504.7)[b]	512.4 (459.5–572.1)[c]	<0.001

[1] Data were analyzed by using Kruskal-Wallis tests. Different superscript letters within a row indicate a difference at $P < 0.05$ (Mann-Whitney test). *Difference for the pregnant group compared with the lactating group, $P < 0.1$. To convert nanograms of folate per milliliter to nanomoles of folate per liter, multiply nanograms per milliliter by 2.266. FA, folic acid; RBC, red blood cell; 5-methyl-THF, 5-methyltetrahydrofolate.

[2] Median; 95% CI in parentheses (all such values).

[3] Serum 5-methyl-THF + FA was used as a proxy for serum total folate because of the lack of serum microbiological assay measurement in the lactating group.

[4] Mean (±SD) urinary FA excretion at baseline was 62.5 ± 96.0 μg/24 h for pregnant women, 39.1 ± 67.8 μg/24 h for lactating women, and 11.2 ± 35.6 μg/24 h for nonpregnant women.

81%, 89%, and 81% of pregnant, lactating, and nonpregnant women, respectively, at study end.

The percentage of serum FA relative to serum 5-methyl-THF + FA (ie, serum total folate) was small and did not vary over the course of the study within physiologic groups ($P = 0.163–0.376$). However, the FA percentage of serum 5-methyl-THF + FA tended to decrease ($P = 0.058$) over the course of the study from 1.6% at baseline to 1.2% at study end in all participants; the FA percentage of serum 5-methyl-THF + FA did not vary by physiologic group ($P = 0.614$) at study end.

Serum 5-methyl-THF + FA

Similar to serum 5-methyl-THF, the consumption of the study folate dose increased (time: $P < 0.001$) serum 5-methyl-THF + FA (ie, total folate) over the course of the study and did not differ by physiologic group (physiologic group × time interaction: $P = 0.275$) after ethnicity, MTHFR C677>T genotype, choline intake, and supplement use before study enrollment were controlled for (Figure 1C). At study end, serum 5-methyl-THF + FA concentrations did not vary by physiologic group ($P = 0.893$; Figure 1C). The study folate intake yielded study-

end serum 5-methyl-THF + FA (ie, total folate) concentration estimates that exceeded 20 ng/mL (Figure 1C), which is an indicator of high-folate status (5).

Urinary folate

Twenty-four-hour urinary folate excretion is a useful folate status indicator because it reflects saturable intracellular retention and renal reabsorption mechanisms related to folate use and metabolism (29). In addition, in contrast to fasting serum folate, which reflects folate status at its lowest point, 24-h urinary folate excretion encompasses the rise and fall of circulating folate and is an indicator of average folate status throughout the 24-h interval (30). Urinary folate species of primary interest in the era of FA fortification (and in individuals who use FA-containing supplements) are 5-methyl-THF, which is the main form in circulation, and FA.

Urinary 5-methyl-THF

The consumption of the study folate dose yielded urinary 5-methyl-THF that differed by physiologic group (physiologic
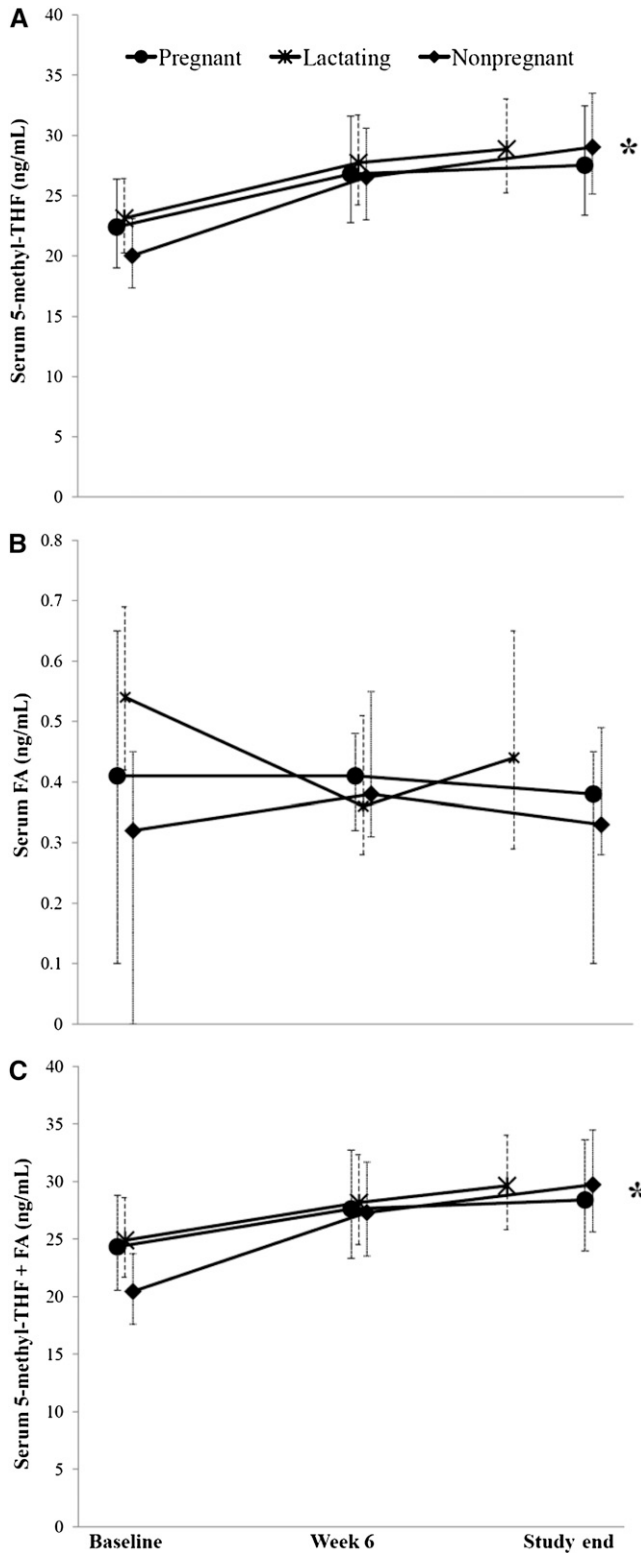
FIGURE 1. Serum concentrations of 5-methyl-THF (A), FA (B), and 5-methyl-THF + FA (C) in third-trimester pregnant women (n = 26), lactating women 5–15 wk postpartum (n = 28), and nonpregnant women of childbearing age (n = 21) who consumed an FA-containing prenatal supplement plus 400 μg natural food folate for 10–12 wk. Statistical analyses for 5-methyl-THF and 5-methyl-THF + FA were performed by using linear mixed models. Statistical analyses for FA were performed by using Kruskal-Wallis and Mann-Whitney tests. All participants were included in the analyses. Plotted data for 5-methyl-THF and 5-methyl-

group $\times$ time: $P < 0.001$) after ethnicity, *MTHFR* C677>T genotype, choline intake, and supplement use before study enrollment were controlled for. In lactating and nonpregnant women, urinary 5-methyl-THF excretion increased (both $P \leq 0.001$) over the course of the study (**Figure 2**A). However in pregnant women, urinary 5-methyl-THF did not change ($P = 1.0$) over the course of the study (Figure 2A). At study end, pregnant women were excreting less 5-methyl-THF than were lactating ($P = 0.006$) and nonpregnant ($P < 0.001$) women, in whom the amounts did not differ ($P = 0.251$) from each other (Figure 2A).

Urinary FA

The consumption of the study folate dose increased ($P < 0.001$) urinary FA excretion in nonpregnant women over the course of the study; however, FA excretion in pregnant and lactating women did not change ($P = 0.162–0.976$) over the course of the study (Figure 2B). At study end, pregnant and lactating women, who did not differ ($P = 0.941$) from each other, excreted less (both $P < 0.001$) urinary FA than did nonpregnant women (Figure 2B). Unmetabolized FA was present in 77%, 89%, and 100% of 24-h urine collections in pregnant, lactating, and nonpregnant women, respectively, at study end.
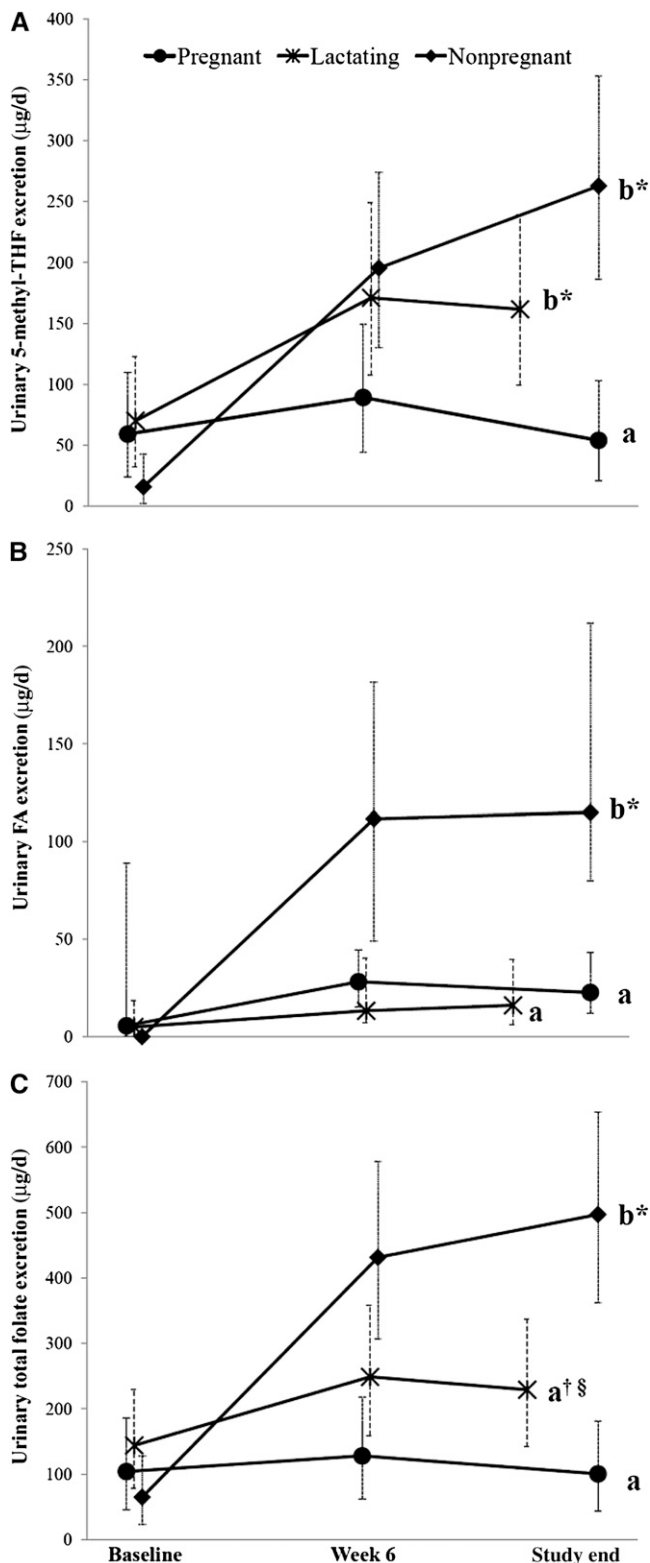
Urinary total folate

Similar to urinary 5-methyl-THF, the consumption of the study folate dose yielded urinary total folate excretion that differed by physiologic group (physiologic group $\times$ time: $P < 0.001$) after ethnicity, *MTHFR* C677>T genotype, choline intake, and supplement use before study enrollment were controlled for. In nonpregnant women, urinary total folate increased ($P < 0.001$) over the course of the study, and in lactating women, urinary total folate tended to increase ($P = 0.093$) over the course of the study (Figure 2C). However, in pregnant women, urinary total folate did not change ($P = 1.0$) over the course of the study (Figure 2C).

At study end, pregnant and lactating women excreted significantly less (both $P \leq 0.004$) total folate than did nonpregnant women, and pregnant women tended to excrete less ($P = 0.075$) total folate than did lactating women (Figure 2C). The study folate intake yielded substantial amounts of urinary folate at study end, with mean urinary total folate excretion equivalent to approximately 9%, 20%, and 43% of the daily total folate intake (1150 μg) for pregnant, lactating, and nonpregnant women, respectively.

Analyses with values adjusted with urinary creatinine excretion (31) to correct for possible incomplete 24-h urine collection

THF + FA are predicted geometric means (95% CIs) derived from linear mixed models; medians (95% CIs) are plotted for FA. Study end was week 10 for lactating participants and week 12 for pregnant and nonpregnant participants. Serum folate concentrations did not differ by physiologic group at study end, $P = 0.251–0.876$. *Serum folate concentrations increased from baseline to study end, $P < 0.001$. The physiologic state did not interact with time for serum 5-methyl-THF and 5-methyl-THF + FA concentrations ($P = 0.251–0.893$); the distribution of serum FA data did not allow this interaction to be tested. To convert nanograms of folate per milliliter to nanomoles of folate per liter, multiply nanograms per milliliter by 2.266. FA, folic acid; 5-methyl-THF, 5-methyltetrahydrofolate.

FIGURE 2. Urinary excretion of 5-methyl-THF (A), FA (B), and total folate (C) in third-trimester pregnant women ($n = 26$), lactating women 5–15 wk postpartum ($n = 28$), and nonpregnant women of childbearing age ($n = 21$) who consumed an FA-containing prenatal supplement plus 400 μg natural food folate for 10–12 wk. Statistical analyses for 5-methyl-THF and total folate were performed by using linear mixed models. Statistical analyses for FA were performed by using Kruskal-Wallis and Mann-Whitney tests. All participants were included in the analyses. Plotted data for 5-methyl-THF and total folate excretion are back-transformed predicted

produced the same results for all urinary folate variables (data not shown).

RBC folate

RBC folate is an indicator of long-term folate status and is less sensitive to acute changes in folate intake (2).

The consumption of the study folate dose yielded RBC folate concentrations that differed by physiologic group (physiologic group $\times$ time: $P = 0.002$) after ethnicity, *MTHFR C677>T* genotype, choline intake, and supplement use before study enrollment were controlled for. In pregnant and nonpregnant women, RBC folate concentrations increased ($P = 0.019$ and 0.044, respectively) slightly ($\sim 10\%$) over the course of the study, whereas in lactating women, RBC folate concentrations did not change ($P = 0.107$) over the course of the study (**Figure 3**).

At study end, lactating women had higher ($P \leq 0.002$) RBC folate than did both pregnant and nonpregnant women, and pregnant women had higher ($P < 0.001$) RBC folate than did nonpregnant women (Figure 3). RBC folate concentrations are related to the 120-d life span of erythrocytes and may take up to 40 wk to achieve a steady state (32). Thus, it is likely that study participants had not fully acclimated to the study folate dose by study end, and differences by physiologic group are not necessarily reflective of differences in physiologic use or requirement.

Breast-milk folate

Breast-milk folate represents a route of folate loss in lactating women and is the primary source of folate for exclusively breastfed infants (2).

The consumption of the study folate dose did not alter breast-milk 5-methyl-THF ($P = 0.232$) or breast-milk total folate ($P = 0.244$) concentrations over the course of the study after ethnicity, *MTHFR C677>T* genotype, choline intake, and supplement use before study enrollment were controlled for (**Figure 4**A; **Table 2**). However, the consumption of the study folate dose resulted in greater (time: $P < 0.001$) breast-milk FA concentrations over the course of the study (Figure 4A; Table 2). Moreover, supplement use before study enrollment interacted ($P < 0.001$) with study week (time) to affect breast-milk FA concentrations.

The relation of supplement use before study enrollment, time, and breast-milk FA concentrations was further explored because previous research has not shown a relation between maternal folate (or FA) intake and breast-milk folate concentrations (33, 34). Interestingly, breast-milk FA concentrations increased ($P = 0.001$) from baseline to study end only in women who did not

means (95% CIs) derived from linear mixed models; medians with 95% CIs are plotted for FA excretion. Study end was week 10 for lactating participants and week 12 for pregnant and nonpregnant participants. The interaction of physiologic state and time was significant ($P < 0.001$) for urinary 5-methyl-THF and total folate excretion; the distribution of the urinary FA data did not allow for this interaction to be tested. Different lowercase letters indicate differences between physiologic groups at study end, $P < 0.01$. *Urinary folate concentrations increased from baseline to study end within designated physiologic groups, $P < 0.01$; †pregnant group and lactating group tended to be different at study end, $P < 0.1$; §folate excretion tended to increase from baseline to study-end within lactating group, $P < 0.1$. FA, folic acid; 5-methyl-THF, 5-methyltetrahydrofolate.
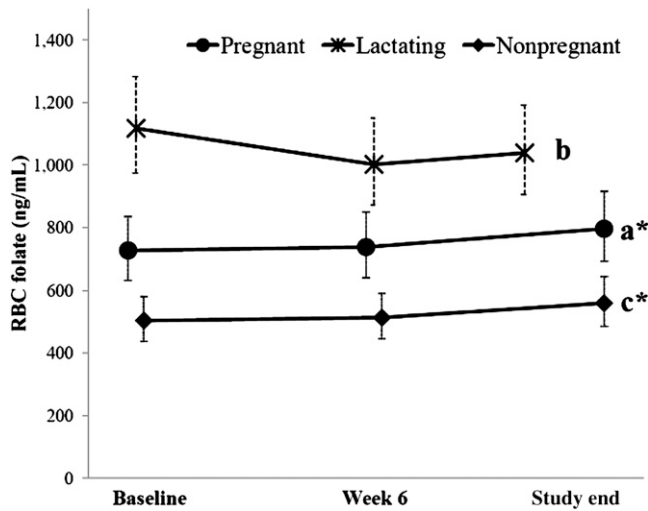
FIGURE 3. Predicted geometric means (95% CIs) of RBC folate concentrations in third-trimester pregnant women ($n = 26$), lactating women 5–15 wk postpartum ($n = 28$), and nonpregnant women of childbearing age ($n = 21$) who consumed an FA-containing prenatal supplement plus 400 μg natural food folate for 10–12 wk. Statistical analyses were performed by using and reported from a linear mixed model. All participants were included in the analyses. Plotted data were derived from linear mixed models. Study end was week 10 for lactating participants and week 12 for pregnant and nonpregnant participants. The interaction of physiologic state and time was significant, $P = 0.002$. Different lowercase letters indicate differences between physiologic groups at study end, $P < 0.01$. *RBC folate increased from baseline to study end within designated physiologic groups, $P < 0.05$. To convert nanograms of folate per milliliter to nanomoles of folate per liter, multiply nanograms per milliliter by 2.266. FA, folic acid; RBC, red blood cell.

take a supplement before study enrollment; there was no change ($P = 1.0$) in breast-milk FA concentrations in women who took an FA-containing supplement at baseline (Figure 4B; Table 2). At baseline, breast-milk FA concentrations were lower ($P < 0.001$) in women who did not consume a supplement before study enrollment; however, at week 6 ($P = 0.324$) and study end ($P = 0.796$), there were no differences in breast-milk FA concentrations on the basis of supplement use before study enrollment (Figure 4B; Table 2). In addition, the ratio of breast-milk FA to breast-milk total folate was significantly lower ($P < 0.001$) in lactating women who did not use a supplement before study enrollment at baseline; however, at week 6 ($P = 0.242$) and study end ($P = 0.174$), there were no differences in the ratio of breast-milk FA to breast-milk total folate ratio on the basis of supplement use before study enrollment (Table 2). The ratio of breast-milk 5-methyl-THF to breast-milk total folate did not change ($P = 0.889$) over the course of the study.

DISCUSSION

To our knowledge, this was the first controlled consumption study to comprehensively assess and compare folate-status response to a known dose (FA-containing prenatal supplement plus 400 μg natural food folate) in third-trimester pregnant women, lactating women 5–15 wk postpartum, and nonpregnant women of childbearing age. The following findings emerged: the study dose yielded supranutritional folate status in all women regardless of physiologic state; folate use was greater during the third trimester of pregnancy than during lactation 5–15 wk

postpartum; and breast-milk folate species were responsive to maternal folate intake.

Consumption of study folate dose yielded supranutritional folate status

Supranutritional folate status is indicated by folate status marker concentrations that far surpass adequacy cutoffs and suggest that metabolic capacity has been exceeded. Such status is achieved with folate intakes that are above what is reachable
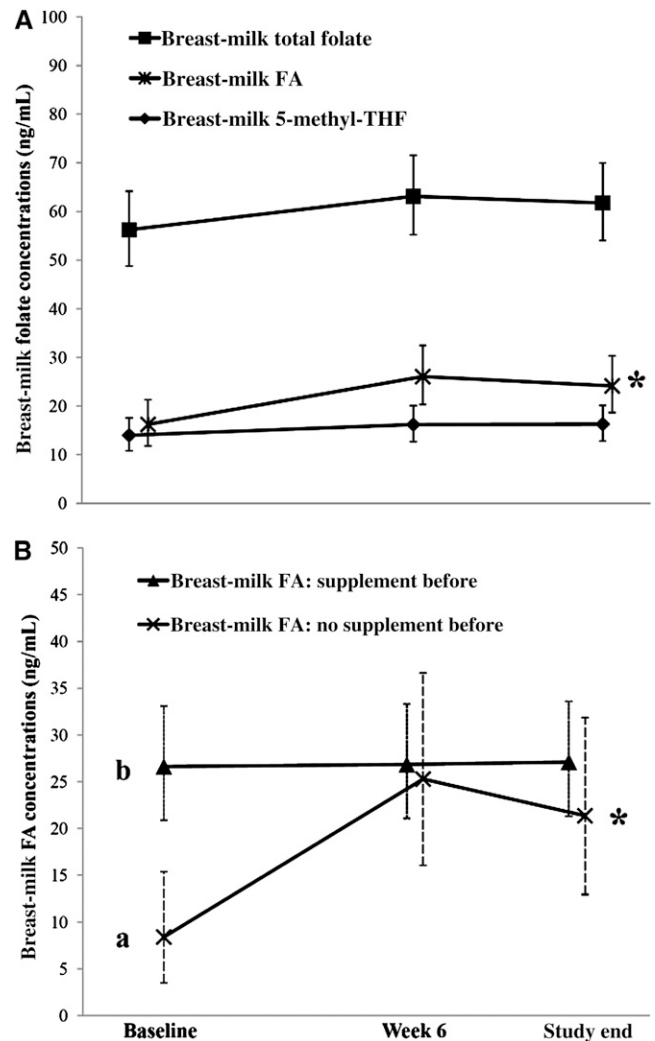
FIGURE 4. A: Back-transformed predicted means (95% CIs) of breast-milk 5-methyl-THF, total folate, and FA concentrations in lactating women ($n = 28$) who consumed an FA-containing prenatal supplement plus 400 μg natural food folate for 10 wk. B: Back-transformed predicted means (95% CIs) of breast-milk FA concentrations in study participants who consumed an FA-containing supplement before study enrollment ($n = 21$) in comparison with study participants who did not consume an FA-containing supplement before study enrollment ($n = 7$). Statistical analyses were performed by using linear mixed models. All participants were included in the analyses. Plotted data were derived from linear mixed models. The interaction of breast-milk FA and supplement use before study enrollment was significant, $P < 0.001$. Different lowercase letters indicate differences between groups at baseline, $P < 0.001$. *Designated breast-milk folate increased from baseline to study end, $P < 0.01$. To convert nanograms of folate per milliliter to nanomoles of folate per liter, multiply nanograms per milliliter by 2.266. FA, folic acid; 5-methyl-THF, 5-methyltetrahydrofolate.

TABLE 2
Breast-milk folate concentrations for lactating women 5–15 wk postpartum (n = 28) at baseline and after consumption of an FA-containing prenatal supplement plus 400 µg naturally occurring food folate for 10 wk[1]

	Baseline	Study end	P
5-methyl-THF (ng/mL)	14.0 (10.8–17.6)	16.3 (12.8–20.2)	0.232
Total folate (ng/mL)	56.2 (48.8–64.2)	61.8 (54.1–70.0)	0.244
FA (ng/mL)			
All participants	16.2 (11.8–21.3)	24.1 (18.7–30.3)	<0.001
No supplement before (n = 7)	8.4 (3.5–15.4)[a]	21.4 (12.9–31.9)	<0.001
Supplement before (n = 21)	26.6 (20.9–33.1)[b]	27.1 (21.3–33.6)	0.984
FA:total folate			
All participants	0.32 (0.26–0.39)	0.40 (0.34–0.46)	0.041
No supplement before (n = 7)	0.18 (0.07–0.30)[a]	0.36 (0.25–0.46)	0.007
Supplement before (n = 21)	0.46 (0.40–0.52)[b]	0.44 (0.38–0.50)	0.814

[1] All values are back-transformed predicted means; 95% CIs in parentheses. Data were analyzed by using linear mixed models; P values refer to baseline compared with study-end Bonferroni-corrected pairwise comparisons derived from linear mixed models. Different superscript letters within a column indicate baseline differences between groups derived from a linear mixed model, $P < 0.001$ (Bonferroni-corrected pairwise comparison). To convert nanograms of folate per milliliter to nanomoles of folate per liter, multiply nanograms per milliliter by 2.266. FA, folic acid; 5-methyl-THF, 5-methyltetrahydrofolate.

through consumption of natural food folate. Several biomarkers examined in the current study showed that the consumption of 400 µg natural food folate plus an FA-containing prenatal supplement yielded supranutritional status. First, estimates of study-end serum folate concentrations far exceeded 20 ng/mL, which is considered a marker of high folate status because it corresponds to the 95th percentile of population-wide folate status before FA fortification (5). Second, at study end, 84% of women had detectable unmetabolized FA in fasted serum, and 100%, 77%, and 89% of nonpregnant, pregnant, and lactating women, respectively, excreted unmetabolized FA in their urine. Third, total urinary folate excretion represented ∼9–43% of total folate intake (ie ∼100–500 µg/d).

The impact of supranutritional folate status on health is unclear; however, most concerns regard the masking of vitamin B-12 deficiency, folate–vitamin B-12 imbalance, putative roles in cancer promotion, and exposure to unmetabolized FA (28). Many over-the-counter prenatal supplements contain 800–1000 µg FA (1360–1700 DFEs) (9), which is an amount that exceeds all recommended intakes for women of childbearing age and approaches or meets the 1000-µg Tolerable Upper Intake Level (2). Because of underlying folate sufficiency (5), current folate and FA intakes (35), and evidence of supranutritional folate status in the current study, a reduction of prenatal supplement FA doses seems prudent.

Although this study was conducted under controlled conditions, the results of this study are broadly generalizable in FA-fortified populations because study participants consumed an over-the-counter prenatal supplement and natural folate from a mixed diet. However, it should be noted that the supranutritional folate status achieved with this study's folate dose should not be extrapolated to less folate-replete populations.

Response by physiologic state

In the current study, intact 24-h urinary folate excretion, which represents an average folate status across the collection period, proved the most informative folate biomarker (compared with fasted serum and RBC folate concentrations) in the comparison of folate use by physiologic group. Third-trimester pregnant women showed the greatest folate use by excreting less urinary 5-methyl-THF and less total folate than did lactating and nonpregnant women at study end. Meanwhile, lactating women showed greater folate use than did nonpregnant women by excreting less urinary total folate and FA at study end. It is notable that nonpregnant women excreted the most unmetabolized FA, which indicated that pregnant and lactating women metabolized more of the FA dose to a physiologic form. The folate dose in the current study far exceeded current RDAs for pregnant and lactating women. Thus to further refine RDA intakes, dose-response studies with intakes approximating current RDAs or Estimated Average Requirements are necessary.

Breast-milk folate

Results of the current study confirmed other findings (33, 34) that breast-milk total folate concentrations are maintained at constant amounts regardless of maternal folate intake; ie, breast-milk total folate concentrations did not vary on the basis of supplement use before study enrollment, and changes were not observed over the course of the study. However, results of the current study indicated, for the first time to our knowledge, that the distribution of folate species in breast milk is related to maternal folate intake.

Previously published studies have reported that 5-methyl-THF and FA make up 20–60% (36, 37) and 8–40% (34, 37), respectively, of breast-milk total folate, which indicates a broad distribution of breast-milk folate species. A significant proportion of breast-milk folate is bound to the protein folate receptor α (FRα) (36, 38). Among amount of FRα in breast milk is positively correlated ($r = 0.71$, $P < 0.001$) (38) with breast-milk total folate concentrations, and FRα may play a role in the regulation of breast-milk folate concentrations (34, 38). Among folate forms, FRα has the highest affinity for FA (39). In the current study, the study FA dose increased breast-milk FA without altering breast-milk total folate concentrations in lactating women who did not consume an FA-supplement before study enrollment. The maintenance of breast-milk total folate

concentrations in the midst of rising breast-milk FA concentrations implies that FA is displacing reduced folate forms from FRα in breast milk. The high percentage of breast-milk FA (∼40%) that arose from the study FA dose may be of concern because some bioavailability studies with milk have suggested that the absorption of FA could be less than that of 5-methyl-THF when bound to FRα (40).

In conclusion, supranutritional folate status resulted from the consumption of an FA-containing prenatal supplement plus 400 μg natural food folate. At study end, serum folate concentrations did not vary by physiologic group, with the increased folate use of pregnant and lactating women apparent only via differences in urinary folate excretion. Breast-milk total folate concentrations did not change in response to the study folate dose; however, the proportion of FA in breast milk increased. Because of unresolved concerns about exposure to excess FA (for women of childbearing age as well as for breastfeeding infants) and the widespread folate adequacy of our post–FA fortification population, it appears prudent to reduce the amount of FA in prenatal supplements to amounts more in line with Dietary Reference Intake recommendations.

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