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Association between serum unmetabolized folic acid concentrations and folic acid from fortified foods

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Abstract

Objective—To investigate the association between serum unmetabolized folic acid (UMFA) concentrations and folic acid from fortified foods and nutrients known as dietary methyl-group donors (folate, methionine, choline, betaine and vitamins B2, B6 and B12) in participants exposed to mandatory fortification of wheat and maize flours with folic acid.

Methods—Cross-sectional study carried out with 144 healthy Brazilian participants, both sexes, supplement nonusers. Serum folate, UMFA, vitamin B12 and total plasma homocysteine (tHcy) were biochemically measured. Dietary intake was assessed by 2 non-consecutive 24-hour dietary recalls (24-HRs) and deattenuated energy-adjusted nutrient data were used for statistical analysis.

Results—Ninety eight (68.1%) participants were women. Median (interquartile range) age was 35.5 (28.0–52.0) years. Elevated serum folate concentrations (>45 nmol/L) were found in 17 (11.8%), while folate deficiency (<7 nmol/L) in 10 (6.9%) participants. No one had vitamin B12 deficiency (<148 pmol/L). An elevated serum UMFA concentration was defined as > 1 nmol/L (90th percentile). UMFA concentrations were positively correlated with folic acid intake and negatively correlated to choline, methionine and vitamin B6 intakes. Participants in the lowest

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC).

Author contributions: C.Z.P., C.P., and E.M.G.S. were responsible for study conception and design. All authors were responsible for generation, collection, assembly, analysis and/or interpretation of data, and drafting or revision of the article. All authors approved the final version of the article.

Ethical Standards Disclosure: This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Institutional Ethics Committees from the Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil. Written informed consent was obtained from all subjects/patients.

quartile of UMFA concentrations had lower dietary intake of total folate (DFEs) and folic acid, and higher dietary intake of methionine, choline and vitamin B6 than participants in the highest quartile of UMFA. Folic acid intake (OR [95% CI] D 1.02 [1.01–1.04)] and being a male (OR [95% CI] = 0.40 [0.19–0.87) were associated with increased and reduced odds for UMFA concentrations > 0.55 nmol/L (median values), respectively.

Conclusion—UMFA concentrations were directly influenced by folic acid intake from fortified foods in a healthy convenience sample of adult Brazilians exposed to mandatory flour fortification with folic acid.

Introduction

Folate is a dietary compound that has a direct influence on both DNA methylation and replication as well as on protein methylation through one-carbon metabolism, therefore affecting the regulation of gene expression from intrauterine development until adulthood [1,2]. The main dietary methyl-group donors are folate, methionine, choline, betaine, and vitamins B2, B6 and B12 [2], which participate as cofactors in folate cycle-dependent enzymes, homocysteine metabolism and synthesis of the methyl donor S-adenosyl-L-methionine [1,2].

From a public health perspective, folate dietary inadequacy is of particular concern given the preventable consequences of its deficiency, such as birth defects, especially neural tube defects (NTDs) [3,4]. As a cheap and effective prevention strategy, mandatory food fortification with synthetic folic acid has been implemented in many countries in order to reduce the prevalence of NTDs and associated morbidity and mortality [5]. Because synthetic folic acid is more stable and has greater bioavailability than natural food folate, this form of vitamin is preferably used for food fortification and supplementation purposes [6].

Latin American countries (except Venezuela) that have adopted programs of mandatory fortification of wheat flour with folic acid, had NTD incidence varying by geographic region from 0.2 to 9.6 per 1,000 live births/year. After the effective implementation of folic acid fortification programs in these countries, NTD prevalence has demonstrated an aggregate percentage decline, ranging from 33% to 59% [7].

In Brazil, wheat and maize flour fortification became effective in July 2004 by adding 150 μg of folic acid and 4.2 mg of iron per 100 g of flour [8]. As a result of folic acid fortification, fetal mortality decreased by 27.3% due to anencephaly and 30% for spina bifida, when comparing the years before and after fortification [9].

Another consequence of food fortification is higher folate status biomarkers (serum and red blood cell) and lower total plasma homocysteine (tHcy) concentrations observed in populations from countries where food folic acid fortification is mandatory [6,10,11], followed by those with voluntary fortification [6]. A recent review of national published studies demonstrated a significant overall increase in serum folate concentrations of Brazilian healthy adults in the postfortification period [11]. A local Brazilian study showed

that the main food contributors of folate intake during postfortification were fortified foods, such as breads, pasta, cakes and cookies [12].

Unlike folate from natural sources, folic acid from fortified foods and supplements relies on dihydrofolate reductase (DHFR) for conversion to tetrahydrofolate for one-carbon metabolism.

It is worth noting that higher nutrient status is found not only in the target group but also in the whole population, including vulnerable groups such as young children and elderly persons [13]. Although no definitive adverse outcomes have been linked to high folic acid intakes in healthy populations to date, there is concern about the possibility of potential adverse effects of folic acid, mainly because this nutrient can modulate epigenetic phenomena [3,5].

Considering that the Brazilian population has been exposed to mandatory fortification of flours with folic acid for at least 10 years, the aims of the present study were to assess folate status biomarkers and unmetabolized folic acid (UMFA) concentrations and investigate the association among UMFA and dietary folic acid intake from fortified foods.

Material and Methods

This was a cross-sectional study carried out with 144 healthy Brazilian participants, both males and females, recruited through convenience sampling. Participants ≥20 years old were from general urban population of São Paulo city, the largest metropolitan area of the country, located in the southeast region of Brazil. The recruitment approach included distributing pamphlets, advertising in the university, and approaching participants one on one in the local community. A personal interview was conducted at enrollment to assess eligibility for participation.

Participants reporting chronic alcohol abuse or the use of drugs that interfere with folate metabolism (such as methotrexate), pregnant women, and participants diagnosed with chronic diseases or a recent or acute infection, as well as those with a history of blood donation or multivitamin/multimineral supplement use in the previous 6 months, were not eligible for the study.

The study protocol was approved by the Institutional Ethics Committees from the Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil (CAEE 04389512.2.000.0067 and 43072215.9.0000.0067), and written consent was obtained from each participant.

Demographic data and anthropometric measurements

Sociodemographic data were obtained through a questionnaire applied to all participants. Body weight was measured with each participant wearing light clothing and no shoes to the nearest 0.1 kg using a digital weight scale (Tanita Corporation of America, Inc., Illinois, USA). Height was measured to the nearest 0.1 cm using a portable stadiometer (Alturexata®, Minas Gerais, Brazil). Body mass index (BMI) was calculated as the weight (kg) divided by height (m^2) squared and used to classify the nutritional status of participants

according to the following categories: underweight (<18.5 kg/m^2), normal weight (18.5 to 24.9 kg/m^2), overweight (25 to <30 kg/m^2) and obese (≥30 kg/m^2) [14].

Blood samples

Blood samples of approximately 50 mL were collected by venipuncture from all participants after an overnight fast of at least 8 hours. Blood cell count was obtained with an electronic counter Pentra 120 DX (Horiba Medical®, Montpellier, France). Reticulocyte count was performed by microscopy using brilliant cresyl blue. High-sensitive C-reactive protein (hs-CRP) was determined by ultrasensitive immunoturbidimetry assay using the Roche-CRPL kit on the Cobas 8000 platform (Roche Diagnostics, Indianapolis, USA). Lactate dehydrogenase was performed by enzymatic assay on the Vitros 250 platform (Ortho Clinical Diagnostics, Rochester, NY).

Serum folate and vitamin B12 concentrations were quantified by microbiological method using strains of *Lactobacillus casei* [15] and *Lactobacillus leishmannii* [16], respectively. Unmetabolized folic acid was determined using high-performance liquid chromatography-tandem mass spectrometry according to the Centers for Disease Control and Prevention [17]. THcy was measured by a chemiluminescence immunoassay method (Immulite 2000 analyzer; Siemens Healthcare, Erlangen, Germany).

Cut-off points

Serum folate concentrations <7.0 nmol/L and >45 nmol/L were defined as folate deficiency and elevated concentrations, respectively [18]. Elevated serum UMFA and tHcy concentrations were defined according to the 90th percentile in the distribution of values obtained from the fasting participants of this study. Serum UMFA concentrations> 1 nmol/L were considered elevated. tHcy concentrations >13.8 μmol/L were characterized as elevated.

Serum vitamin B12 concentrations <148 pmol/L were defined as deficient [19]. Anemia was defined as hemoglobin concentrations <120 g/L for women and <130 g/L for men [20].

Dietary intake assessment

Dietary intake was measured using 2 non-consecutive 24-hour dietary recalls (24-HRs), collected by trained interviewers according to the multiple pass method [21, 22]. In brief, detailed information concerning types of foods and beverages with their corresponding household units of intake consumed during the previous day of the interview were recorded following standardized questions.

The first recall was obtained on the day of blood collection through a face-to-face interview, and a second recall was obtained after a median of 45 days through personal interview or phone call. A randomization schedule for 24-HR collections was created in order to allow dietary intake representativeness of every day of the week, including weekends. Among the 144 study participants, 138 (95.83 %) and 122 (84.72 %) answered the first and second 24-HRs, respectively. In order to obtain nutritional information from 24-HRs, household units of food, beverage and recipes intakes were initially converted in weight and volume units following regional reference of standard recipes and food preparations [23,24].

The data were entered into Nutrition Data System for Research software Version 2014, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis. This software uses the U.S. Department of Agriculture's food composition table as its main database, from which the folate naturally present in foods, the dietary folic acid added to fortified foods, and the total dietary folate expressed as dietary folate equivalents (DFEs) are calculated. The more recent food composition database from Brazil does not distinguish these forms of folate or betaine and choline values, which were crucial to perform the analysis in this study. Furthermore, dietary folic acid and consequently DFE values were corrected to account for differences in levels of mandatory fortification of flours between Brazil (150 μg per 100 grams of flour) and the United States (140 μg per 100 grams of flour) [8]. These variables were corrected using Stata (StataCorp, College Station, TX), generating 2 new variables: (1) new variable folic acid in micrograms and (2) new total folate in DFEs.

Multiple Source Method (Ver, 1.0.1. Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke) was used to correct the effect of variability and to estimate usual dietary intake distributions (deattenuated data) [25]. An estimated average requirement (EAR) cutoff method was used to estimate the prevalence of inadequate micronutrient intake [26].

We adopted The Dietary Reference Intake (DRI), which contains a set of nutrition recommendations for healthy populations from the Institute of Medicine (IOM) of the National Academies and is the most accurate scientific knowledge regarding nutrients needs [26]. Energy-adjusted nutrient intakes were obtained through the regression residual method, as proposed by Willett [27].

Statistical analysis

Statistical analyses were carried out using SPSS Statistics for Windows, Version 22.0 (SPSS Inc., Chicago, Il). Statistical significance was set as $p < 0.05$. For descriptive analysis, categorical variables were described as number of participants and percentage and continuous variables were presented as median and interquartile ranges.

Regarding dietary food intake, deattenuated data were used for descriptive purposes, and also to estimate the prevalence of inadequate micronutrient intakes, while deattenuated energy-adjusted nutrient data were used for statistical tests.

Spearman's correlation coefficients were calculated to measure the strength of association between UMFA concentrations and dietary nutrients as total dietary folate, food folate, dietary folic acid, methionine, choline, betaine, and vitamins B2, B6 and B12. The Mann-Whitney *U* test was used to assess significantly differences in nutrient intakes between the lowest and highest quartiles of UMFA concentrations as well as differences between women and men with regard to serum folate, UMFA, tHcy, and vitamin B12 concentrations.

A model of backward stepwise logistic regression was performed using UMFA as the dependent variable, considering values above vs equal or under the median value of 0.55 nmol/L. The following independent variables were included in the model: sex, age, and total folate (expressed as DFEs), natural food folate, and folic acid intakes.

Results

Ninety eight (68.1 %) participants were women. Median values and interquartile ranges for age and BMI of all participants were 35.5 (28.0 – 52.0) years and 24.9 (22.3 – 27.3) kg/m^2, respectively. Among the participants, 80 (55.6%) were young adults (20 – 39 years), 41 (28.5%) were middle-aged (40 – 59 years) and 23 (16%) were ≥60 years of age. Three (2.1%) participants were underweight, 70 (48.6%) participants had normal BMI, 55 (38.2%) were overweight, and 16 (11.1%) were obese. The majority of participants finished high school and have been to college, with the median and interquartile range for years of education being 15 (11 – 19).

The profile of percentiles of serum folate, UMFA, vitamin B12, and tHcy is presented in Table 1. Folate deficiency was seen in 10 (6.9%) participants, being 8 men, while elevated serum folate concentrations were found in 17 (11.8%) participants, being 16 women. None of the participants had vitamin B12 deficiency. Participants with concentrations above the 90th percentile for UMFA (>1.00nmol/L) and tHcy (>13.8 μmol/L) had values in the range of 1.04 to 1.63 nmol/ L and 13.9 to 34.2 μmol/L, respectively.

Serum folate concentrations were significant higher in women (median and interquartile range: 26.5 [17.8 – 42.2] nmol/L) compared to men (median and interquartile range: 15.8 [10.2 – 23.4] nmol/L), $p < 0.001$. UMFA concentrations were also greater in women (median and interquartile range: 0.58 [0.44 – 0.77] nmol/L] than in men (median and interquartile range: 0.46 [0.33 – 0.56] nmol/L], p <0.001.

Women (median and interquartile range: 9.1 [7.8 – 10.8] μmol/L) had lower tHcy concentrations that men (median and interquartile range: 10.8 [9.2 – 12.5] μmol/L), $p < 0.001$, and no difference was observed in vitamin B12 concentrations between the 2 groups.

With regards to hematological parameters, the median and interquartile range was 138 (128 – 148) g/L for hemoglobin, 89.6 (86.1 – 91.8) fL for mean corpuscular volume, 6.6 (5.3 – 7.3) x10^9/L for leukocytes, 214 (184 – 241) x 10^9/L for platelets, and 0.8% (0.7% – 1.1%) for reticulocytes, showing normal hematological profiles. After analyzing hemoglobin concentrations according to sex, anemia was found in 7.6% (11) of participants. Among these participants with anemia, 5 were men and only one presented folate deficiency (6.8 nmol/L); the 6 women with anemia had adequate serum folate concentrations. It is noting that anemia has several different causes.

The participants had normal cellular duplication (lactate dehydrogenase: median and interquartile range: 416 [370 – 463] U/L) and had no inflammatory process (hs-CRP: median and interquartile range: 0.17 [0.09 – 0.40] mg/dL).

Deattenuated food intake data and prevalence of inadequate nutrient intake, calculated according to the EAR method and tolerable upper intake level (UL), are presented in Table 2. Dietary folic acid from fortified foods represented almost half (48.4 %) of total dietary folate intake, expressed as DFEs. Regarding the forms of folate (DFEs, natural food folate, and folic acid), no differences were observed when comparing men's and women's energy

adjusted intake. The EAR for folate is given as micrograms of DFEs, which considers the bioavailability of natural food folate and the folic acid in fortified foods. None of the participants had intakes above the UL for vitamin B6 (100 mg/d) or folate (1 mg/d); the UL for folate only applies to folic acid present in fortified foods or supplements [26]. For vitamins B2 and B12, ULs have not yet been determined. For choline, only adequate intakes for women and men have been set [26], and no dietary reference intake levels have been established for betaine or methionine. Thus, no prevalence of inadequacy was calculated for these nutrients.

Serum UMFA concentrations were positively correlated with dietary folic acid (r =0.303; $p < 0.001$; N = 138) and negatively correlated with choline (r = −0.212; $p = 0.013$; N =138), methionine (r = − 0.185; $p = 0.030$; N=138) and vitamin B6 (r = − 0.195; $p = 0.022$; N =138) intakes.

Table 3 shows that participants in the lowest quartile of UMFA concentrations had a lower dietary intake of folic acid and total folate and higher dietary intakes of methionine, choline, and vitamin B6 than participants in the highest quartile. These findings corroborate those described in the correlation analysis.

An increase in dietary folic acid intake was significantly associated with increased odds of UFMA concentrations ≥0.55 nmol/L (odds ratio [OR] = 1.02; 95% confidence interval [CI], 1.01 – 1.04). Being male was significantly associated with decreased odds of UMFA concentrations ≥ 0.55 nmol/L (OR = 0.40; 95% CI, 0.19–0.87; Table 4).

Discussion

In this convenience sample of healthy Brazilian adults exposed to mandatory flour fortification with folic acid, we found some participants with folate deficiency (6.9%) and some with elevated serum folate concentrations (11.8 %). Detectable but small UMFA concentrations (≤2 nmol/L) were found in all participants, likely as a result of dietary folic acid intake from fortified foods. Although the participants had small background concentrations of UMFA, this does not mean that the finding is linked to adverse outcomes in a population exposed to food fortification with folic acid.

Serum folate concentrations are an indicator of short-term status, closely related to recent dietary intake, particularly to folic acid from fortified foods and supplements, which has a higher bioavailability than natural food folate [6]. In the pre-fortification era, some Brazilian studies conducted in healthy adults using different folate methodologies showed mean ± SD serum folate concentrations of 12.1 ± 4.3 nmol/L [28] and 13.6 ± 0.9 nmol/L [29]. We observed higher serum folate concentrations in our postfortification study, presenting a mean ± SD of 26.7 ±17.6 nmol/L. Our result is similar to those published in a recent review of serum folate concentrations in healthy adults after fortification [11]. We also found a folate deficiency rate of almost 7%, which is higher than in the United States, where deficiency is almost nonexistent [30]. Indeed, it is crucial to point out that folate deficiency is not the appropriate measure to determine whether fortification is effective, because the main purpose of food fortification with folic acid is not to prevent megaloblastic anemia but to

prevent NTDs. All of the Brazilian studies cited above included small samples and were not population based, reinforcing the need for larger and controlled studies to assess the impact of folic acid fortification in Brazil.

Serum UMFA concentrations are a result of folic acid intake, either from fortified foods, from supplements, or both. It is suggested that an intake of this synthetic form above 200 μg/meal exceeds the enzyme capacity of DHFR to reduce folic acid to tetrahydrofolate, the bioactive form of folate [6, 31]. A recent study also pointed to another possible explanation for UMFA systemic circulation, chronic liver exposure to folic acid in humans, which may induce DHFR saturation [32].

Little is known about possible side effects of elevated UMFA concentrations. In healthy, postmenopausal women, reduced natural killer cell cytotoxicity was observed among those who consumed a folate-rich diet in addition to folic acid supplements (>400 μg/d) compared to those consuming a low-folate diet and no supplements [33].

Furthermore, there is still a lack of consensus regarding cut-off points for determining elevated UMFA concentrations, and it is also important to highlight that there are no health outcomes associated with any of these cut-offs. Data from the National Health and Nutrition Examination Survey 2007–2008 selected UMFA >1 nmol/L, which reflected approximately the 75th percentile for fasting (from food and supplements) individuals (supplement users and nonusers together) [34]. In our study, we adopted the 90th percentile as a reference (>1 nmol/L), which is the same as the cut-off point from the National Health and Nutrition Examination Survey [34] and lower when compared to Kalmbach et al., who defined high UMFA as ≥1.35 nmol/L, considering the 85th percentile for the Framingham Heart Study [35].

The Framingham cohort demonstrated that among nonsupplement users, the prevalence of high circulating folic acid was 2.16 times greater in the highest quartile of folic acid intake than in the lowest quartile category after multivariate adjustment (95% CI, 1.22, 3.84) and also 2.84 times greater for participants in the highest quartile compared to the lowest quartile category for total folate intake as DFEs (95% CI, 1.51, 5.35) [35].

In the US population, the odds ratio of having UMFA concentrations >1 nmol/L in fasting adults ≥20 years of age was 1.5 times higher for a 200 μg/d increase in total folic acid intake from diet and supplements, 1.2 times higher for a 200 nmol/L increase in red blood cell folate, and 1.3 times higher for a 1-drink/d decrease in alcohol intake. Furthermore, adults age 20–59 years were 2 times less likely to have UMFA concentrations >1 nmol/L compared to participants ≥60 years of age [36]. Though our model explored only a few variables of interest, we found sex and dietary folic acid intake to be independent variables that explain UMFA concentrations ≥ 0.55 nmol/L.

One possible explanation why being male appeared to be a protective factor for UMFA concentrations is that we found a higher prevalence of folate deficiency in males (8 of 10 participants). Lower concentrations of serum folate may be due to dietary intake and other factors such as polymorphisms in genes related to absorption and metabolism of folate, which were not assessed in this study.

A Brazilian study with dietary assessment methods similar to ours, showed that in the years before fortification, mean ± SD total folate intake in men and women was 285 ± 66 and 198 ± 63 μg DFEs/d, respectively, and the prevalence of inadequate folate intake was 76% and 95%, respectively. In the years postfortification, total folate intake increased in men and women to 568 ± 171 and 410 ± 198 μg DFEs/day, respectively, and the prevalence of inadequate folate intake decreased to 6% and 38%, respectively [12]. Our study also found mean DFE intake values above 400 μg/d for both sexes and a total prevalence of inadequate folate intake of 14.2% in relation to the EAR cutoff point from the IOM [26]. When stratified by sex, 4.3% of men and 20.6% of women had inadequate folate intake, which was lower compared to data in the prefortification era [12].

For women capable of becoming pregnant, IOM recommends a daily intake of 400 μg of folic acid from fortified foods or supplements or both, in order to reduce the risk of an NTD-affected pregnancy. The folate UL for adults (≥19 years old) is set at 1 mg/d, covering only the synthetic form of the vitamin, folic acid, found in fortified foods or supplements, because no adverse effects have been associated with folate naturally present in some food sources [26]. None of our participants had a folic acid intake above the UL. Studies concerning UMFA concentrations and dietary intake have shown a direct relation between UMFA concentrations, total folate (DFEs) and dietary folic acid [34, 35]. To the best of our knowledge, this is the first study to explore the relation of UMFA concentrations in a convenience sample of healthy Brazilian adults with dietary methyl donor nutrients. Interestingly, we observed a negative correlation between UMFA concentrations and other nutrients, such as choline, methionine and vitamin B6, as well as higher intakes of choline, methionine and vitamin B6 in participants in the lowest quartile of UMFA concentrations. Thus, it seems that these micronutrients may also take part in UMFA metabolism or, more likely, higher folic acid intake was found among those who consumed more fortified products instead of fruits and vegetables, essentially reaching the target population at risk for low folates and higher NTD risk. This finding is corroborated by an interventional study conducted with elderly people (>65 years) not exposed to food fortification with folic acid. The investigators gave the participants either a daily supplement of folic acid or folic acid plus cyanocobalamin and pyridoxine. After supplementation, participants who received the B-complex had lower postintervention serum UMFA concentrations and prevalence of UMFA ≥0.21 nmol/L, compared to the folic acid group, suggesting that vitamins B6 and B12 are needed to efficiently metabolize folic acid and natural folate [36].

A limitation of the current study was the moderate sample size and the nature of convenience sample. On the other hand, the strengths of this study were the certainty that the participants were not taking any supplements, the measurement of UMFA concentrations by liquid chromatography/tandem mass spectrometry, and the rigorous dietary intake assessment. Although it is known that dietary intake methods are still subject to errors, 24 HRs adjusted for usual intake are fairly reliable tools for assessing dietary intake and minimize possible measurement errors.

In conclusion, UMFA concentrations are found to be directly associated with folic acid intake from fortified foods in a Brazilian healthy cohort exposed to mandatory fortification of flours with folic acid. It seems that dietary intake of methyl donor nutrients also may be

associated with UMFA concentrations, requiring more investigation into the relationship between UMFA and betaine, choline, methionine, and vitamins B2, B6 and B12.

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References

1. Chango A, Pogribny IP. Considering maternal dietary modulations for epigenetic regulation and programming of the fetal epigenome. Nutrients. 2015; 7(4):2748–2770. [PubMed: 25875118]
2. McKay JA, Mathers JC. Diet induced epigenetic changes and their implication for health. Acta Physiol. 2011; 202:103–118.
3. Barua S, Kuizon S, Junaid MA. Folic acid supplementation in pregnancy and implications in health and disease. J Biomed Sci. 2014; 21:77. [PubMed: 25135350]
4. Pitkin RM. Folate and neural tube defects. Am J Clin Nutr. 2007; 85:285S–288S. [PubMed: 17209211]
5. Crider KS, Bailey LB, Berry RJ. Folic acid food fortification – its history, effect, concerns and future directions. Nutrients. 2011; 3:370–384. [PubMed: 22254102]
6. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF 3rd, Mills JL, Pfeiffer CM, Fazili Z, Zhang M, Ueland PM, Molloy AM, Caudill MA, Shane B, Berry RJ, Bailey RL, Hausman DB, Raghavan R, Raiten DJ. Biomarkers of nutrition for development-Folate review. J Nutr. 2015; 145(7):1636S–1680S. [PubMed: 26451605]
7. Rosenthal J, Casas J, Taren D, Alverson CJ, Flores A, Frias A. Neural tube defects in Latin American and the impact of fortification: a literature review. Public Health Nutr. 2014; 17(3):537–550. [PubMed: 23464652]
8. Brasil. Agência Nacional de Vigilância Sanitária. Aprova o Regulamento Técnico para a Fortificação das Farinhas de Trigo e das Farinhas de Milho com Ferro e Ácido Fólico. Diário Oficial da União, Poder Executivo; Brasília: 2002. Resolução RDC n' 344
9. Schuler-Faccini L, Sanseverino MTV, de Azevedo LMR, Moorthie S, Alberg C, Chowdhury S, Sagoo GS, Burton H, Nacul LC. Health needs assessment for congenital anomalies in middle-income countries : Examining the case for neural tube defects in Brazil. J Community Genet. 2014; 5:147–155. [PubMed: 23990401]
10. Scorsatto M, Uehara SK, Luiz RR, de Oliveira GM, Rosa G. Fortification of flours with folic acid reduces homocysteine levels in Brazilian women. Nutr Res. 2011; 31(12):889–895. [PubMed: 22153514]
11. Britto JC, Cançado R, Guerra-Shinohara EM. Concentrations in blood folate in Brazilian studies prior to and after fortification of wheat and cornmeal (maize flour) with folic acid. Rev Bras Hematol Hemoter. 2014; 36(4):275–286. [PubMed: 25031167]
12. Marchioni DM, Verly E Jr, Steluti J, Cesar CL, Fisberg RM. Folic acid intake before and after mandatory fortification: a population-based study in São Paulo, Brazil. Cad Saude Publica. 2013; 29(10):2083–2092. [PubMed: 24127102]
13. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999–2000. Am J Clin Nutr. 2005; 82(2):442–450. [PubMed: 16087991]
14. WHO. WHO Technical Report Series 854. Geneva: World Health Organization; 1995. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee.
15. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol. 1992; 45(4):344–347. [PubMed: 1577973]

16. Kelleher BP, Walshe KG, Scott JM, O'Broin SD. Microbiological assay for vitamin B12 with use of a colistin-sulfate-resistant organism. Clin Chem. 1987; 33(1):52–54. [PubMed: 3542297]
17. Fazili Z, Whitehead RD Jr, Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem. 2013; 405:4549–4560. [PubMed: 23462981]
18. World Health Organization. Serum and Red Blood Cell Folate Concentrations for Assessing Folate Status in Populations. Geneva: World Health Organization; 2012.
19. Gibson, RS. Principles of nutritional assessment. New York, NY: Oxford University Press; 1990.
20. World Health Organization Scientific Group. Nutritional anaemias. Report of a WHO scientific group. World Health Organ Tech Rep Ser. 1968; 405:5–37. [PubMed: 4975372]
21. Moshfegh AJ, Borrud L, Perloff B, LaComb R. Improved method for the 24-hour dietary recall for use in national surveys [abstract]. FASEB J. 1999; 13:A603.
22. Moshfegh AJ, Raper N, Ingwersen L, et al. An improved approach to 24-hour dietary recall methodology [abstract]. Ann Nutr Metab. 2001; 45(suppl):156.
23. Fisberg, RM., Marchioni, DML. Manual de Avaliação do Consumo Alimentar em Estudos Populacionais : a Experiência do Inquérito de Saúde em São Paulo (ISA)/ Universidade de São Paulo. São Paulo: Faculdade de Saúde Pública da USP; 2012.
24. Fisberg, RM., Villar, BS. Manual de Receitas e Medidas Caseiras para Cálculo de Inquéritos Alimentares: Manual Elaborado para Auxiliar o Processamento de Dados de Inquéritos Alimentares. São Paulo: Signus; 2002.
25. Harttig U, Haubrock J, Knüppel S, Boeing H. Consortium E. The MSM program: Web-based statistics package for estimating usual dietary intake using the multiple source method. Eur J Clin Nutr. 2011; 65(Suppl 1):S87–91. [PubMed: 21731011]
26. Institute of Medicine, National Academy of Sciences. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin and choline. Washington, DC: National Academy Press; 1998.
27. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. Am J Clin Nutr. 1997; 65(Suppl 4):1220S–1228S. [PubMed: 9094926]
28. Pereira AC, Schettert IT, Morandini Filho AA, Guerra-Shinohara EM, Krieger JE. Methylenetetrahydrofolate reductase (MTHFR) c677t gene variant modulates the homocysteine folate correlation in a mild folate-deficient population. Clin Chim Acta. 2004; 340(1–2):99–105. [PubMed: 14734201]
29. Helfenstein T, Fonseca FA, Relvas WG, Santos AO, Dabela ML, Matheus SC, D'Almeida V, Tufik S, Souza FG, Rodrigues PR, Taglieri R, Sousa EF, Izar MC. Prevalence of myocardial infarction is related to hyperhomocysteinemia but not influenced by C677T methylenetetrahydrofolate reductase and A2756G methionine synthase polymorphisms in diabetic and non-diabetic subjects. Clin Chim Acta. 2005; 355(1–2):165–172. [PubMed: 15820491]
30. U.S. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 2012. Atlanta (GA): National Center for Environmental Health; 2012.
31. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in participants consuming fortified food and supplements. Am J Clin Nutr. 1997; 65(6): 1790–1795. [PubMed: 9174474]
32. Patanwala I, King MJ, Barrett DA, Rose J, Jackson R, Hudson M, Philo M, Dainty JR, Wright AJ, Finglas PM, Jones DE. Folic acid handling by the human gut: implications for food fortification and supplementation. Am J Clin Nutr. 2014; 100(2):593–599. [PubMed: 24944062]
33. Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, Wood B, Selhub J, McTiernan A, Yasui Y, Oral E, Potter JD, Ulrich CM. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr. 2006; 136(1):189–194. [PubMed: 16365081]
34. Pfeiffer CM, Sternberg MR, Fazili Z, Yetley EA, Lacher DA, Bailey RL, Johnson CL. Unmetabolized folic acid is detected in nearly all serum samples from US children, adolescents, and adults. J Nutr. 2015; 145(3):520–531. [PubMed: 25733468]

35. Kalmbach RD, Choumenkovitch SF, Troen AM, D'Angostino R, Jacques PF, Selhub J. Circulating folic acid in plasma: relation to folic acid fortification. Am J Clin Nutr. 2008; 88:763–768. [PubMed: 18779294]

36. Obeid R, Kirsch SH, Dilmann S, Klein C, Eckert R, Geisel J, Herrmann W. Folic acid causes higher prevalence of detectable unmetabolized folic acid in serum than B-complex: a randomized trial. Eur J Nutr. 2016; 55(3):1021–1028. [PubMed: 25943647]

Table 1

Serum folate, UMFA, vitamin B12 and tHcy concentrations in 144 healthy Brazilian participants, São Paulo, Brazil.

	Median (25th–75th Percentiles)	Geometric mean (95% CI)	Percentiles			
			2.5	10	90	97.5
Serum folate (nmol/L)	22.8 (14.4 – 38.4)	21.7 (19.3 – 24.3)	5.7	9.2	46.6	58.4
UMFA (nmol/L)	0.55 (0.40 – 0.69)	0.55 (0.50 – 0.61)	0.23	0.32	1.00	1.43
Vitamin B_{12} (pmol/L)	368 (292 – 500)	377 (355 – 400)	176	231	600	763
tHcy (μmol/L)	9.5 (8.2 – 11.4)	9.7 (9.3 – 10.2)	5.6	6.5	13.8	19.1

UMFA = unmetabolized folic acid, tHcy = total plasma homocysteine, CI = confidence interval.

Table 2

Dietary intake of 138 healthy Brazilian participants, São Paulo, Brazil

Dietary intake	Median (25th –75th Percentiles)	< EAR (%)	> UL[a]
Energy (Kcal)	1876 (1570 – 2170)	----	----
Total folate (μg DFE/d)[b]	403 (353 – 473)	14.2	
Natural food folate (μg/d)	208 (179 – 235)	----	----
Folic acid (μg/d)	115 (89 – 141)	----	0
Vitamin B2 (mg/d)	1.5 (1.3 – 1.8)	4.1	ND
Vitamin B6 (mg/d)	1.6 (1.4 – 1.8)	2.0	0
Vitamin B12 (μg/d)	3.6 (2.8 – 5.0)	11.7[c]	ND
Methionine (g/d)	1.9 (1.6 – 2.2)	ND	ND
Choline (mg/d)	312 (268 – 375)	ND	ND
Betaine (mg/d)	121 (94 – 155)	ND	ND

EAR= Estimated Average Requirement; UL = Tolerable Upper Intake Levels; ND = not determined.

[a] Two outliers were excluded before estimating the prevalence of inadequate intake for vitamin B12.

[b] Presented as dietary folate equivalents (DFEs). 1 μg of DFEs = 1 μg of natural folate sources = 0.6 μg of folic acid from fortified foods.

[c] Presented as number of participants above the UL. The UL for folate applies only to folic acid present in fortified foods or supplements.

Table 3

Dietary nutrient intake variables of healthy Brazilian participants according to the lowest and highest quartiles of unmetabolized folic acid (UMFA) concentrations**,** São Paulo, Brazil

Variables[a]	Quartiles of UMFA		*p*[b]
	1 (Lowest) (N = 35)	4 (Highest) (N = 37)	
UMFA concentrations (nmol/L)	0.34 (0.28 – 0.36)	0.89 (0.74 – 1.09)	-
Total folate (μg DFEs/d)[c]	383 (327 – 431)	408 (377 – 469)	**0.015**
Natural food folate (μg/d)	211 (184 – 225)	201 (182 – 228)	0.642
Folic acid (μg/d)	95 (76 – 125)	136 (104 – 148)	**0.001**
Vitamin B2 (mg/d)	1.6 (1.3 – 1.7)	1.6 (1.4 – 1.6)	0.892
Vitamin B6 (mg/d)	1.7 (1.6 – 1.8)	1.6 (1.5 – 1.7)	**0.035**
Vitamin B12 (μg/d)	4.2 (3.6 – 5.2)	3.5 (2.7 – 4.6)	0.088
Methionine (g/d)	2.0 (1.7 – 2.4)	1.9 (1.6 – 2.0)	**0.037**
Choline (mg/d)	348 (306 – 388)	310 (283 – 329)	**0.009**
Betaine (mg/d)	124 (99 – 164)	125 (107 – 148)	0.883

[a] Described as median and interquartile range. All variables were compared using the Mann-Whitney U test.

[b] Significant *p* values shown in bold font.

[c] Presented as dietary folate equivalents (DFEs). 1 μg of DFEs = 1 μg of natural folate sources = 0.6 μg of folic acid from fortified foods.

Table 4

Backward stepwise logistic regression analysis for the dependent variable unmetabolized folic acid (UMFA) concentrations in healthy Brazilian participants, São Paulo, Brazil[a].

Variables	β	SE	OR	95% CI	*p*
Dietary folic acid (μg/d)	0.024	0.008	1.02	1.01 – 1.04	0.004
Sex (male)	−0.912	0.396	0.402	0.185 – 0.874	0.021

UMFA= unmetabolized folic acid, OR = odds ratio; CI = confidence interval.

[a]For the dependent variable UMFA, values were considered above vs. equal or under the median value of 0.55 nmol/L. Independent variables for UMFA concentrations include sex, age, total folate (expressed as dietary folate equivalents [DFEs]), natural folate and dietary folic acid intakes.