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Demographic, physiologic, and lifestyle characteristics observed with serum total folate differ among folate forms: cross-sectional data from fasting samples in the National Health and Nutrition Examination Survey 2011–2016

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Abstract

Background: Serum folate forms were measured in the US population during recent National Health and Nutrition Examination Surveys (NHANES) to assess folate status.

Objective: We describe post-folic acid-fortification concentrations of serum folate forms in the fasting US population ≥1 y from the NHANES 2011–2016.

Design: We measured 5 biologically active folates and 1 oxidation product (MeFox) of 5-methyltetrahydrofolate. We calculated geometric means of 5-methyltetrahydrofolate, unmetabolized folic acid (UMFA), non-methyl folate (sum of tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methenyltetrahydrofolate), total folate (sum of above biomarkers), and MeFox by demographic, physiologic, and lifestyle variables; estimated the magnitude of variables on biomarker concentrations after covariate adjustment; and determined the prevalence of UMFA >2 nmol/L.

Results: After demographic adjustment, age, sex, and race-Hispanic origin were significantly associated with most folate forms. MeFox increased with age, while 5-methyltetrahydrofolate, UMFA, and non-methyl folate displayed an U-shaped age pattern. Compared with non-Hispanic Whites, non-Hispanic Blacks had 23% lower predicted 5-methyltetrahyrofolate but comparable

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UMFA; non-Hispanic Asians had comparable 5-methyltetrahydrofolate but 28% lower UMFA; Hispanics, non-Hispanic Asians, and non-Hispanic Blacks had ~20% lower MeFox. After additional physiologic and lifestyle adjustment, predicted UMFA and MeFox concentrations were 43% and 112% higher, respectively in adults with chronic kidney disease and 17% and 15% lower, respectively in adults consuming daily 1–<2 alcoholic beverages; 5-methyltetrahydrofolate concentrations were 20% lower in adult smokers. The prevalence of UMFA >2 nmol/L was highest in persons ≥70 y (9.01%) and lowest in 12–19 y olds (1.14%). During 2011–2014, the prevalence was 10.6% in users and 2.22% in non-users of folic acid-containing supplements.

Conclusions: In fasting persons ≥1 y, the demographic, physiologic, and lifestyle characteristics observed with serum total folate differed among folate forms, suggesting biological and/or genetic influences on folate metabolism. High UMFA was mostly observed in supplement users and older persons.

Keywords

NHANES; 5-methyltetrahydrofolate; unmetabolized folic acid; folate oxidation product; MeFox; LC-MS/MS

Introduction

The folate status of the US population has been continuously assessed through the National Health and Nutrition Examination Surveys (NHANES)[7] since the 1970s by measuring serum and red blood cell “total” folate (1). During the post-folic acid-fortification period, clinical folate deficiency (i.e., megaloblastic anemia) has nearly disappeared (2). However, monitoring folate insufficiency in women of reproductive age is still of public health concern, because it increases the risk of neural tube birth defects in their offspring (3). Furthermore, interest emerged to assess concentrations of serum folate forms, including unmetabolized folic acid (UMFA) (1). This form appears in circulation when intake of folic acid exceeds the limited ability of the human gut to reduce and methylate folic acid (4-6).

The introduction of liquid-chromatography tandem-mass-spectrometry (LC-MS/MS) to public health laboratories allowed the measurement of 5-methyltetrahydrofolate (5-methylTHF) and UMFA in serum samples from NHANES 2007–2008 (1/3 subset) (7). UMFA was detected in nearly all samples and concentrations >1 nmol/L were largely explained by fasting status and by total folic acid intake from diet and supplements. During 2011–2012, additional non-methyl folate forms [tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-formylTHF), and 5,10-methenyltetrahydrofolate (5,10-methenylTHF)] and an oxidation product of 5-methylTHF (pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF, so called MeFox) were measured for the first time in a full set of NHANES (8). We observed mostly higher concentrations of serum folate forms in non-fasting individuals. Given that plasma folate concentrations respond rapidly to dietary intake, non-fasting concentrations need to be interpreted with caution (9). In NHANES 2011–2012, the associations between each folate form and demographic, physiologic, and lifestyle variables were similar to serum total folate except for MeFox, suggesting the possibility of altered folate metabolism dependent on biological characteristics (e.g., BMI, kidney function, smoking status) (8). The same serum folate forms were measured during 2

recent NHANES survey cycles: 2013–2014 and 2015–2016. We later discovered that the UMFA measurements in 2011–2012 and 2013–2014 were biased ~25% higher due to issues with folic acid calibrator solubility (10). The UMFA calibration bias has been corrected mathematically in NHANES 2011–2014 prior to data release (11) and UMFA results produced during NHANES 2015–2016 are based on a modified procedure that avoided the calibration bias (10).

Our objectives were to describe post-fortification concentrations of serum folate forms for the first time in the fasting US population ≥1 y by various demographic, physiologic, and lifestyle variables using a large data set from NHANES 2011–2016. We also estimated the prevalence of high UMFA concentrations (>2 nmol/L) overall and stratified by use of folic acid-containing supplements (limited to 2011–2014). Lastly, we defined a serum total folate cutoff value associated with high UMFA to aid investigations that may not have UMFA measurements.

METHODS

Participants and study design.

The National Center for Health Statistics at the Centers for Disease Control and Prevention (CDC) conducts the NHANES. The continuous survey collects cross-sectional data on the health and nutritional status of the civilian non-institutionalized US population through home-based interviews combined with medical and physical examinations at a Mobile Examination Center (MEC). NHANES uses a stratified, multistage, probability sample designed to represent the US population. Interview and examination response rates for each survey period are publicly available (12). All respondents gave their informed consent, and the National Center for Health Statistics Research Ethics Review Board approved the NHANES protocol.

Biomarker measurements.

The CDC laboratory analyzed NHANES 2011–2016 serum samples from participants ≥1 y for 5 biologically active folate forms and MeFox using LC-MS/MS (13). Serum total folate was defined as the sum of the 5 biologically active folate forms excluding MeFox which is biologically inactive. Imputed values [limit of detection (LOD) divided by the √2] were used if any folate form result was <LOD. No serum total folate was calculated when 1 of the folate form results was missing. Long-term quality control CVs were <3% for 5-methylTHF, and mostly <10% for other folate forms (Supplementary Table 1). For information on the UMFA calibration bias, see Supplementary Text 1.

Study variables.

We categorized the demographic variables as follows: 7 age groups (1–5, 6–11, 12–19, 20–39, 40–59, 60–69, and ≥70 y), sex (males and females), and 4 race-Hispanic origin groups [Hispanic, non-Hispanic Asian (NHA), non-Hispanic Black (NHB), and non-Hispanic White (NHW)]. We categorized physiologic and lifestyle variables as follows: inflammation as determined by C-reactive protein [CRP; <5 mg/L no inflammation and ≥5 mg/L inflammation (14); only available in NHANES 2015–2016 for persons ≥1 y and measured as

high sensitivity CRP]; kidney function as determined by estimated glomerular filtration rate [eGFR; 0–<60 chronic kidney disease (moderate or severe decrease in eGFR, including kidney failure), 60–<90 mild decrease in eGFR, and ≥90 mL/(min×1.73 m^2) normal eGFR (15,16); available for persons ≥12 y]; BMI [<18.5 underweight, 18.5–<25 normal, 25–<30 overweight, and ≥30 kg/m^2 obese (17); available for persons ≥2 y]; body surface area [BSA, calculated as square root of (height in cm × weight in kg/3600); <1.5 generally represents children, 1.5–<1.8, 1.8–<2.0, and ≥2.0 m^2 generally represents adult men (18); available for persons ≥2 y]; smoking status as determined by serum cotinine [≤10 μg/L nonsmoker and >10 μg/L smoker (19); available for persons ≥3 y]; and alcohol intake as determined by average daily number of "standard" drinks (~15 g alcohol per drink) [no drinks, <1 (not 0), 1–<2, and ≥2 drinks per day (20); available for persons ≥18 y]. In analyses limited to NHANES 2011–2014, we categorized data by use of folic acid-containing dietary supplements (self-reported use during the 24 h prior to visiting the MEC; yes and no; supplement use information not available for 2015–2016).

Statistical analyses.

Statistical analyses were performed using SAS for Windows software version 9.4 (SAS Institute, Cary, NC) and SAS callable SUDAAN software version 11 (RTI, Research Triangle Park, NC) to account for the complex survey design. We calculated non-methyl folate as the sum of 3 minor forms (THF, 5-formylTHF, and 5,10-methenylTHF; non-methyl folate <LOD if all minor forms <LOD and ≥LOD if at least 1 minor form ≥LOD). Using descriptive analysis, we first assessed the concentration distribution of folate forms during each survey cycle for all participants ≥1 y using the 2-y MEC survey weights to account for unequal probabilities of selection and adjustment for non-response (Supplementary Figure 1). We found comparable median concentrations for total folate and 5-methylTHF and only small significant differences for the minor forms present in low concentrations (UMFA, non-methyl folate, and MeFox) (Supplementary Table 2 and Supplementary Methods 1) and thus decided to combine the data for the 3 survey cycles.

Because we noted differences in concentrations of some serum folate forms by fasting status in our previous analysis limited to 2011–2012 (8), we focused the current combined 2011–2016 analysis on samples from fasting persons (no food intake for the past ≥8 h prior to blood draw). Our analytical sample (n=10,070) consisted of fasting persons ≥1 y with complete data for all serum folate forms (including MeFox) and excluded pregnant and lactating women (n=274) (Supplemental Figure 1). We created combined 6-y MEC survey weights to calculate geometric mean concentrations (95% CI) for total folate and each folate form overall and by demographic, physiologic, and lifestyle variables. We assessed unadjusted differences for each variable using a Wald F test. We used multiple linear regression after log transforming the dependent variable, to estimate the magnitude of demographic differences after adjusting for age, sex, and race-Hispanic origin (in fasting persons ≥1 y) and physiologic and lifestyle differences after additionally for demographic variables and eGFR, BMI, serum cotinine, and alcohol intake (in fasting persons ≥20 y). We limited the model including physiologic and lifestyle variables to adults because certain variables were unavailable for children (e.g., alcohol intake, creatinine) and we did not include inflammation because CRP data were only available for 2015–2016. To facilitate the

interpretation of the log-transformed model, we back-transformed the estimated β coefficients, which can be interpreted as the difference between a pair of predicted values. We assessed significance of each variable in the model using a Satterthwaite F test. We report adjusted pairwise comparisons to the reference category; to account for multiple comparisons, the type-I error (α=0.05) was controlled using the sequentially rejective Bonferroni procedure of Hochberg (21) using the Wald F P values from the regression coefficients for each comparison.

We estimated the mean percent contribution of 5-methylTHF, UMFA, and non-methyl folate to serum total folate by demographic variables by first calculating the percent contribution for each participant. We also calculated the mean percent contribution of MeFox to serum total folate plus MeFox. Furthermore, we calculated the mean absolute concentrations and mean percent contributions of 5-methylTHF, UMFA, and non-methyl folate to serum total folate by weighted decile of serum total folate. The decile categories were: 1st (<19.2); 2nd (19.2–<24.3); 3rd (24.3–<29.2); 4th (29.2–<33.9); 5th (33.9–<39.2); 6th (39.2–<45.1); 7th (45.1–<52.4); 8th (52.4–<62.3); 9th (62.3–<76.2); and 10th (≥76.2).

We determined the prevalence of high UMFA concentrations (>2 nmol/L) in our analytical sample by demographic characteristics. We chose this UMFA concentration because it was the 95th percentile in fasting persons ≥1 y in NHANES 2007–2008 and thus represents an unusual concentration (7). The 2007–2008 data were not affected by the UMFA calibration bias and thus did not require adjustment (22). To estimate a cutoff for serum total folate that is associated with high UMFA concentrations (>2 nmol/L), we conducted a Receiver operating characteristic (ROC) analysis using sample data from fasting persons ≥1 y. To identify an optimal cutoff, we minimized the Euclidean distance between the ROC curve and the (0,1) point. To assess the consistency of the serum total folate cutoff, we repeated the ROC analysis in the overall population ≥1 y as a sensitivity analysis.

In exploratory analyses, we limited the NHANES data to 2011–2014 to assess another variable, namely use of folic acid-containing supplements. All statistical comparisons were evaluated at a 2-sided significance level of α=0.05.

Results

Folate biomarker concentrations by demographic characteristics

Of the 23,682 persons ≥1 y with complete serum folate forms data and no missing fasting status data, 42.5% (unweighted; 10,070 persons) reported to have been fasting for at least 8 h prior to the blood draw (Table 1). There was no significant difference between fasting and non-fasting persons with regards to sex and race-Hispanic origin; however, there was a significant difference in age distribution driven by children <12 y who are not requested to fast. There was also a significant difference with regards to all physiologic and lifestyle characteristics except for alcohol intake, likely for the same reason.

In fasting persons, age, sex, and race-Hispanic origin were significantly associated with the various folate forms before (Figure 1) and after controlling for demographic variables (Table 2), except for non-methyl folate (no significant association with sex and race-Hispanic

origin) and MeFox (no significant association with sex after covariate adjustment). We noted U-shaped age patterns for all serum folate forms where concentrations decreased from the 1–5 y age group through the 20–39 y age group and then increased with age. The only exception was for MeFox where concentrations increased with age. Percentiles presented by age, sex, and race-Hispanic origin generally showed the greatest variation by age group (Supplementary Table 3). The overall central 95% reference intervals (2.5^{th}–97.5^{th} percentile, nmol/L) were: serum total folate 13.3–103; 5-methylTHF 11.7–96.6; UMFA 0.27–3.24; non-methyl folate <LOD–4.71; and MeFox 0.38–4.39.

The 3 demographic variables explained a small portion of the biomarker's total variability: serum total folate (R^2=10%), 5-methylTHF (R^2=10%), UMFA (R^2=5.1%), non-methyl folate (R^2=1.9%), and MeFox (R^2=14%) (Table 2). After controlling for demographic variables, children <12 y had ~80–90% higher serum total folate and 5-methylTHF concentrations, while they had ~10% higher UMFA, 20–45% higher non-methyl folate, and ~35% lower MeFox compared with the reference group. Predicted serum total folate, 5-methylTHF, UMFA, and MeFox concentrations were ~20% higher in persons 60–69 y but ~40–50% higher in persons ≥70 y compared with the reference group. Females had significantly higher predicted serum total folate (10%), 5-methylTHF (11%), and UMFA (7%) concentrations, but comparable non-methyl folate and MeFox to males. NHB persons had 21% and 23% lower predicted serum total folate and 5-methylTHF concentrations, respectively, but comparable UMFA with NHW persons. NHA persons on the other hand had comparable predicted serum total folate and 5-methylTHF concentrations but 28% lower UMFA compared with NHW persons. We observed no race-Hispanic origin differences for non-methyl folate. Hispanic, NHA, and NHB persons all had ~20% lower predicted MeFox concentrations compared with NHW persons. An exploratory analysis limited to NHANES 2011–2014 where we additionally adjusted for use of folic acid-containing supplements produced similar results as mentioned above except that Hispanic persons no longer had significantly higher predicted total folate, 5-methylTHF, and UMFA concentrations compared with NHW persons (data not shown). Controlling for supplement use in addition to demographic variables explained approximately twice the variability in biomarker concentration around its mean except for MeFox where we observed no change (data not shown).

Folate biomarker concentrations by physiologic and lifestyle characteristics

In fasting persons ≥1 y, all physiologic and lifestyle variables were significantly associated with total folate, 5-methylTHF, non-methyl folate, and MeFox, while only kidney function and alcohol intake were significantly associated with UMFA (Supplementary Table 4). After controlling for covariates (age, sex, race-Hispanic origin, kidney function, BMI, smoking, and alcohol intake) in persons ≥20 y, kidney function was no longer significantly associated with total folate and 5-methylTHF; BMI, smoking, and alcohol intake were no longer significantly associated with non-methyl folate; and the association between alcohol intake and UMFA became significant (Table 3). In adults with chronic kidney disease, predicted UMFA (43%) and MeFox (112%) concentrations were substantially higher compared with adults with normal kidney function. In obese adults, predicted total folate and 5-methylTHF concentrations were ~14% lower, while MeFox was 13% higher compared with adults with

normal BMI. In smokers, predicted total folate and 5-methylTHF concentrations were ~20% lower, while MeFox was 8.4% higher compared with nonsmokers. Daily consumption of 1–<2 alcoholic beverages resulted in lower predicted UMFA (17%) and MeFox (15%) concentrations compared with no alcohol consumption. The addition of physiologic and lifestyle variables to the model explained only slightly more of the biomarker's total variability compared to demographic variables only: serum total folate (R^2=11%), 5-methylTHF (R^2=11%), UMFA (R^2=7.3%), non-methyl folate (R^2=1.7%), and MeFox (R^2=18%).

Contribution of folate forms to serum total folate

In fasting persons, 5-methylTHF (93.7%) was the biggest contributor to serum total folate, whereas UMFA (2.4%) and non-methyl folate (3.9%) contributed only small amounts (Supplementary Table 5). Although there was a significant age effect for the relative contribution of folate forms to serum total folate, only MeFox showed a clear age pattern with increasing contribution from 1.3% (children 1–5 y) to 4.2% (persons ≥70 y) to total folate plus MeFox. There was no significant sex difference in the relative contribution of UMFA to serum total folate and of MeFox to serum total folate plus MeFox. Although the relative contribution varied significantly by race-Hispanic origin, differences were small.

When we categorized concentrations of folate forms by decile of serum total folate, we noted a mostly linear increase in 5-methylTHF concentrations, while UMFA, non-methyl folate, and MeFox concentrations were mostly constant up to the 8th or 9th decile but showed a clear increase in the 10th decile (Figure 2 and Supplementary Table 6). The relative contribution of folate forms to serum total folate by decile showed some fluctuations for 5-methylTHF (89.2–95.6%) and UMFA (1.8–3.7%), while non-methyl folate showed a decrease with increasing decile (7.1–2.3%) (Supplementary Table 6). In NHANES 2011–2014 (Supplementary Figure 2), about half of the people in the 10th decile of serum total folate were ≥60 y (48%, panel A) and about 2/3 were supplement users (64%, panel D) compared to only 19% and 7.4% in the first decile, respectively.

Prevalence of high UMFA and cutoff for serum total folate to define high UMFA

The overall prevalence of UMFA concentrations >2 nmol/L was 4.18% in the fasting US population ≥1 y in 2011–2016 and 4.67% in 2011–2014 (Table 4). While the prevalence was comparable in males and females (~4%), it differed by age group (from ~1% in persons 12–19 y to ~10% in persons ≥70 y) and race-Hispanic origin (from ~2% in Hispanic persons to ~5% in NHW persons). In 2011–2014, ~10% of supplement users compared to only ~2% of non-users had high UMFA and the prevalence in older supplement users (≥70 y) was 20.3% (Table 4).

In the fasting population ≥1 y, the optimal cutoff for serum total folate obtained by ROC analysis was 56 nmol/L with a sensitivity of 78% and a specificity of 76%; the area-under-the-curve was 0.834 (data not shown). When we repeated the analysis using the overall population ≥1 y, we obtained very similar results: serum total folate optimal cutoff of 54 nmol/L, sensitivity of 78%, specificity of 76%, and area-under-the-curve of 0.848 (data not shown).

Discussion

The uniqueness of this paper is that it describes nationally representative estimates for post-fortification concentrations of serum folate forms and total folate measured by LC-MS/MS in the fasting US population over a 6-y period of NHANES (2011–2016). The demographic, physiologic, and lifestyle characteristics noted for serum total folate differed among folate forms.

Most folate forms displayed an U-shaped age pattern except for MeFox where concentrations increased with age, suggesting more MeFox generation or accumulation in older compared with younger persons. After we adjusted for the effect of age in our model, we saw that impaired kidney function was still associated with higher MeFox concentrations (nearly double in adults with chronic kidney disease), which may suggest impaired clearance as a reason for high MeFox concentrations in older persons. Given that predicted MeFox concentrations were also higher in obese adults and smokers, and that unadjusted MeFox concentrations were higher in inflammation, MeFox may be an indicator of negative health factors. The lower predicted MeFox in Hispanic and NHA persons compared to NHW persons relative to the similar 5-methylTHF concentrations in these 3 groups may indicate different metabolism possibly due to genetic differences. These findings, while mostly consistent with our previous report (8), can be interpreted more reliably as they represent a large fasting data set.

We are unaware of other reports discussing MeFox by various characteristics. Of note is a recent case-control study of daily supplementation with 5 mg folic acid in Brazilian patients with hereditary spherocytosis which found a much higher ratio of MeFox to 5-methylTHF in supplemented patients (~8 nmol/L to ~60 nmol/L) compared to healthy controls (~1 nmol/L to ~30 nmol/L, which was similar to our study) (23). These observations strengthen our working hypothesis that MeFox in serum may not be entirely generated post-blood collection from 5-methylTHF oxidation. We therefore question the approach of using a sum indicator called “methylated folate” (sum of 5-methylTHF and MeFox) to interpret folate status (24). Instead, we suggest to separately report results for 5-methylTHF and MeFox to provide broader utility to investigators. In addition to providing relevant information regarding the quality of sample handling, MeFox data may also provide insights into folate metabolism. While it is still debatable whether to include MeFox as part of the total folate, it only contributes 3.6% and not including MeFox represents a more conservative approach to folate status assessment because it avoids underestimating the prevalence of low folate concentrations.

UMFA concentrations were not proportional to total folate concentrations across race-Hispanic origin groups. Of note are the lower predicted UMFA concentrations in NHA compared with NHW persons relative to the similar total folate in these 2 groups; this holds true even after additionally adjusting for supplement use in NHANES 2011–2014 (data not shown). Also of note are the comparable predicted UMFA concentrations in NHB compared with NHW persons despite NHB persons having lower total folate, which also holds true after supplement use adjustment. These race-Hispanic origin differences in UMFA seem to be independent of supplement use and may point to differences in uptake, reduction, and/or

methylation of folic acid in different population groups. Previous studies found a limited ability of the human gut to reduce and methylate folic acid (4), human liver has been shown to have low and variable dihydrofolate reductase activity (25), and Kalmbach *et al.* found a polymorphism of dihydrofolate reductase which may limit folic acid assimilation into cellular folate stores (26). The higher predicted UMFA concentrations with decreasing kidney function likely indicate impaired clearance, while the lower predicted UMFA and MeFox concentrations with increasing alcohol intake may be a result of increased clearance and/or altered metabolism (27).

Only limited UMFA data are available from other populations. In the UK National Diet and Nutrition Survey Rolling Programme (Years 1 to 4), the median (95% central reference interval; % undetectable) UMFA concentration in 19–64 y olds was 0.33 nmol/L [0.06–1.12 nmol/L; ~30%; (28)] compared with 0.65 nmol/L (0.27–3.24 nmol/L; ~1%) in fasting persons ≥1 y in our study. Other reports of small convenience samples also showed lower median (% undetectable) UMFA concentrations: 0.34 nmol/L (6%) in fasting older Irish persons [voluntary fortification; (29)]; 0.10 nmol/L (80%) in non-pregnant German women who were not using supplements [no fortification; (30)]; and 0.08 nmol/L (74%) in fasting older German persons who were not using supplements [no fortification; (31)].

Interestingly non-methyl folate did not show sex or race-Hispanic origin differences, nor did it show an association with BMI, smoking status, or alcohol intake after covariate adjustment; it only showed a significant positive association with kidney function. This folate component is mostly comprised of THF. If THF is due to UMFA reduction during absorption, one may expect higher THF with higher UMFA concentrations. Earlier we found higher UMFA concentrations in non-fasting *vs.* fasting persons at the upper tail of the distribution [95th percentile of 13.7 *vs.* 2.47 nmol/L in persons ≥1 y, (8)], but comparable non-methyl folate concentrations [5.29 *vs.* 4.99 nmol/L, (8)], suggesting that the appearance of non-methyl folate may be rate-limited.

The prevalence of high UMFA was ~10% or less regardless of demographic subgroup and it was highest among supplement users ≥70 y (~20% in 2011–2014). In 2011–2016, 47.3% of persons with high UMFA belonged to the 10th decile of serum total folate and 79.2% belonged to the 8th decile or higher (data not shown). Our serum total folate cutoff of 56 nmol/L associated with high UMFA (>2 nmol/L) was similar to the cutoff found in a recent case-control study in Brazilian patients with hereditary spherocytosis supplemented with a daily 5 mg dose of folic acid [54 nmol/L, (23)]. The availability of such a cutoff may help other investigators predict the proportion of participants with high UMFA in the absence of measured UMFA data. However, the sensitivity (78%) and specificity (76%) found in our study indicate that there are some false positives and false negatives with this approach. The higher sensitivity (100%) and specificity (91.7%) found in the Brazil study may have been a result of the larger proportion of high UMFA due to the high folic acid dose (23). Confirmation from other studies would be desirable.

The major strength of this study is the use of a large nationally representative, racially and ethnically diverse survey spanning 6 y of NHANES. Because of the large sample size, we were able to focus on the fasting US population and explore folate status across a variety of

variables, which we could not do previously due to sample size limitations (8). For example, in our previous report (overall population) UMFA was significantly associated with BMI and smoking status (8), while in the current analysis (fasting persons) it is not, likely due to the clearance of the circulating UMFA compared to the nonfasting state. Furthermore, MeFox is significantly associated with smoking status in current report, but was not previously (8), likely because of the confounding effect of nonfasting on the association between MeFox and smoking. These examples show that in some instances the interpretation based on the overall population can be misleading. Another strength of this study is that it used accurately calibrated or adjusted UMFA data. While it would be of interest to characterize concentrations of folate forms by intake and/or supplement use, we conducted exploratory analyses by supplement use where this variable was available. Although the clinical significance of serum total folate is well understood, the clinical interpretation of folate forms is yet to be defined. In conclusion, these findings identify population groups susceptible to higher concentrations of folate forms, including UMFA. This in turn may help identify at-risk-populations for potential cause-and-effect relationships between excess folate and adverse health outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

5-formylTHF	5-formyltetrahydrofolate
5,10-methenylTHF	5,10-methenyl-tetrahydrofolate
5-methylTHF	5-methyltetrahydrofolate
BSA	body surface area
CDC	Centers for Disease Control and Prevention
CRP	C-reactive protein
eGFR	estimated glomerular filtration rate
LOD	limit of detection
MEC	Mobile Examination Center

MeFox	pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF
MEC	Mobile Examination Center
NHA	non-Hispanic Asian
NHANES	National Health and Nutrition Examination Survey
NHB	non-Hispanic Black
NHW	non-Hispanic White
ROC	receiver operating characteristic
THF	tetrahydrofolate
UMFA	unmetabolized folic acid

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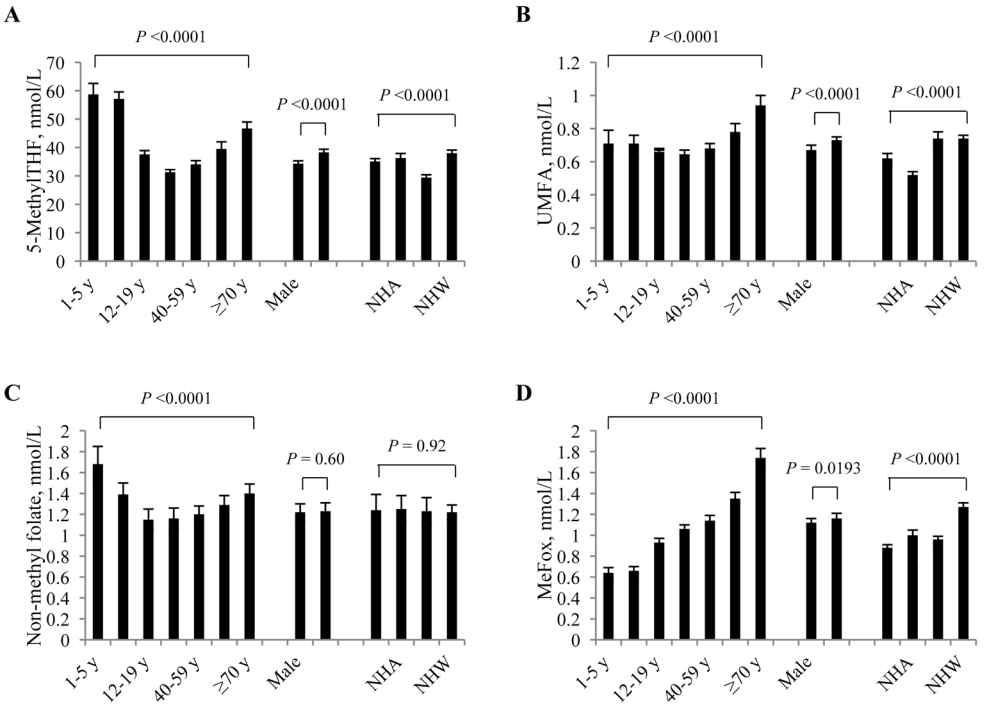

Figure 1.

Weighted geometric mean concentrations of serum folate forms by age, sex, and race-Hispanic origin for the fasting US population ≥1 y, NHANES 2011–2016. Error bars represent 95% CI. Sample sizes by age group, sex, and race-Hispanic origin are same as in Table 1, fasting persons ≥1 y. Non-methyl folate represents the sum of 3 minor forms tetrahydrofolate, 5-formyl-tetrahydrofolate and 5,10-methenyltetrahydrofolate. 5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; UMFA, unmetabolized folic acid.

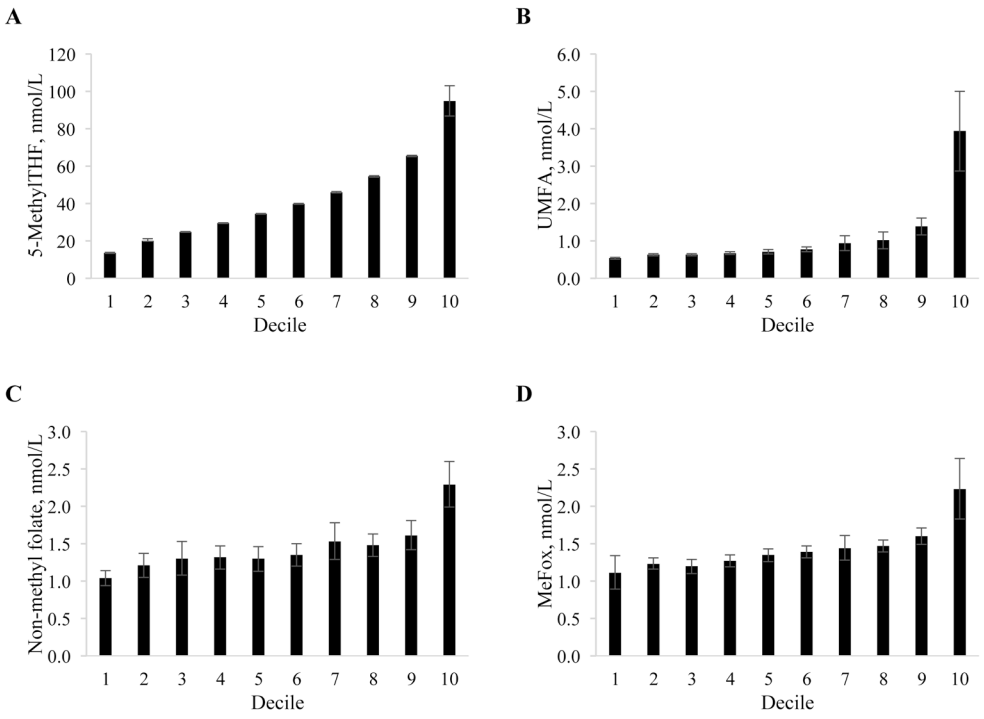

Figure 2.

Weighted mean absolute concentration of 5-methylTHF (panel A), UMFA (panel B), non-methyl folate (panel C), and MeFox (panel D) by weighted decile of serum total folate in the fasting US population ≥1 y of age, NHANES 2011–2016. Error bars represent 95% CI. Sample sizes by decile are same as in Supplementary Table 6 (between 961 and 1041 persons per decile). Serum total folate represents the sum of 5-methylTHF, UMFA, and non-methyl folate. Non-methyl folate represents the sum of 3 minor forms tetrahydrofolate, 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. 5-MethylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolized folic acid.

Table 1.

Participant characteristics by variable categories in the US population ≥1 y, NHANES 2011–2016[1]

	Persons ≥1 y		Fasting persons ≥1 y		Non-fasting persons ≥1 y		
Variables	**Sample size**	**Estimate**	**Sample size**	**Estimate**	**Sample size**	**Estimate**	***P* value[2]**
	n	*%*	*n*	*%*	*n*	*%*	
Overall	23,682	100	10,070	100	13,612	100	NA
Age group, y							<0.0001
1–5	2118	4.5	265	1.1	1853	7.4	
6–11	3021	7.0	706	3.2	2315	10.1	
12–19	3435	10.9	1660	11.3	1775	10.5	
20–39	5017	27.1	2475	28.6	2542	25.8	
40–59	5145	29.2	2545	31.8	2600	27.0	
60–69	2552	11.5	1293	13.9	1259	9.5	
≥70	2394	9.8	1126	10.1	1268	9.6	
Sex							0.88
Male	11,840	49.6	5028	49.7	6812	49.6	
Female	11,842	50.4	5042	50.3	6800	50.4	
Race-Hispanic origin							0.23
Hispanic	6646	17.0	2725	16.4	3921	17.4	
Non-Hispanic Asian	2599	5.1	1174	5.2	1425	5.0	
Non-Hispanic Black	5475	11.5	2359	11.6	3116	11.4	
Non-Hispanic White	7948	63.0	3467	63.7	4481	62.5	
Inflammation[3]							0.0195
CRP <5	6364	81.4	2583	79.8	3781	82.7	
CRP ≥5	1371	18.6	625	20.2	746	17.3	
Kidney function[4]							0.0215
eGFR 0–<60	1326	6.4	579	5.7	747	7.1	
eGFR 60–<90	4691	29.3	2229	29.4	2462	29.3	
eGFR ≥90	12368	64.3	6213	64.9	6155	63.6	
BMI[5]							<0.0001

Variables	Persons ≥1 y		Fasting persons ≥1 y		Non-fasting persons ≥1 y		
	Sample size	Estimate	Sample size	Estimate	Sample size	Estimate	*P* value[2]
Underweight	3867	10.1	913	5.3	2954	14.2	
Normal weight	7036	29.9	3168	30.1	3868	29.8	
Overweight	5741	28.5	2745	30.1	2996	27.1	
Obese	6356	31.5	3118	34.5	3238	28.9	
BSA[6], cm×kg							<0.0001
<1.5	5724	14.8	1499	8.5	4225	20.1	
1.5–1.8	6204	26.7	2958	28.0	3246	25.5	
1.8–2	4963	24.6	2433	26.3	2530	23.2	
≥2	6109	33.9	3054	37.1	3055	31.1	
Smoking status[7]							0.0003
Cotinine ≤10	18605	79.8	7916	77.6	10689	81.6	
Cotinine >10	3952	20.2	2001	22.4	1951	18.4	
Alcohol intake[8]							0.25
No drinks	4823	26.3	2362	26.5	2461	26.1	
<1 (not 0)	7992	59.2	3969	59.3	4023	59.2	
1–<2	1007	8.9	490	8.4	517	9.4	
≥2	656	5.5	352	5.8	304	5.3	

[1]Estimates are weighted percentages. Fasting refers to no food intake for the past ≥8 h prior to blood draw; only participants ≥12 y were requested to fast prior to the blood drawing. Table is limited to participants with no missing fasting status data and with complete serum folate forms data to allow calculation of total serum folate, excluding pregnant women and women who were lactating and/or breastfeeding. BSA, body surface area; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate.

[2]Chi-square Wald *F P* value tests the null hypothesis of no difference in distribution of the proportions for each variable between fasting to non-fasting persons.

[3]CRP (mg/L) used to assess inflammation; measured as serum high sensitivity CRP in participants ≥1 y; only available in NHANES 2015–1016; CRP <5 (no inflammation), CRP ≥5 (inflammation).

[4]eGFR [mL/(min×1.73 m^2)] used to assess kidney function; available for persons ≥12 y; eGFR 0–<60 (chronic kidney disease), eGFR 60–90 (mild decrease in kidney function); eGFR ≥90 (normal kidney function).

[5]BMI (kg/m^2): calculated for participants ≥2 y; <18.5 (underweight); 18.5–>25 (normal weight); 25–<30 (overweight); and ≥30 (obese).

[6]BSA used to assess body size; calculated as square root of [(height in cm × weight in kg)/3600] or square root of [(height in inches × weight in pounds)/3131]; calculated for participants ≥2 y.

[7]Serum cotinine (μg/L) used as biomarker of tobacco smoke exposure; calculated for participants ≥3 y; cotinine ≤10 (nonsmoker), cotinine >10 (smoker).

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[8] Calculated for participants ≥18 y as average daily number of "standard" drinks [(quantity × frequency) / 365.25]; 1 drink ≈ 15 g ethanol.

Table 2.

Model-adjusted percent difference in biomarker concentrations for demographic variables in the fasting US population ≥1 y, NHANES 2011–2016[1]

Variable	Total folate	5-MethylTHF	UMFA	Non-methyl folate	MeFox
			%		
Age group, y					
1–5	90 (78, 102)*	92 (81, 105)*	11 (−1.8, 26)	45 (32, 59)*	−36 (−41, −30)*
6–11	81 (73, 89)*	85 (77, 94)*	12 (4.7, 20)*	20 (13 27)*	−35 (−39, −31)*
12–19	19 (15, 23)*	21 (17, 25)*	2.6 (−1.9, 7.3)	−0.2 (−5.4, 5.4)	−12 (−16, −7.7)*
20–39[2]	Reference	Reference	Reference	Reference	Reference
40–59	7.7 (4.3, 11)*	7.7 (4.2, 11)*	3.4 (−1.5, 8.6)	4.2 (−0.5, 9.0)	5.3 (1.6, 9.2)*
60–69	23 (16, 31)*	23 (15, 31)*	19 (11, 28)*	12 (5.7, 19)*	22 (15, 28)*
≥70	44 (37, 51)*	44 (37, 52)*	41 (31, 51)*	22 (15, 28)*	54 (45, 64)*
P_{model}[3]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Sex					
Male[2]	Reference	Reference	Reference	Reference	Reference
Female	10 (7.4, 14)*	11 (7.8, 14)*	7.0 (3.2, 11)*	0.0 (−2.2, 2.3)	3.0 (−0.2, 6.4)
P_{model}[3]	<0.0001	<0.0001	00004	0.99	0.06
Race-Hispanic origin					
Hispanic	−6.5 (−9.7, −3.2)*	−6.8 (−10, −3.2)*	−12 (−17, −7.8)*	3.1 (−6.4, 14)	−24 (−27, −21)*
Non-Hispanic Asian	−2.1 (−6.3, 2.2)	−1.9 (−6.4, 2.8)	−28 (−31 −24)*	4.1 (−2.9, 12)	−18 (−22, −14)*
Non-Hispanic Black	−21 (−24, −18)*	−23 (−26, −20)*	3.2 (−2.0, 8.6)	1.9 (−5.9, 10)	−20 (−23, −16)*
Non-Hispanic White[2]	Reference	Reference	Reference	Reference	Reference
P_{model}[3]	<0.0001	<0.0001	<0.0001	0.76	<0.0001
R^2 (%)[4]	10	10	5.1	1.9	14

[1] Values are weighted percent difference (95% CI) in the adjusted geometric mean (or predicted value) relative to the reference category. Fasting refers to no food intake for the past ≥8 h prior to blood draw. Total folate is the sum of biologically active folate forms (5-methylTHF, UMFA, and non-methyl folate), not including MeFox. Non-methyl folate is the sum of 3 minor forms (tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methenyltetrahydrofolate). 5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; UMFA, unmetabolized folic acid.

[2] For every comparison to the reference category, the type-I error was controlled using the sequentially rejective Bonferroni procedure of Hochberg (21) using the Wald *F P* values from the regression coefficients; asterisked when $P \leq 0.05$.

[3] P_{model} is the Satterthwaite F P value adjusted for age, sex, and race-Hispanic origin, but not for multiple comparisons; tests the null hypothesis of no difference in the geometric means across the categories for each demographic variable.

[4] The model R^2 indicates the percent of the biomarker's (on the log scale) total variability explained by a model that includes age, sex, and race-Hispanic origin.

Table 3.

Model-adjusted percent difference in biomarker concentrations for physiological and lifestyle variables in the fasting US population ≥20 y, NHANES 2011–2016[1]

Variable	Total folate	5-MethylTHF	UMFA	Non-methyl folate	MeFox
			%		
Kidney function[2]					
eGFR 0–<60	2.9 (−6.8, 14)	0.8 (−8.6, 11)	43 (28, 60)*	12 (3.2, 21)	112 (90, 138)*
eGFR 60–<90	−0.6 (−5.2, 4.2)	−1.1 (−5.8, 3.8)	8.0 (2.0, 14)*	4.3 (−0.8, 9.7)	14 (9.4, 20)*
eGFR ≥90[3]	Reference	Reference	Reference	Reference	Reference
P_{model}[4]	0.72	0.84	<0.00001	0.0302	<0.00001
BMI[5]					
Underweight	−3.6 (−13, 7.0)	−3.1 (−13, 8.2)	1.6 (−7.9, 12)	2.1 (−7.6, 13)	−6.4 (−17, 5.4)
Normal weight[3]	Reference	Reference	Reference	Reference	Reference
Overweight	−5.4 (−9.4, −1.1)	−5.4 (−9.6, −0.9)	−1.5 (−5.9, 3.1)	2.5 (−1.2, 6.3)	3.7 (−2.4, 10)
Obese	−13 (−16, −9.9)*	−14 (−17, −10)*	−5.2 (−10, −0.0)	4.8 (−0.4, 10)	13 (7.4, 20)*
P_{model}[4]	<0.00001	<0.00001	0.08	0.24	<0.00001
Smoking status[6]					
Cotinine ≤10[3]	Reference	Reference	Reference	Reference	Reference
Cotinine >10	−19 (−22, −15)*	−20 (−24, −17)*	0.8 (−4.8, 6.8)	−2.3 (−7.1, 2.8)	8.4 (2.7, 14)*
P_{model}[4]	<0.00001	<0.00001	0.78	0.37	0.0042
Alcohol intake[7]					
No drinks[3]	Reference	Reference	Reference	Reference	Reference
<1 (not 0)	−3.4 (−6.5, −0.1)	−3.4 (−6.6, −0.0)	−9.3 (−13, −5.2)*	−0.3 (−4.9, 4.5)	−9.9 (−13, −6.6)*
1–<2	−9.6 (−16, −3.3)*	−10 (−17, −3.8)*	−17 (−25, −9.0)*	4.3 (−3.6, 13)	−15 (−20, −11)*
≥2	−2.5 (−10, 6.2)	−2.5 (−11, 6.8)	−18 (−26, −9.6)*	7.3 (−2.0, 18)	−12 (−19, −3.9)*
P_{model}[4]	0.0323	0.0254	<0.0001	0.26	<0.00001
R^2 (%)[8]	11	11	7.3	1.7	18

[1] Values are weighted percent difference (95% CI) in the adjusted geometric mean (or predicted value) relative to the reference category. Fasting refers to no food intake for the past ≥8 h prior to blood draw. Total folate is the sum of biologically active folate forms (5-methylTHF, UMFA, and non-methyl folate), not including MeFox. Non-methyl folate is the sum of 3 minor forms (tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methenyltetrahydrofolate). 5-MethylTHF, 5-methyltetrahydrofolate; eGFR, estimated glomerular filtration rate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; UMFA, unmetabolized folic acid.

[2] eGFR [mL/(min×1.73 m^2)] used to assess kidney function; available for persons ≥12 y; eGFR 0–<60 (chronic kidney disease), eGFR 60–90 (mild decrease in kidney function); eGFR ≥90 (normal kidney function).

[3] For every comparison to the reference category, the type-I error was controlled using the sequentially rejective Bonferroni procedure of Hochberg (21) using the Wald *F P* values from the regression coefficients; asterisked when $P \leq 0.05$.

[4] P_{model} is the Satterthwaite *F P* value adjusted for age, sex, race-Hispanic origin, eGFR, BMI, serum cotinine, and alcohol intake, but not for multiple comparisons; tests the null hypothesis of no difference in the geometric means across the categories for each demographic variable.

[5] BMI (kg/m^2): calculated for participants ≥2 y; <18.5 (underweight); 18.5–>25 (normal weight); 25–<30 (overweight); and ≥30 (obese).

[6] Serum cotinine (μg/L) used as biomarker of tobacco smoke exposure; calculated for participants ≥3 y; cotinine ≤10 (nonsmoker), cotinine >10 (smoker).

[7] Calculated for participants ≥18 y as average daily number of “standard” drinks [(quantity × frequency) / 365.25]; 1 drink ≈ 15 g ethanol

[8] The model R^2 indicates the percent of the biomarker’s (on the log scale) total variability explained by a model that includes age, sex, race-Hispanic origin, eGFR, BMI, serum cotinine, and alcohol intake.

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Table 4.

Prevalence of high serum UMFA concentrations by demographic variables and stratified by folic acid-containing supplement use for the fasting US population ≥1 y, NHANES 2011–2016[1]

Variable	2011–2016 Overall	2011–2014 Overall	2011–2014 Supplement users	2011–2014 Supplement non-users
		%		
Overall	4.18 (3.63, 4.81) *10,070*	4.67 (3.96, 5.49) *6781*	10.6 (8.98, 12.6) *1705*	2.22 (1.65, 2.97) *5076*
Age group, y				
1–5	4.08 (1.75, 9.22) *265*	5.57 (2.15, 13.7) *165*	4.22 (1.04, 15.6) *41*	5.98 (1.85, 17.6) *124*
6–11	3.48 (1.93, 6.20) *706*	4.41 (2.24, 8.50) *481*	6.52 (2.19, 17.8) *97*	3.74 (1.91, 7.21) *384*
12–19	1.14 (0.70, 1.84) *1660*	1.15 (0.61, 2.16) *1140*	2.04 (0.44, 8.95) *168*	0.99 (0.53, 1.84) *972*
20–39	2.99 (2.12, 4.21) *2475*	3.59 (2.41, 5.31) *1708*	9.46 (5.99, 14.6) *384*	1.70 (0.93, 3.11) *1324*
40–59	4.27 (3.26, 5.59) 2545	5.00 (3.61, 6.89) *1724*	10.2 (7.38, 13.9) *497*	2.59 (1.53, 4.34) *1227*
60–69	5.55 (3.71, 8.21) *1293*	4.56 (2.66, 7.71) *833*	9.38 (5.71, 15.0) *253*	1.60 (0.46, 5.47) *580*
≥70	9.01 (6.87, 11.70) *1126*	11.1 (8.08, 15.0) *739*	20.3 (14.3, 28.0) *265*	4.53 (2.31, 8.68) *465*
P value[2]	<0.0001	<0.0001	0.0004	0.0004
Sex				
Male	4.45 (3.67, 5.39) *5028*	4.81 (3.80, 6.07) *3379*	11.9 (9.28, 15.2) *782*	2.22 (1.51, 3.24) *2597*
Female	3.91 (3.27, 4.68) *5042*	4.52 (3.77, 5.41) *3402*	9.58 (7.33, 12.4) *923*	2.21 (1.56, 3.14) *2479*
P value[2]	0.30	0.64	0.25	0.10
Race-Hispanic origin				
Hispanic	1.72 (1.26, 2.35) *2725*	1.96 (1.39, 2.75) *1672*	5.74 (3.47, 9.35) *304*	1.08 (0.63, 1.85) *1368*
Non-Hispanic Asian	2.33 (1.52, 3.56) *1174*	2.53 (1.54, 4.11) *814*	10.7 (7.27, 15.4) *345*	1.18 (0.53, 2.59) *594*
Non-Hispanic Black	4.46 (3.58, 5.54) *2359*	4.32 (3.16, 5.87) *1628*	11.8 (9.71, 14.2) *779*	2.60 (1.67, 4.04) *1283*
Non-Hispanic White	5.02 (4.24, 5.94) 3467	5.67 (4.71, 6.83) *2453*	6.12 (3.42, 10.7) *220*	2.63 (1.88, 3.67) *1674*
P value[2]	<0.0001	<0.0001	0.0054	0.0071

[1] Values are weighted percent (95% CI) and sample size (in italics). Fasting refers to no food intake for the past ≥8 h prior to blood draw. UMFA, unmetabolized folic acid.

[2] Chi-square *P* value tests the null hypothesis of no difference in prevalence across the categories for each demographic variable.