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Folate status and concentrations of serum folate forms in the US population: National Health and Nutrition Examination Survey 2011–2

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Abstract

Serum and erythrocyte (RBC) total folate are indicators of folate status. No nationally representative population data exist for folate forms. We measured the serum folate forms (5-methyltetrahydrofolate (5-methylTHF), unmetabolised folic acid (UMFA), non-methyl folate (sum of tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-formylTHF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF)) and MeFox (5-methylTHF oxidation product)) by HPLC–MS/MS and RBC total folate by microbiologic assay in US population ≥1 year (*n* approximately 7500) participating in the National Health and Nutrition Examination Survey 2011–2. Data analysis for serum total folate was conducted including and excluding MeFox. Concentrations (geometric mean; detection rate) of 5-methylTHF (37·5 nmol/l; 100 %), UMFA (1·21 nmol/l; 99·9 %), MeFox (1·53 nmol/l; 98·8 %), and THF (1·01 nmol/l; 85·2 %) were mostly detectable. 5-FormylTHF (3·6 %) and 5,10-methenylTHF (4·4 %) were rarely detected. The biggest contributor to serum total folate was 5-methylTHF (86·7 %); UMFA (4·0 %), non-methyl folate (4·7 %) and MeFox (4·5 %) contributed smaller amounts. Age was positively related to MeFox, but showed a U-shaped pattern for other folates. We generally noted sex and race/ethnic biomarker differences and weak (Spearman's $r < 0{\cdot}4$) but significant ($P<0{\cdot}05$) correlations with physiological and lifestyle variables. Fasting, kidney function, smoking and alcohol intake showed negative associations. BMI and body surface area showed positive associations with MeFox but negative associations with other folates. All biomarkers showed significantly higher concentrations with recent folic acid-containing dietary supplement use. These first-time population data for serum folate forms generally show similar associations with demographic, physiological and lifestyle variables as serum total folate. Patterns observed for MeFox may suggest altered folate metabolism dependent on biological characteristics.

Key words: National Health and Nutrition Examination Survey: Folate vitamers: 5-Methyltetrahydrofolate: Unmetabolised folic acid: Non-methyl folate: Folate oxidation products: Liquid chromatography–MS/MS

The assessment of folate status has a long tradition in the National Health and Nutrition Examination Survey (NHANES). It has been carried out since 1974 with the use of different measurement procedures and serum and erythrocyte (RBC) folate as biomarkers[1]. Early measurements were conducted by microbiologic assay (1974–8), followed by two variants of a radio protein-binding assay (1978–1991 and 1991–2006), and more recently by a much improved microbiologic assay (2007–2010). Folate assays have had continued issues with comparability across laboratories and methods, necessitating the adjustment of data to allow the assessment of trends over time[1,2]. The NHANES 2011–2 survey assessed folate status in the US population for the first time by a combination of two analytical methods: serum folate forms were measured by HPLC–MS/MS, while whole-blood folate was measured by microbiologic assay. RBC folate was then calculated using the data from both assays. This approach was the result of a 2010 expert roundtable that advised Centers for Disease Control

Abbreviations: 5-formylTHF, 5-formyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; BSA, body surface area; CDC, Centers for Disease Control and Prevention; LOD, limit of detection; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF; NHANES, National Health and Nutrition Examination Survey; RBC, erythrocytes; THF, tetrahydrofolate; UMFA, unmetabolised folic acid.

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and Prevention (CDC) on folate biomarkers and methods for future NHANES surveys[1]. It allows clinicians, public health practitioners and researchers to obtain information on the full profile of folate forms, including unmetabolised folic acid (UMFA). This is important for monitoring purposes in the post-fortification era where an extremely low prevalence of folate deficiency is paired with higher folic acid intake from dietary supplements[3]. HPLC–MS/MS is currently the best tool to measure individual folate forms with high sensitivity, specificity and accuracy[1].While this analytical method produced results fully comparable to those of the microbiologic assay for serum folate, this was not the case for the more complex analysis of whole-blood folate[1]. Because these assay differences need to be better understood, HPLC–MS/MS was not used for whole blood folate.

Despite the interest in serum folate forms, currently only information on total folate can be interpreted clinically in the context of folate status. Clearly, the sum of biologically active folate vitamers (5-methyltetrahydrofolate (5-methylTHF), UMFA, tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-formylTHF) and 5,10-methenyltetrahydrofolate (5,10-methenylTHF)) constitutes total folate. However, it remains to be determined whether MeFox (pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF) should be included in the calculation of total folate. This compound is an oxidation product of 5-methylTHF that lacks vitamin biologic activity[4] and is therefore not captured as part of the total folate measurement with the microbiologic assay. While it has been shown that MeFox can be formed *in vitro* after blood collection as a result of suboptimal sample handling[5,6], its possible existence *in vivo* is unclear[5]. If the formation of MeFox occurs solely *in vitro* (i.e. after blood collection), then a small part of the formerly active folate pool is lost and MeFox should be included in the total folate calculation to avoid underestimating the biologically active amount of folate. However, if some or all of the MeFox may already be present *in vivo* for an extended period of time, including it in the total folate calculation may slightly overestimate the biologically active amount of folate (i.e. folate status will appear better than it is because a small part of the total folate is not biologically available).

Our main objective was to describe serum concentrations of several folate forms in the US population ≥1 year participating in the NHANES 2011–2 by selected demographic, physiological, and lifestyle variables. Our other objective was to update information on total folate status and to provide the first nationally representative data for non-Hispanic Asians. We report information on serum total folate with and without the inclusion of MeFox to provide much needed insight on this new topic.

Methods

Participants and study design

The NHANES is conducted by the CDC and collects cross-sectional data on the health and nutritional status of the civilian non-institutionalised US population by use of a stratified, multistage, probability sample design. In addition to obtaining information in a home interview setting, participants undergo a physical examination and blood draw in a mobile examination centre. In 2011–2, the NHANES oversampled Asian and Hispanic persons in addition to oversampling some other population groups[7–9]. The unweighted response rates for participants ≥1 years of age were 72·2 % for the interview component and 69 % for the examination component[10]. All respondents gave their informed consent, and the NHANES protocol was reviewed and approved by the CDC Research Ethics Review Board.

Biomarker measurement

Serum and whole-blood haemolyate samples from participants ≥1 year were analysed by the CDC laboratory for serum folate forms (folate cofactors and MeFox) by use of HPLC–MS/MS[11–13] and for RBC total folate by use of microbiologic assay[14–16], respectively. We did not obtain valid final results for a few samples (<30), resulting in different sample sizes among compounds: serum 5-methylTHF (*n* 7454), UMFA (*n* 7462), THF (*n* 7461), 5-formylTHF (*n* 7466), 5,10-methenylTHF (*n* 7466), MeFox (*n* 7469), and total folate including MeFox (*n* 7442), and RBC total folate (*n* 7867). Sample sizes for the folate biomarkers by covariate categories are presented in Table 1. Because concentrations of the three minor folate forms THF, 5-formylTHF, and 5,10-methenylTHF were often below the limit of detection (LOD) and can be a result of folate interconversions at slightly acidic pH during sample preparation[17], we calculated the sum of these three forms as non-methyl folate. Serum total folate was calculated as the sum of the six folate forms including MeFox. CDC released results <LOD as imputed values (LOD divided by the $\sqrt{2}$); we used the imputed values in our calculation when the folate form result was <LOD[11]. The serum total folate including MeFox result was missing if one of the folate forms was missing. We calculated serum total folate excluding MeFox by subtracting MeFox from total folate including MeFox. RBC total folate was calculated from the measured whole-blood folate concentration after subtracting the serum total folate including MeFox concentration (as determined by HPLC–MS/MS) and adjusting for RBC volume[15]. Assay performance is summarised in the online Supplementary Table S1 and has been described previously with regard to international reference materials for the HPLC–MS/MS [1,18] and microbiologic assay[14].

Study variables

We categorised the demographic variables as follows: age (1–5, 6–11, 12–19, 20–39, 40–59, and ≥60 years), sex (males and females), and race–ethnicity (Hispanic (Mexican American + other Hispanic), non-Hispanic Asian, non-Hispanic Black, and non-Hispanic White; other racial/ethnic groups were included in overall estimates). We also reported separate estimates for Mexican Americans to allow comparison to previous reports. We examined physiological and lifestyle variables previously shown to be associated with folate concentrations[19,20]: fasting

Table 1. Unweighted sample sizes for serum folate forms and serum and erythrocyte (RBC) total folate by selected demographic, physiological and lifestyle variable categories for the US population ≥1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2*

	Serum					RBC
Variable categories	5-MethylTHF	UMFA	Non-methyl folate	MeFox	Total folate	Total folate
All	7454	7462	7458	7469	7442	7867
Age (years)						
1–5	568	573	570	575	563	708
6–11	955	957	959	960	953	1044
12–19	1073	1074	1074	1074	1073	1127
20–39	1730	1730	1730	1730	1730	1762
40–59	1604	1604	1605	1606	1603	1655
≥60	1524	1524	1520	1524	1520	1571
Sex						
Male	3739	3745	3746	3750	3735	3944
Female	3715	3717	3712	3719	3707	3923
Race–ethnicity†						
Hispanic	1798	1803	1803	1806	1794	1919
Mexican American	989	993	995	996	987	1071
Other Hispanic	809	810	808	810	807	848
Non-Hispanic Asian	947	947	948	951	944	999
Non-Hispanic Black	2025	2027	2027	2027	2025	2185
Non-Hispanic White	2401	2402	2398	2402	2397	2471
Other	283	283	282	283	282	293
Fasting time (h)						
<3	2476	2479	2475	2482	2469	2644
3–<8	1633	1637	1635	1637	1631	1744
≥8	3345	3346	3348	3350	3342	3479
eGFR stage‡ (ml/(min × 1·73 m^2))						
0–<60	467	467	466	467	466	466
60–<90	2032	2032	2031	2033	2030	2039
≥90	3382	3383	3383	3384	3381	3428
BMI§ (kg/m^2)						
Underweight	1203	1206	1204	1209	1197	1382
Normal weight	2297	2301	2301	2303	2295	2415
Overweight	1854	1854	1855	1855	1854	1882
Obese	1921	1921	1918	1921	1918	1970
BSA‖ (cm × kg)						
<1·5	1756	1762	1761	1766	1750	1971
1·5–1·8	1989	1990	1989	1991	1987	2069
1·8–2	1596	1596	1596	1597	1595	1640
≥2	1934	1934	1932	1934	1932	1969
Serum cotinine¶ (μg/l)						
≤10	5938	5941	5937	5945	5929	6068
>10	1240	1240	1240	1241	1239	1259
Alcohol intake** (g)						
No drinks	1474	1474	1472	1474	1472	1530
<1 (not 0)	2540	2540	2539	2541	2538	2602
1–<2	342	342	342	342	342	349
≥2	235	235	234	235	234	243
Supplement use††						
Yes	1303	1305	1302	1306	1299	1370
No	5567	5573	5574	5579	5561	5882

5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF; eGFR, estimated glomerular filtration rate; BSA, body surface area.

* Serum folate forms and serum total folate (sum of all forms including MeFox) were measured by HPLC–MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methenyltetrahydrofolate.

† Hispanic sub-group represents sum of Mexican American and other Hispanic ethnicity; other sub-group includes persons with multi-ethnic background.

‡ Used to assess renal function; available for persons ≥12 years; impaired renal function was defined as eGFR <60 ml/(min × 1·73 m^2).

§ BMI definitions: <18·5 kg/m^2 (underweight); 18·5–>25 kg/m^2 (normal weight); 25–<30 kg/m^2 (overweight); and ≥30 kg/m^2 (obese).

‖ Calculated as √((height in cm × weight in kg)/3600) or √((height in inches × weight in pounds)/3131).

¶ Biomarker of tobacco smoke exposure; concentrations >10 μg/l are considered to represent smokers.

** Calculated for participants ≥18 years as average daily number of 'standard' drinks ((quantity × frequency)/365·25); 1 drink approximately 15 g ethanol.

†† Folic acid-containing dietary supplements used during the last 24 h prior to visiting the Mobile Examination Center.

time (<3, 3–<8 and ≥8 h), kidney function as determined by estimated glomerular filtration rate (0–<60, 60–<90, and ≥90 ml/(min × 1·73 m^2))[21], BMI (<18·5 kg/m^2 (underweight), 18·5–<25 kg/m^2 (normal), 25–<30 kg/m^2 (overweight) and ≥30 kg/m^2 (obese))[22], body surface area (BSA, calculated as √(height in cm × weight in kg/3600); <1·5, 1·5–<1·8, 1·8–<2·0, and ≥2·0 m^2)[23], smoking (serum cotinine ≤10 μg/l (nonsmoker) and >10 μg/l (smoker))[24], alcohol

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intake (average daily number of 'standard' drinks (one drink approximately 15 g alcohol): no drinks, <1 (not 0), 1– < 2, and ≥2 drinks/d; only available for participants ≥18 years)[19], and use of folic acid-containing dietary supplements (self-reported use during the 24 h prior to visiting the mobile examination centre (self-reported use during the last 30 d is no longer available in NHANES 2011–2); yes and no). We presented the association between folate concentrations and body size by two variables: BMI, used traditionally in nutrition studies, and BSA, used in exposure studies to provide insight on 'body burden'. A BSA value $<1{\cdot}5\,m^2$ generally represents children, while a value $\geq 2{\cdot}0\,m^2$ generally represents adult men. This additional variable may shed light on the distinction between metabolism *v.* 'dilution effects' due to body size.

Statistical analysis

We applied no exclusion criteria to our data analysis and used pairwise deletion for missing values in a particular analysis. We used the mobile examination centre weights to account for differential non-response or non-coverage and to adjust for oversampling of some groups. We calculated the mean percent contribution of each folate form to serum total folate including MeFox. We also calculated the mean absolute and percent contribution of each folate form to serum total folate including MeFox by weighted decile of serum total folate including MeFox. Bivariate associations between geometric mean folate biomarker concentrations (to normalise for right-skewed distributions) and each study variable were described. Geometric means were compared across the categories without (Wald F *P* value) and with (Satterthwaite F *P* value) controlling for additional covariates (age, sex and race–ethnicity). We used Spearman's coefficients to assess pairwise correlations among folate biomarkers as well as between folate biomarkers and selected physiological and lifestyle variables. We assessed the distributions (geometric means and selected percentiles (95 % CI)) for each folate biomarker among all participants, fasted (≥8 h) participants and non-fasted (<8 h) participants ≥1 year by demographic variables. Significance was defined as a two-sided *P* value of <0·05. Statistical analyses were performed using SAS (version 9; SAS Institute, Inc.) and SUDAAN (version 9.2; RTI) software.

Results

A summary of the characteristics of the study population for each variable of interest is given in the online Supplementary Table S2. Among US population ≥1 year in the unweighted NHANES 2011–2 sample, 27 % were children (1–11 years), 14 % were adolescents (12–19 years) and 59 % were adults (≥20 years). Half of the participants were female and almost one-third of the participants were non-Hispanic white. Approximately half (43 %) of the participants were fasted for ≥8 h, 8 % had an impaired estimated glomerular filtration rate ($<60\,ml/min/1{\cdot}73\,m^2$), 24 % were obese, 30 % had a small BSA ($<1{\cdot}5\,m^2$), 17 % were considered smokers (serum cotinine concentrations >10 μg/l), one-third of participants ≥18 years reported not consuming any alcoholic beverage, and about 20 % of participants reported using folic acid-containing dietary supplements during the last 24 h.

Folate biomarker concentrations and contribution of folate forms to serum total folate

The concentration ranges of serum total folate excluding MeFox, serum total folate including MeFox and RBC total folate were 3·26–375, 3·50–377 and 149–5490 nmol/l, respectively. Concentration ranges of serum folate forms were: 1·88–295 nmol/l for 5-methylTHF, <LOD (0·14)–282 nmol/l for UMFA, <LOD (0·37)–11·5 nmol/l for THF, <LOD (0·30)–31·6 nmol/l for 5-formylTHF, <LOD (0·34)–4·38 nmol/l for 5,10-methenylTHF and <LOD (0·34)–20·4 nmol/l for MeFox. Concentrations of 5-methylTHF (100 %), UMFA (99·9 %), MeFox (98·8 %) and THF (85·2 %) were detectable in all or most samples, while concentrations of 5-formylTHF (3·6 %) and 5,10-methenylTHF (4·4 %) were detectable in only a few samples.

On average, 5-methylTHF (86·7 %) was the biggest contributor to serum total folate including MeFox, while UMFA (4·0 %), non-methyl folate (4·7 %), and MeFox (4·5 %) contributed smaller amounts. When we calculated the contribution of these folate forms by decile of serum total folate including MeFox (Fig. 1 and online Supplementary Table S3), we noted some fluctuation in the proportion of 5-methylTHF (79·9–90·2 %), a decreasing proportion of non-methyl folate (9·1 % in the first, 2·8 % in the last decile) and MeFox (6·6 % in the first, 3·0 % in the last decile), and a generally U-shaped proportion of UMFA (4·5 % in the first, approximately 3 % in the fifth, and 10·4 % in the last decile) with increasing decile of serum total folate including MeFox.

Folate biomarker concentrations by demographic characteristics

We noted approximately U-shaped age patterns for all serum folate forms except MeFox, for which the concentration was significantly higher in persons ≥60 years compared to all other age groups and the proportion of MeFox relative to serum total folate including MeFox was significantly higher in persons ≥60 years compared to the three youngest age groups (Table 2 and online Supplementary Fig. S1). Age was a significant factor for all serum folate forms as well as for serum total folate including and excluding MeFox, and RBC total folate whether or not we controlled for other demographic covariates (sex and race–ethnicity). While females had significantly higher serum total folate including and excluding MeFox, RBC total folate, 5-methylTHF and UMFA concentrations with and without controlling for age and race–ethnicity, there were no sex differences for non-methyl folate and MeFox concentrations. All folate biomarker concentrations except non-methyl folate varied significantly by race–ethnicity, with non-Hispanic whites having the highest concentrations and non-Hispanic Asians having similar concentrations compared to Hispanics. These observations did not change after we controlled for age and sex. The concentration difference between geometric means of serum total

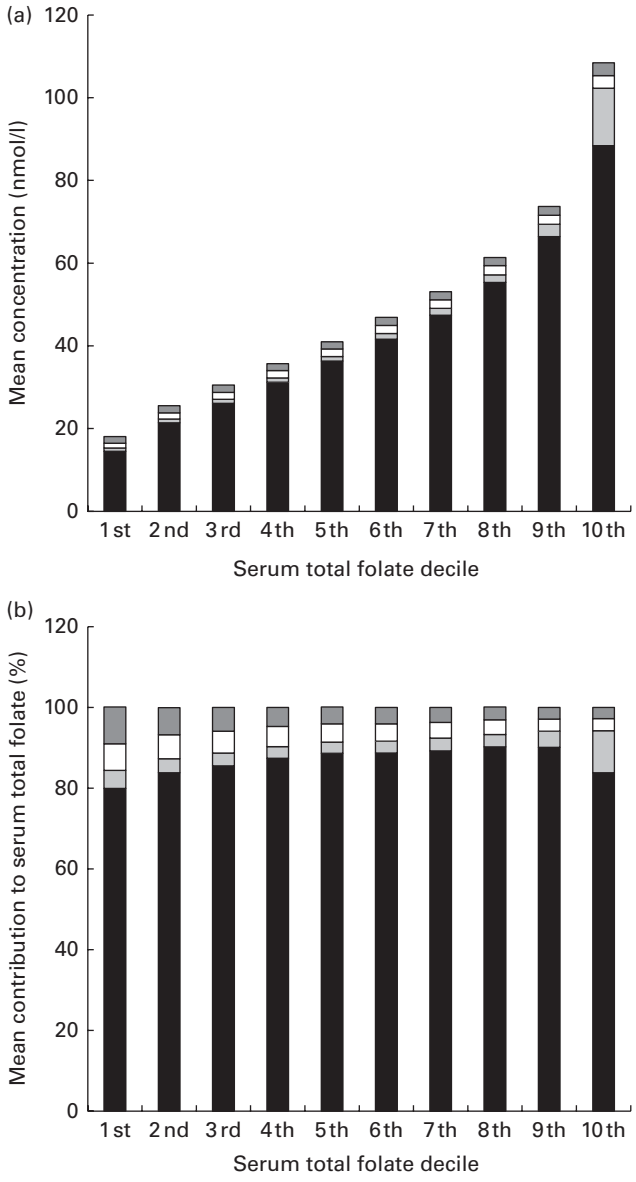

Fig. 1. Mean absolute (a) and relative (b) contribution of folate forms to serum total folate by weighted decile of serum total folate in the US population ≥1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2. Serum folate forms and serum total folate (sum of folate forms including MeFox (pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF, □)) were measured by HPLC–MS/MS. Non-methyl folate (▨) represents the sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. 5-MethylTHF, 5-methyltetrahydrofolate (■); UMFA, unmetabolised folic acid (▪).

folate excluding *v.* including MeFox was approximately 2 nmol/l and it was reasonably consistent across demographic groups.

Correlations among folate biomarkers

Spearman's correlations were significant for most pairwise comparisons of folate biomarkers in persons ≥1 year (see online Supplementary Table S4). We observed significant and strong ($r \geq 0{\cdot}7$) correlations for 5-methylTHF with serum total folate including MeFox (r 0·99) and for THF with non-methyl folate (r 1·0). We observed significant and moderate ($0{\cdot}4 \leq r < 0{\cdot}7$) correlations for 5-methylTHF with RBC total folate (r 0·59), for UMFA with 5-methylTHF (r 0·44) and serum total folate including MeFox (r 0·50), and for serum total folate including MeFox with RBC total folate (r 0·59). We observed significant but weak ($r < 0{\cdot}4$) correlations for 5-methylTHF with MeFox (r 0·25). After stratifying by age group, we noted a strengthening of the correlations in persons ≥60 years (trace covered a larger area than for persons 1–19 or 20–59 years), but the same patterns overall (Fig. 2).

Associations between folate biomarkers and selected physiological and lifestyle characteristics

We observed generally significant but weak Spearman's correlations between folate biomarkers and the continuous physiological and lifestyle variables (Table 3). Fasting, kidney function, smoking and alcohol intake were negatively associated with most folate biomarkers. BMI and BSA showed positive associations with MeFox and negative associations with other folates. We noted generally significant differences in concentrations between the levels of the categorical variables, including categorised versions of the physiological and lifestyle variables, whether or not we controlled for demographic covariates (age, sex and race–ethnicity) (Table 4). All folate biomarkers showed significantly higher concentrations with recent folic acid-containing dietary supplement use whether or not we controlled for demographic covariates. The concentration difference between serum total folate excluding *v.* including MeFox was approximately 2 nmol/l and it was again reasonably consistent across categories of variables.

Reference intervals and distributions of folate biomarker concentrations

Because fasting was a significant factor for most folate biomarkers, we calculated the central 95 % reference intervals (2·5th–97·5th percentile) for all, fasted, and non-fasted 'generally healthy' persons ≥1 year (Table 5). Reference intervals were fairly comparable among these three groups for 5-methylTHF, non-methyl folate, serum total folate excluding and including MeFox, and RBC total folate. However, we noted a lower upper end of the reference interval in fasted persons for UMFA and MeFox.

Selected percentiles (5th–95th) presented by age, sex and race–ethnicity for all, fasted, and non-fasted persons ≥1 year generally showed the greatest variation by age group (see online Supplementary Tables S5–S11). We observed distinct differences in the distributions of serum total folate excluding and including MeFox, RBC total folate and 5-methylTHF by age group. We also observed differences in the upper end of the distribution of UMFA by age group, higher non-methyl folate concentrations at the lower end of the distribution for children 1–5 years compared to any other age group, and a right-shift in the distribution of MeFox with increasing age group. The differences we observed in the central

Table 2. Concentrations of serum folate forms and serum and erythrocyte (RBC) total folate by demographic variable categories for the US population ≥1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2*

(Geometric mean values and 95 % confidence intervals)

	Serum (nmol/l)												RBC (nmol/l)	
	5-MethylTHF		UMFA		Non-methyl folate		MeFox		Total folate without MeFox		Total folate with MeFox		Total folate	
Variable	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI
All	37·5	36·0, 39·1	1·21	1·15, 1·28	1·59	1·32, 1·92	1·53	1·45, 1·61	41·4	40·1, 42·9	43·4	42·0, 44·9	1050	1010, 1090
Age (years)														
1–5	53·6	50·5, 57·0	1·72	1·46, 2·02	1·92	1·68, 2·18	1·31	1·12, 1·52	59·5	56·1, 63·2	61·2	57·7, 65·0	1030	985, 1070
6–11	53·2	50·3, 56·4	1·55	1·36, 1·78	1·75	1·44, 2·13	1·39	1·27, 1·51	58·5	55·4, 61·8	60·4	57·2, 63·7	1060	1020, 1090
12–19	37·1	34·8, 39·5	1·10	1·02, 1·17	1·44	1·18, 1·75	1·33	1·19, 1·48	40·5	38·4, 42·7	42·2	39·9, 44·5	933	899, 969
20–39	32·0	30·6, 33·3	1·04	.968, 1·12	1·55	1·22, 1·96	1·41	1·32, 1·51	35·5	34·2, 36·8	37·3	36·0, 38·6	961	922, 1000
40–59	34·3	32·3, 36·4	1·14	1·04, 1·24	1·49	1·25, 1·78	1·51	1·43, 1·60	38·1	36·0, 40·3	40·0	37·8, 42·3	1060	1000, 1110
≥60	44·4	42·1, 46·8	1·49	1·36, 1·64	1·77	1·49, 2·11	1·98	1·86, 2·11	49·0	46·7, 51·5	51·7	49·3, 54·2	1260	1200, 1330
P	<0·0001		<0·0001		<0·0001		<0·0001		<0·0001		<0·0001		<0·0001	
P adjusted	<0·0001		<0·0001		0·0002		<0·0001		<0·0001		<0·0001		<0·0001	
Sex														
Male	35·8	34·0, 37·6	1·17	1·09, 1·25	1·57	1·30, 1·90	1·50	1·40 1·60	39·6	38·0, 41·2	41·5	39·8, 43·2	1030	982, 1070
Female	39·2	37·6, 40·9	1·26	1·18, 1·34	1·60	1·33, 1·94	1·55	1·48, 1·64	43·3	41·7, 45·1	45·4	43·7, 47·2	1070	1040, 1110
P	0·0002		0·0438		0·15		0·18		0·0004		0·0005		0·0040	
P adjusted	0·0001		0·0349		0·25		0·23		0·0002		0·0002		0·0039	
Race–ethnicity†														
Hispanic	36·1	34·3, 38·0	1·01	0·924, 1·12	1·73	1·26, 2·38	1·26	1·20, 1·33	40·0	38·1, 42·0	41·7	39·8, 43·6	961	937, 984
MA	37·4	34·8, 40·2	0·984	0·872 1·11	1·78	1·28, 2·47	1·21	1·13, 1·29	41·2	38·9, 43·5	42·8	40·5, 45·1	973	940, 1010
NH Asian	37·2	35·4, 39·1	0·945	0·864, 1·03	1·60	1·28, 2·00	1·59	1·45, 1·74	40·9	39·1, 42·8	43·1	41·2, 45·1	952	911, 994
NH Black	29·8	28·2, 31·4	1·19	1·12, 1·26	1·83	1·35, 2·46	1·21	1·16, 1·26	34·0	32·2, 35·8	35·6	33·8, 37·4	860	830, 892
NH White	39·4	38·0, 40·9	1·30	1·23, 1·37	1·52	1·29, 1·79	1·66	1·59, 1·74	43·3	41·9, 44·9	45·5	44·0, 47·0	1130	1080, 1170
P	<0·0001		<0·0001		0·35		<0·0001		<0·0001		<0·0001		<0·0001	
P adjusted	<0·0001		<0·0001		0·21		<0·0001		<0·0001		<0·0001		<0·0001	

5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF; MA, Mexican American; NH, non-Hispanic.

* Serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC–MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methenyltetrahydrofolate; for sample sizes, see Table 1; *P* value is the unadjusted Wald F *P*-value, while *P* value adjusted is the Satterthwaite F *P* value adjusted for age, sex and race–ethnicity.

† Hispanic sub-group represents the sum of MA and other Hispanic ethnicity; *P* values for race–ethnicity show comparison of Hispanic, NH White, NH Black, NH Asian and other (not shown).

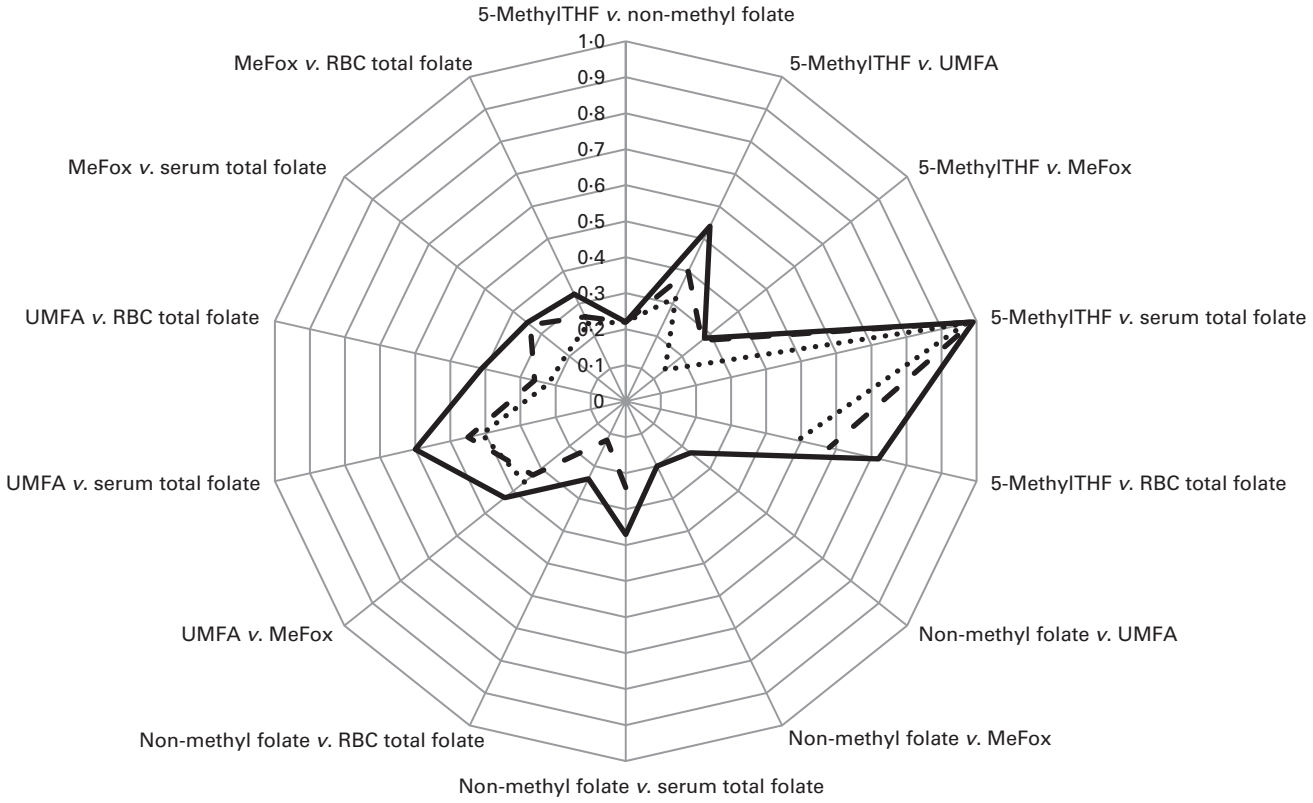

Fig. 2. Spearman's correlation between various folate biomarkers by age group in the US population ≥1 years, National Health and Nutrition Examination Survey (NHANES) 2011–2. Only statistically significant correlations are shown (see online Supplementary Table S4 for complete information). Serum folate forms and serum total folate (sum of folate forms including MeFox (pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF)) were measured by HPLC–MS/MS. Non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. Erythrocyte (RBC) total folate was measured by microbiologic assay. 5-MethylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid. ···, 1–19 years; - - -, 20–59 years; —, ≥60 years.

95 % reference intervals between all and fasted persons were also notable in the entire distribution of folate concentrations.

Folate status time trend

Serum total folate excluding MeFox concentrations was similar in 2011–2 (geometric mean 41·4 (95 % CI 40·1, 42·9) nmol/l) compared to that in the previous two survey cycles, when the microbiologic assay was used, which does not respond to MeFox: 2007–8 (geometric mean 39·5 (95 % CI 37·7, 41·3) nmol/l) and 2009–2010 (geometric mean 38·2 (95 % CI 37·2, 39·3) nmol/l). RBC total folate concentrations measured in all three survey cycles by microbiologic assay also appeared to be similar: 2007–8 (geometric mean 1120 (95 % CI 1070, 1160) nmol/l), 2009–2010 (geometric mean 1040 (95 % CI 1010, 1070) nmol/l) and 2011–2 (geometric mean 1050 (95 % CI 1010, 1090) nmol/l). As in the previous two survey cycles, <1 % of the US population in NHANES 2011–2 had serum (<10 nmol/l) or RBC total folate (<340 nmol/l) concentrations at risk for deficiency[25].

Discussion

The present study provides the first national reference information for serum folate forms measured by HPLC–MS/MS in a population exposed to folic acid fortification. It also offers a better understanding of variables associated with concentrations of serum folate forms. Based on the newest serum and RBC total folate concentrations from NHANES 2011–2, the folate status of the US population was comparable to that in the previous years and non-Hispanic Asians had similar folate concentrations compared to Hispanics.

Previous studies that assessed the profile of serum folate forms used convenience samples and were small in size (mostly <100 subjects). Most studies investigated special population subgroups such as pregnant women, older adults or haemodialysis patients[26–30], while a few studies measured serum folate forms in apparently healthy US, German or Norwegian adults, though generally as part of method validations[5,17,31–34]. Given that the population in the present study was exposed to folic acid fortification and known to have a historical prevalence of folic acid supplement use of approximately 35 %[35], the higher 5-methylTHF (38·5 nmol/l) and UMFA (0·991 nmol/l) median concentrations compared to the small convenience sample reports for German (15·8 and 0·10 nmol/l, respectively,[34]) or Norwegian (16·4 and 0·0 nmol/l, respectively[5] adults from countries with no folic acid fortification were not surprising. However, caution should be used when comparing data from different

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Table 3. Spearman's correlations between various folate biomarkers and selected physiological and lifestyle variables for the US population ≥1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2*

	Serum						RBC
Variable categories	5-MethylTHF	UMFA	Non-methyl folate	MeFox	Total folate without MeFox	Total folate with MeFox	Total folate
Fasting time							
Spearman's *r*	−0·02	−0·19	0·01	−0·34	−0·04	−0·06	0·02
P	0·10	<0·0001	0·39	<0·0001	0·0093	0·0005	0·07
eGFR†							
Spearman's *r*	−0·07	−0·17	−0·07	−0·23	−0·07	−0·08	−0·19
P	<0·0001	<0·0001	0·07	<0·0001	<0·0001	<0·0001	<0·0001
BMI‡							
Spearman's *r*	−0·22	−0·08	−0·02	0·08	−0·22	−0·22	0·07
P	<0·0001	0·0021	0·31	<0·0001	<0·0001	<0·0001	0·0020
BSA§							
Spearman *r*	−0·27	−0·10	−0·05	0·04	−0·27	−0·26	0·04
P	<0·0001	0·0005	0·0241	0·0040	<0·0001	<0·0001	0·0344
Serum cotinine‖							
Spearman's *r*	−0·25	−0·14	−0·02	−0·06	−0·25	−0·24	−0·18
P	<0·0001	<0·0001	0·71	0·0097	<0·0001	<0·0001	<0·0001
Alcohol intake¶							
Spearman's *r*	−0·10	−0·14	−0·04	−0·13	−0·10	−0·11	−0·03
P	0·0022	<0·0001	0·05	<0·0001	0·0022	0·0012	0·21

RBC, erythrocyte; 5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF; eGFR, estimated glomerular filtration rate; BSA, body surface area.

* Serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC–MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyl-tetrahydrofolate, and 5,10-methenyltetrahydrofolate; for sample sizes, see Table 1.

† Used to assess renal function; available for persons ≥12 years; impaired renal function was defined as eGFR <60 ml/(min × 1·73 m^2).

‡ BMI definitions: <18·5 kg/m^2 (underweight); 18·5–>25 kg/m^2 (normal weight); 25–<30 kg/m^2 (overweight); and ≥30 kg/m^2 (obese).

§ Calculated as √((height in cm × weight in kg)/3600) or √((height in inches × weight in pounds)/3131).

‖ Biomarker of tobacco smoke exposure; concentrations >10 μg/l are considered to represent smokers.

¶ Calculated for participants ≥18 years as average daily number of 'standard' drinks ((quantity × frequency)/365·25); 1 drink approximately 15 g ethanol.

populations, in part due to potential method differences that have historically plagued folate analyses[36].

The 5-methylTHF concentration was the biggest and a constant contributor to serum total folate regardless of the population (86·7 % in US (this study) compared to 87·2 % in German[34] and 85·8 % in Norwegian persons[5]). The higher mean UMFA concentration (13·5 nmol/l) and relative contribution (10·2 %) in the highest decile of serum total folate including MeFox compared to the lower deciles (0·78–2·87 nmol/l, 2·75–4·45 %) in the present study are likely due to the larger intake of folic acid from dietary supplements and/or fortified foods and the incomplete conversion of folic acid to 5-methylTHF upon absorption[37–39]. A previous report from NHANES 2007–8 showed that UMFA concentrations >1 nmol/l were largely explained by total folic acid intake from diet and supplements apart from fasting status[40]. Not surprisingly, we found significantly higher serum folate forms as well as serum and RBC total folate concentrations in persons who reported consuming folic acid-containing dietary supplements during the last 24 h.

Given that MeFox is an oxidation product of 5-methylTHF, the correlation between these two folate forms (*r* 0·25) was lower than expected. This may indicate that factors beyond the amount of circulating 5-methylTHF may influence the generation of MeFox. Thus, the relevance of MeFox in relation to folate status is likely of interest in any population, regardless of whether they have high folate status as a result of fortification or supplementation or not. The high correlations between 5-methylTHF and serum total folate (*r* 0·99) and between THF and non-methyl folate (*r* 1·00) were expected, as these two folate forms were the major contributors to serum total folate and non-methyl folate, respectively. We found a correlation between UMFA and 5-methylTHF (*r* 0·54 for persons ≥60 years) similar to that reported for older German adults (*r* 0·42 at baseline and *r* 0·56 after supplementation with folic acid)[30]. We found lower correlations between UMFA and THF (*r* 0·22) or between 5-methylTHF and THF (*r* 0·30) in US older persons compared to the report in German older adults (at baseline: *r* 0·39 and *r* 0·51, respectively; after supplementation: *r* 0·45 and *r* 0·56, respectively)[30].

Among demographic variables studied, we found interesting patterns with age. While most folate forms displayed the typical U-shaped age pattern previously documented with serum and RBC total folate[41], concentrations of MeFox showed a linear pattern and were highest in persons ≥60 years. The distribution of MeFox concentrations showed a right-shift with increasing age group, resulting in higher detection rates of MeFox in persons ≥60 years (99·7 % compared to 95·5 % in children 1–5 years). Conversely, detection rates of THF were highest in children 1–5 years (96·1 % compared to 82·9–89·1 % for other age groups). These observations may indicate altered folate metabolism, possibly as a result of ageing, and will have to be confirmed in other studies. It is interesting though to note that older age was associated with less bioactive folate (THF) and more biologically inactive folate (MeFox), possibly pointing to an increased catabolism.

Table 4. Concentrations of serum folate forms and serum and erythrocyte (RBC) total folate by selected physiological and lifestyle variables for the US population ≥1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2*

(Geometric mean values and 95 % confidence intervals)

	Serum (nmol/l)												RBC (nmol/l)	
	5-MethylTHF		UMFA		Non-methyl folate		MeFox		Total folate without MeFox		Total folate with MeFox		Total folate	
Variable categories	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI
Fasting time (h)														
<3	39·1	37·5, 40·7	1·52	1·44, 1·61	1·57	1·31, 1·88	1·93	1·84, 2·03	43·7	42·2, 45·3	46·2	44·6, 47·8	1040	1000, 1080
3–<8	37·8	35·8, 40·0	1·40	1·29, 1·52	1·66	1·37, 2·00	1·96	1·83, 2·10	42·2	40·1, 44·4	44·8	42·5, 47·1	1050	988, 1120
≥8	36·3	34·7, 37·9	0·978	0·923, 1·04	1·57	1·29, 1·92	1·17	1·10, 1·25	39·6	38·2, 41·1	41·1	39·6, 42·7	1060	1020, 1090
P	<0·0001		<0·0001		0·19		<0·0001		<0·0001		<0·0001		0·25	
P adjusted	0·0108		<0·0001		0·44		<0·0001		0·0170		0·0006		0·43	
eGFR† (ml/(min × 1·73 m^2))														
0–<60	42·3	39·1, 45·8	1·74	1·57, 1·92	1·80	1·46, 2·22	2·89	2·66, 3·15	47·5	43·8, 51·4	51·3	47·6, 55·2	1330	1240, 1430
60–<90	36·5	34·8, 38·3	1·24	1·15, 1·33	1·57	1·31, 1·89	1·61	1·54, 1·68	40·4	38·7, 42·2	42·5	40·7, 44·3	1100	1060, 1140
≥90	34·6	33·0, 36·3	1·05	0·988, 1·13	1·52	1·24, 1·88	1·37	1·26, 1·48	38·1	36·6, 39·7	39·9	38·3, 41·5	982	939, 1030
P	0·0001		<0·0001		0·0406		<0·0001		<0·0001		<0·0001		<0·0001	
P adjusted	0·48		0·0006		0·26		<0·0001		0·42		0·11		0·0019	
BMI‡														
Underweight	49·3	46·3, 52·4	1·62	1·43, 1·83	1·76	1·49, 2·07	1·36	1·22, 1·52	54·7	51·7, 57·8	56·6	53·5, 59·7	1010	982, 1040
Normal weight	38·8	36·9, 40·8	1·21	1·14, 1·30	1·55	1·28, 1·88	1·47	1·36, 1·59	42·8	41·0, 44·8	44·7	42·8, 46·8	1010	973, 1050
Overweight	36·7	35·4, 38·2	1·21	1·14, 1·29	1·55	1·28, 1·87	1·52	1·44, 1·61	40·5	39·2, 41·9	42·4	41·0, 43·9	1060	1020, 1110
Obese	33·3	31·6, 35·0	1·09	0·998, 1·18	1·61	1·30, 1·99	1·65	1·56, 1·76	36·9	35·2, 38·6	39·0	37·3, 40·8	1090	1040, 1150
P	<0·0001		0·0002		0·0048		0·0001		<0·0001		<0·0001		0·0068	
P adjusted	0·0004		0·0015		0·73		0·0046		0·0004		0·0006		0·0026	
BSA§ (cm×kg)														
<1·5	50·2	47·8, 52·8	1·55	1·39, 1·72	1·78	1·50, 2·10	1·41	1·31, 1·52	55·5	53·1, 58·0	57·4	54·9, 60·0	1040	1020, 1060
1·5–1·8	38·5	36·2, 40·9	1·23	1·15, 1·32	1·56	1·28, 1·90	1·53	1·43, 1·64	42·6	40·2, 45·1	44·6	42·2, 47·2	1020	975, 1060
1·8–2	36·0	33·9, 38·1	1·15	1·09, 1·22	1·55	1·27, 1·89	1·53	1·43, 1·63	39·6	37·5, 41·8	41·6	39·4, 43·8	1050	995, 1110
≥2	33·0	31·3, 34·8	1·11	1·02, 1·20	1·56	1·29, 1·89	1·57	1·48, 1·67	36·6	35·0, 38·2	38·6	36·9, 40·2	1080	1030, 1140
P	<0·0001		<0·0001		0·0019		0·0009		<0·0001		<0·0001		0·24	
P adjusted	0·0053		0·0131		0·63		0·0159		0·0036		0·0040		0·0309	
Serum cotinine‖ (μg/l)														
≤10	39·6	37·8, 41·4	1·25	1·19, 1·32	1·60	1·33, 1·93	1·54	1·46, 1·63	43·6	42·0, 45·3	45·6	43·9, 47·4	1080	1040, 1130
>10	29·3	27·6, 31·2	1·04	0·917, 1·17	1·52	1·22, 1·89	1·49	1·38, 1·61	32·9	31·3, 34·7	34·8	33·1, 36·7	928	877, 981
P	<0·0001		0·0025		0·26		0·24		<0·0001		<0·0001		<0·0001	
P adjusted	<0·0001		0·0052		0·68		0·22		<0·0001		<0·0001		<0·0001	
Alcohol intake¶ (g)														
No drinks	38·2	36·6, 40·0	1·33	1·25, 1·42	1·68	1·36, 2·06	1·81	1·73, 1·89	42·5	40·6, 44·4	44·8	42·9, 46·8	1090	1030, 1140
<1 (not 0)	35·7	33·9, 37·6	1·18	1·09, 1·27	1·54	1·26, 1·89	1·53	1·44, 1·61	39·5	37·7, 41·4	41·4	39·6, 43·4	1060	1010, 1100
1–<2	31·7	28·0, 35·9	1·06	0·949, 1·18	1·60	1·34, 1·91	1·45	1·30, 1·61	35·3	31·6, 39·5	37·2	33·3, 41·4	1110	981, 1250
≥2	30·5	27·6, 33·6	0·855	0·732, 0·998	1·52	1·19, 1·93	1·35	1·14, 1·59	33·6	30·7, 36·7	35·3	32·4, 38·5	1010	940, 1080
P	0·0021		0·0003		0·14		<0·0001		0·0018		0·0010		0·28	
P adjusted	0·0162		0·0002		0·57		0·0017		0·0108		0·0074		0·39	
Supplement use**														
Yes	52·9	51·0, 54·9	2·10	1·92, 2·29	1·78	1·49, 2·13	1·79	1·67, 1·92	59·2	56·9, 61·5	61·5	59·2, 63·9	1360	1290, 1430
No	33·6	32·1, 35·2	1·02	0·964, 1·09	1·53	1·26, 1·87	1·44	1·37, 1·52	37·0	35·7, 38·4	38·9	37·5, 40·4	973	940, 1010
P	<0·0001		<0·0001		0·0043		<0·0001		<0·0001		<0·0001		<0·0001	
P adjusted	<0·0001		<0·0001		0·0008		0·0028		<0·0001		<0·0001		<0·0001	

5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF; eGFR, estimated glomerular filtration rate; BSA, body surface area.

* Serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC–MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyl-tetrahydrofolate and 5,10-methenyltetrahydrofolate; for sample sizes, see Table 1; *P* value is the unadjusted Wald F *P*-value, while *P* value adjusted is the Satterthwaite F *P* value adjusted for age, sex and race–ethnicity.

† Used to assess renal function; available for persons ≥12 years; impaired renal function was defined as eGFR <60 ml/(min × 1·73 m^2).

‡ BMI definitions: <18·5 kg/m^2 (underweight); 18·5–>25 kg/m^2 (normal weight); 25–<30 kg/m^2 (overweight); and ≥30 kg/m^2 (obese).

§ Calculated as $\sqrt{}$((height in cm × weight in kg)/3600) or $\sqrt{}$((height in inches × weight in pounds)/3131).

‖ Biomarker of tobacco smoke exposure; concentrations >10 μg/l are considered to represent smokers.

¶ Calculated for participants ≥18 years as average daily number of 'standard' drinks ((quantity × frequency)/365·25); 1 drink approximately 15 g ethanol.

** Folic acid-containing dietary supplements used in the last 24 h prior to visiting the Mobile Examination Center.

The associations of serum folate forms with physiological and lifestyle variables were generally consistent with reports for serum total folate from NHANES 2003–6[19,20]. While most serum folate forms showed higher concentrations in non-fasted persons, we found no difference in RBC total folate concentrations by fasting time, consistent with the prevailing knowledge that RBC total folate concentrations are not affected by fasting[42]. The negative association of serum folate forms and of serum and RBC total folate with decreasing kidney function was no longer significant for several folate biomarkers (5-methylTHF, non-methyl folate, total folate excluding and including MeFox) after we controlled for demographic covariates. Most serum folate forms showed lower concentrations in smokers and in persons with higher alcohol intake.

Some observations with physiological and lifestyle variables, particularly for MeFox, were unexpected and may indicate altered folate metabolism, possibly challenging the notion that MeFox occurs solely *in vitro*. BMI and BSA were positively associated with MeFox, but negatively with 5-methylTHF concentrations. This is counter-intuitive if one assumes that higher 5-methylTHF concentrations will lead to higher MeFox concentrations. It may in fact indicate a greater 'loss' of active folate in obesity. Whether this has any bearing on epidemiologic observations linking obesity with a higher rate of neural tube defects[43] is unclear, particularly if one considers that in the present study obese *v.* normal weight persons had approximately 5·5 nmol/l lower 5-methylTHF, but only approximately 0·2 nmol/l higher MeFox concentrations. We need a better understanding of these relationships to appropriately interpret them. The lower serum total folate (and higher RBC total folate) concentrations reported previously with higher BMI led to the hypothesis that cellular uptake and tissue distribution of folate may be altered by BMI[44]. The existing data by BSA category may instead imply a 'dilution effect' whereby most folate forms and serum total folate concentrations were lower in larger persons. This interpretation was also consistent with the finding that children <11 years had higher 5-methylTHF and serum total folate concentrations compared to all other age groups.

We found a median MeFox concentration (1·49 nmol/l) similar to that reported in Norwegian adults (2·3 nmol/l; the authors called this compound 4α-hydroxy-5-methylTHF but stated that it was the pyrazino-*s*-triazine derivative[5]), suggesting that concentrations of this oxidation product are low as long as the blood is processed under controlled conditions. Yet, we found apparent inconsistencies regarding MeFox and 5-methylTHF concentrations (Table 6), which raised the possibility that MeFox may be formed *in vivo* rather than solely *in vitro* as part of 5-methylTHF oxidation. In rats, administration of radio-labelled folic acid resulted in the excretion of several labelled products: a precursor of MeFox (4α-hydroxy-5-methylTHF), 5-methylTHF, 10-formyl-folic acid, and *p*-aminobenzoyl-L-glutamate, a folate breakdown product[45]. It is therefore conceivable that a small portion of MeFox measured in serum may already be in circulation at the time of the blood draw. We know that MeFox can be formed during the pre-analytical phase

Table 5. Central 95 % reference intervals for serum folate forms and for serum and erythrocyte (RBC) total folate in the US population ≥ 1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2*

	All persons				Fasted persons				Non-fasted persons			
Analyte	2·5th	95 % CI	97·5th	95 % CI	2·5th	95 % CI	97·5th	95 % CI	2·5th	95 % CI	97·5th	95 % CI
Serum 5-methylTHF (nmol/l)	12·3	11·4, 13·2	95·4	92·0, 99·8	11·7	10·8, 12·5	96·1	91·4, 104	13·2	11·6, 13·9	94	91·5, 96·6
Serum UMFA (nmol/l)	0·410	0·368, 0·449	17·2	14·3, 21·0	0·383	0·341, 0·416	4·93	2·99, 9·70	0·450	0·397, 0·480	28·0	219, 37·7
Serum non-methyl folate (nmol/l)		<LOD	5·94	5·31, 7·53		<LOD	5·89	5·06, 7·69		<LOD	6·08	5·50, 7·62
THF		<LOD	5·38	4·74, 7·16		<LOD	5·37	4·55, 7·37		<LOD	5·39	4·83, 7·34
5-FormylTHF		<LOD	0·340	<LOD, 0·392		<LOD	0·336	<LOD, 0·406		<LOD	0·343	0·317, 0·383
5,10-MethenylTHF		<LOD	0·478	0·414, 0·570		<LOD	0·480			<LOD	0·629	0·532, 0·775
Serum MeFox (nmol/l)	0·445	0·401, 0·475	6·13	5·70, 6·77	0·384	<LOD, 0·424	4·75	4·20, 5·55	0·536	0·496, 0·565	7·01	6·66, 7·59
Serum total folate (nmol/l)												
Excluding MeFox	14·7	13·6, 15·9	107	103, 112	14·1	13·3, 14·8	103	98·6, 111	16·0	14·5, 17·0	111	104, 124
Including MeFox	16·0	14·5, 16·9	109	106, 115	14·7	13·8, 16·0	105	100, 113	17·1	16·4, 18·0	114	108, 126
RBC total folate	477	460, 493	2320	2200, 2520	476	445, 505	2340	2250, 2480	477	460, 492	2300	2100, 2560

5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid; THF, tetrahydrofolate; 5-formylTHF, 5-formyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; MeFox, pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF.

* Serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC–MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: THF, 5-formylTHF, and 5,10-methenylTHF; for sample sizes, see Table 1.

Table 6. Inconsistencies between serum MeFox (pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methylTHF) and 5-methyltetrahydrofolate (5-methylTHF) concentrations in the US population ≥1 year, National Health and Nutrition Examination Survey (NHANES) 2011–2

Variable	Finding
Correlation between MeFox and 5-methylTHF	Unexpectedly low (*r* 0·25) considering that MeFox is an oxidation product of 5-methylTHF
Age	MeFox concentrations seemed to increase linearly with age, while 5-methylTHF concentrations showed a U-shaped age pattern
Sex	MeFox concentrations did not differ by sex, while 5-methylTHF concentrations were higher in females
Fasting status	MeFox concentrations were much lower in fasted (≥8 h) compared to non-fasted (<3 h) persons (39 %), while 5-methylTHF concentrations were only a little bit lower (7 %); the central 95 % reference intervals for serum total folate excluding and including MeFox were almost the same in fasted persons, but not in non-fasted persons
Kidney function	MeFox concentrations were much higher (111 %) in persons with poor (eGFR < 60 ml/(min × 1·73 m^2) compared to good (eGFR ≥90 ml/(min × 1·73 m^2) kidney function, while 5-methylTHF concentrations were only a little bit higher (22 %)
BMI	MeFox concentrations were higher (21 %) with higher BMI (obese *v.* underweight), while 5-methylTHF concentrations were lower (32 %)
BSA	MeFox concentrations were higher (11 %) with higher BSA (≥2 *v.* < 1·5 cm × kg), while 5-methylTHF concentrations were lower (34 %)
Smoking status	MeFox concentrations did not differ by smoking status, while 5-methylTHF concentrations were lower (26 %) in smokers

eGFR, estimated glomerular filtration rate; BSA, body surface area.

(blood collection and processing)[5,6], leading to a small loss of 'active' folate after blood is collected. We have shown that the analytical phase of the CDC LC–MS/MS method does not generate additional MeFox[11]. Thus, it appears that failure to include MeFox as part of serum total folate may slightly underestimate folate status if MeFox is mostly formed *in vitro*, while including it may slightly overestimate folate status if MeFox is mostly formed *in vivo*. Mechanistic studies that further explore the origins of this oxidation product are needed.

The present study is subject to some limitations. The data are based on only one NHANES survey period limiting our ability to generalise findings for some stratifications. While we evaluated the association of folate biomarkers with recent use of folic acid-containing dietary supplements as part of selected lifestyle factors, evaluating dietary folate intake overall and according to intake sources (fortified cereal-grain foods, ready-to-eat cereals, supplements, and combinations thereof) was beyond the scope of the present study. Lastly, because these are the first national estimates of folate vitamers, we are limited in our ability to compare findings to other studies of similar magnitude.

In summary, these novel data on serum folate forms generally show associations between these compounds and selected demographic, physiological and lifestyle variables similar to those reported previously for serum total folate. However, particularly for MeFox, we observed distinct patterns with the variables studied that may suggest altered folate metabolism dependent on biological characteristics. Thus, measuring MeFox as part of the folate profile may provide relevant information in populations with high or low folate status. While we cannot as yet answer the question of whether it is more accurate to include or exclude MeFox from the total folate, the difference between the two approaches is rather small (approximately 5 %). Based on the findings of the present study and for practical reasons, we suggest that until an unequivocal answer is found, MeFox should not be included in the calculation of serum total folate, but should be separately reported to allow an assessment of the quality of sample handling as well as potential insight into folate metabolism. This approach has two advantages: the total folate without MeFox can be directly compared to the microbiologic assay and other assays that do not measure this biologically inactive form and one errs on the side of caution with the interpretation of folate status by slightly underestimating it. The new reference intervals for serum folate forms in a population that has been exposed to folic acid fortification for over 15 years provide a much-needed benchmark to researchers and public health officials in those nations in which folic acid fortification has already occurred (e.g. the USA and Canada) and where folic acid intakes are significant contributors to total folate intakes, but also to nations that consider folic acid fortification (e.g. the UK).

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114515001142

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References

1. Yetley EA, Pfeiffer CM, Phinney KW, *et al.* (2011) Biomarkers of folate status in the National Health and Nutrition Examination Survey (NHANES): a roundtable summary. *Am J Clin Nutr* **94**, 303S–312S.
2. Pfeiffer CM, Hughes JP, Durazo-Arvizu RA, *et al.* (2012) Changes in measurement procedure from a radioassay to a microbiologic assay necessitates adjustment of serum and RBC folate concentrations in the U.S. population from the NHANES 1988–2010. *J Nutr* **142**, 894–900.
3. Yang Q, Cogswell ME, Hamner HC, *et al.* (2010) Folic acid source, usual intake, and folate and vitamin B_{12} status in US adults: National Health and Nutrition Examination Survey (NHANES) 2003–2006. *Am J Clin Nutr* **91**, 64–72.
4. Gregory JF III (1989) Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folates. *Adv Food Nutr Res* **33**, 1–101.
5. Hannisdal R, Ueland PM & Svardal A (2009) Liquid chromatography–tandem mass spectrometry analysis of folate and folate catabolites in human serum. *Clin Chem* **55**, 1147–1154.
6. Hannisdal R, Ueland PM, Eussen SJ, *et al.* (2009) Analytical recovery of folate degradation products formed in human serum and plasma at room temperature. *J Nutr* **139**, 1415–1418.
7. Centers for Disease Control and Prevention (2013) *National Health and Nutrition Examination Survey: Analytic Guidelines, 2011–2012*. Washington, DC: Centers for Disease Control and Prevention. http://www.cdc.gov/nchs/data/nhanes/analytic_guidelines_11_12.pdf (accessed October 2014).
8. Johnson CL, Dohrmann SM, Burt VL, *et al.* (2014) National Health and Nutrition Examination Survey: sample design, 2011–2014. *Vital Health Stat* **2**, 1–33.
9. Mirel LB, Mohadjer LK, Dohrmann SM, *et al.* (2013) National Health and Nutrition Examination Survey: Estimation procedures, 2007–2010. *Vital Health Stat* **2**, 1–25.
10. Centers for Disease Control and Prevention (2014) *NHANES Response Rates and Population Totals*. Washington, DC: Centers for Disease Control and Prevention. http://www.cdc.gov/nchs/nhanes/response_rates_CPS.htm (accessed October 2014).
11. Fazili Z & Pfeiffer CM (2013) Accounting for an isobaric interference allows correct determination of folate vitamers in serum by isotope dilution–liquid chromatography–tandem mass spectrometry. *J Nutr* **143**, 108–113.
12. Centers for Disease Control and Prevention (2014) *Laboratory Procedure Manual: Folate Forms, Serum/Whole Blood, by Liquid Chromatography Tandem Mass Spectrometry*. Washington, DC: Centers for Disease Control and Prevention. http://www.cdc.gov/nchs/data/nhanes/nhanes_11_12/FOLFMS_G_met_FOLATE_FORMS.pdf (accessed October 2014).
13. Centers for Disease Control and Prevention (2014) *National Health and Nutrition Examination Survey: 2011–2012 Data Documentation, Codebook, and Frequencies. Serum Folate Forms (FOLFMS_G)*. Washington, DC: Centers for Disease Control and Prevention. http://www.cdc.gov/nchs/nhanes/nhanes2011-2012/FOLFMS_G.htm (accessed October 2014).
14. Pfeiffer CM, Zhang M, Lacher DA, *et al.* (2011) Comparison of serum and red blood cell folate microbiologic assays for national population surveys. *J Nutr* **141**, 1402–1409.
15. Centers for Disease Control and Prevention (2014) *Laboratory Procedure Manual: Total Folate, Serum/Whole Blood, by Microbiologic Assay*. Washington, DC: Centers for Disease Control and Prevention. http://www.cdc.gov/nchs/data/nhanes/nhanes_11_12/FOLATE_G_met_RBC.pdf (accessed October 2014).
16. Centers for Disease Control and Prevention (2014) *National Health and Nutrition Examination Survey: 2011–2012 Data Documentation, Codebook, and Frequencies. RBC Folate (FOLATE_G)*. Washington, DC: Centers for Disease Control and Prevention. http://www.cdc.gov/nchs/nhanes/nhanes2011-2012/FOLATE_G.htm (accessed October 2014).
17. Fazili Z & Pfeiffer CM (2004) Measurement of folates in serum and conventionally prepared whole blood lysates: Application of an automated 96-well plate isotope-dilution tandem MS method. *Clin Chem* **50**, 2378–2381.
18. Fazili Z, Whitehead RD Jr, Paladugula N, *et al.* (2013) A high-throughput LC–MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. *Anal Bioanal Chem* **405**, 4549–4560.
19. Pfeiffer CM, Sternberg MR, Schleicher RL, *et al.* (2013) Dietary supplement use and smoking were strongly related to biomarkers of water-soluble vitamin status after adjusting for socioeconomic and lifestyle factors in a representative sample of US adults. *J Nutr* **143**, 957S–965S.
20. Haynes BMH, Pfeiffer CM, Sternberg MR, *et al.* (2013) Selected pre-analytical and physiological factors are weakly to moderately associated with twenty-nine biomarkers of diet and nutrition, NHANES 2003–2006. *J Nutr* **143**, 1001S–1010S.
21. Stevens LA, Coresh J, Greene T, *et al.* (2006) Assessing kidney function – measured and estimated glomerular filtration rate. *N Engl J Med* **354**, 2473–2483.
22. World Health Organization (2000) *Obesity: Preventing and Managing the Global Epidemic. Report of a WHO Consultation. WHO Technical Report Series* no. 894. Geneva: WHO.
23. Mosteller RD (1987) Simplified calculation of body-surface area. *N Engl J Med* **317**, 1098.
24. Pirkle JL, Flegal KM, Bernert JT, *et al.* (1996) Exposure of the US population to environmental tobacco smoke. *J Am Med Assoc* **275**, 1233–1240.
25. de Benoist B (2008) Conclusions of a WHO technical consultation on folate and vitamin B_{12} deficiencies. *Food Nutr Bull* **29**, S238–S244.
26. Ghandour H, Bagley PJ, Shemin D, *et al.* (2002) Distribution of plasma folate forms in hemodialysis patients receiving high daily doses of L-folinic or folic acid. *Kidney Int* **62**, 2246–2249.
27. Wang Y, Zhang H-Y, Liang Q-L, *et al.* (2008) Simultaneous quantification of 11 pivotal metabolites in neural tube defects by HPLC–electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **863**, 94–100.
28. Bodnar LM, Himes KP, Venkataramanan R, *et al.* (2010) Maternal serum folate species in early pregnancy and risk of preterm birth. *Am J Clin Nutr* **92**, 864–871.
29. Obeid R, Kasoha M, Kirsch SH, *et al.* (2010) Concentrations of unmetabolized folic acid and primary folate forms in pregnant women at delivery and in umbilical cord blood. *Am J Clin Nutr* **92**, 1416–1422.

30. Obeid R, Kirsch SH, Kasoha M, *et al.* (2011) Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults. *Metabolism* **60**, 673–680.
31. Summers CM, Mitchell LE, Stanislawska-Sachadyn A, *et al.* (2010) Genetic and lifestyle variables associated with homocysteine concentrations and the distribution of folate derivatives in healthy premenopausal women. *Birth Defects Res (Part A)* **88**, 679–688.
32. Pfeiffer CM, Fazili Z, McCoy LF, *et al.* (2004) Determination of folate vitamers in human serum by stable-isotope dilution tandem mass spectrometry and comparison to radioassay and microbiologic assay. *Clin Chem* **50**, 423–432.
33. Fazili Z, Pfeiffer CM & Zhang M (2007) Comparison of serum folate species analyzed by LC–MS/MS with total folate measured by microbiologic assay and BioRad radioassay. *Clin Chem* **53**, 781–784.
34. Kirsch SH, Knapp J-P, Herrmann W, *et al.* (2010) Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography–tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **878**, 68–75.
35. Bailey RL, Dodd KW, Gahche JJ, *et al.* (2010) Total folate and folic acid intake from foods and dietary supplements in the United States: 2003–2006. *Am J Clin Nutr* **91**, 231–237.
36. Shane B (2011) Folate status assessment history: implications for measurement of biomarkers in NHANES. *Am J Clin Nutr* **94**, 337S–342S.
37. Kelly P, McPartlin J, Goggins M, *et al.* (1997) Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. *Am J Clin Nutr* **65**, 1790–1795.
38. Sweeney MR, McPartlin J, Weir DG, *et al.* (2006) Postprandial serum folic acid response to multiple doses of folic acid in fortified bread. *Br J Nutr* **95**, 145–151.
39. Patanwala I, King MJ, Barrett DA, *et al.* (2014) Folic acid handling by the human gut: implications for food fortification and supplementation. *Am J Clin Nutr* **100**, 593–599.
40. Pfeiffer CM, Sternberg MR, Fazili Z, *et al.* (2015) Unmetabolized folic acid is detected in nearly all serum samples from US children, adolescents, and adults. *J Nutr* **145**, 520–531.
41. Pfeiffer CM, Hughes JP, Lacher DA, *et al.* (2012) Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988–2010. *J Nutr* **142**, 886–893.
42. Pfeiffer CM, Schleicher RL & Caldwell KL (2013) Biochemical indices. In *Encyclopedia of Human Nutrition*, 3rd ed., pp. 156–174 [B Caballero, editor]. Waltham, MA: Academic Press vol 1,.
43. Rasmussen SA, Chu SY, Kim SY, *et al.* (2008) Maternal obesity and risk of neural tube defects: a metaanalysis. *Am J Obstet Gynecol* **198**, 611–619.
44. Tinker SC, Hamner HC, Berry RJ, *et al.* (2012) Does obesity modify the association of supplemental folic acid with folate status among nonpregnant women of childbearing age in the United States? *Birth Defect Res A Clin Mol Teratol* **94**, 749–755.
45. Barford PA, Staff FJ & Blair JA (1978) The metabolic fate of [2-^{14}C]folic acid and a mixture of [2-^{14}C]- and [3′,5′,9-^{3}H]-folic acid in the rat. *Biochem J* **174**, 579–583.