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Relationship of several serum folate forms with kidney function and albuminuria: cross-sectional data from the National Health and Nutrition Examination Surveys (NHANES) 2011–2018

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Abstract

We aim to examine the relation of several folate forms (5-methyltetrahydrofolate (5-mTHF), unmetabolised folic acid (UMFA) and MeFox) with kidney function and albuminuria, which remained uncertain. The cross-sectional study was conducted in 18 757 participants from National Health and Nutrition Examination Survey 2011–2018. The kidney outcomes were reduced estimated glomerular filtration rate (eGFR) (<60 ml/min/1·73 m^2), microalbuminuria (albumin:creatinine ratio (ACR) of 30–299 mg/g) and macroalbuminuria (ACR ≥ 300 mg/g). Overall, there were significant inverse associations between serum 5-mTHF and kidney outcomes with significant lower prevalence of reduced eGFR (OR, 0·71; 95 % CI: 0·57, 0·87) and macroalbuminuria (OR, 0·65; 95 % CI: 0·46, 0·91) in participants in quartiles 3–4 (*v.* quartiles 1–2; both $P_{\text{for trend}}$ across quartiles <0·05). In contrast, there were significant positive relationship between serum UMFA and kidney outcomes with significant higher prevalence of reduced eGFR in participants in quartiles 2–4 (*v.* quartile 1; OR, 2·12; 95 % CI: 1·45, 3·12; $P_{\text{for trend}}$ <0·001) and higher prevalence of macroalbuminuria in participants in quartile 4 (*v.* quartiles 1–3; OR, 1·46; 95 % CI: 1·06, 2·01; $P_{\text{for trend}}$ <0·001). However, there was no significant associations of 5-mTHF and UMFA with microalbuminuria. In addition, there were significant positive relationships of serum MeFox with reduced eGFR, microalbuminuria and macroalbuminuria (all $P_{\text{for trend}}$ <0·01). In conclusion, higher 5-mTHF level, along with lower UMFA and MeFox level, was associated with lower prevalence of kidney outcomes, which may help counsel future clinical trials and nutritional guidelines regarding the folate supplement.

Key words: **5-methyltetrahydrofolate: Unmetabolised folic acid: MeFox: Reduced eGFR: Albuminuria**

Chronic kidney disease (CKD) is a foremost worldwide public health problem and substantially increases the risk of progression to end-stage renal disease, CVD and mortality[1,2]. The age-standardised global prevalence of CKD in adults is 11·8 % in women and 10·4 % in men[3]. It is estimated that over 30 million American adults have CKD, and the prevalence of recognised CKD has steadily risen year after year across all stages of CKD[4]. Therefore, new treatment approaches to this problem are critically needed, and non-traditional risk factors have received considerable interest.

Several observational studies have shown a significant association between high concentrations of plasma total homocysteine and the risk of developing albuminuria and CKD[5–8]. However, only one interventional study found that homocysteine-lowering folic acid treatment can significantly delay the progression of CKD among hypertensive patients without folic acid fortification[9], and others using high doses of folic acid have shown no benefit or harmful effects on renal outcomes in regions with folic acid fortification[10,11]. One interpretation is that chronic exposure to folic acid or high intake of folic acid may induce saturation of the capacity to convert folic acid to the metabolically active 5-methyltetrahydrofolate (5-mTHF), resulting in the presence of unmetabolised folic acid (UMFA) in the circulation[12,13], which may reduce natural killer cell cytotoxicity[14,15].

Abbreviations: ACR, albumin:creatinine ratio; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; 5-mTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; NHANES, National Health and Nutrition Examination Surveys; UMFA, unmetabolised folic acid.

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Most importantly, previous studies have found that treatment with 5-mTHF *v.* folic acid improved survival rate in haemodialysis patients[16]. Therefore, it is speculated that increased risks associated with UMFA may mask the benefit of 5-mTHF on kidney function. However, to date, few studies have been conducted to assess the association between serum folate forms and the risk of CKD.

To address the above important knowledge gaps, using data from continuous National Health and Nutrition Examination Surveys (NHANES) 2011–2018, we aimed to examine the relation of several serum folate forms (5-mTHF, UMFA and MeFox (pyrazino-s-triazine derivative of 4*α*-hydroxy-5-methyltetrahydrofolate, an oxidation product of 5-mTHF)) with the risk of CKD and examine any possible effect modifiers in a large and nationally representative sample of USA adults.

Methods

Study design and population

In order to monitor the health and nutritional status of the USA civilian non-institutionalised population, NHANES was conducted every year and released on a 2-year basis by the National Center for Health Statistics of the Centers for Disease Control and Prevention, with a stratified multistage probability sample. Detailed survey operation manuals, consent documents and brochures of each period are available on the NHANES website[17]. NHANES was approved by the National Center for Health Statistics Institutional Review Board, and all participants signed an informed consent.

In this study, we analysed data from NHANES 2011–2018 (n 39 156), where a comprehensive list of serum folate forms has been measured by isotope-dilution HPLC coupled to tandem mass spectrometry (LC–MS/MS), and the folate forms concentrations were comparable over time (online Supplementary Table 1)[18].We restricted our analysis to persons who were ≥ 18 years without pregnancy, as well as not receiving dialysis. Of the 23 492 participants, 4735 were excluded due to missing information on serum folate forms concentrations or serum creatinine or urine albumin:creatinine ratio (ACR) at baseline. Therefore, a total of 18 757 subjects were enrolled in our present analysis (online Supplementary Fig. 1).

Measurements of serum folate forms

Specimen donors were recommended fast prior to specimen collection, but fasting is not required. Details on fasting before blood draw were collected via questionnaire before blood draw. Serum samples were analysed for 5-mTHF, UMFA and MeFox using LC–MS/MS by the Centers for Disease Control and Prevention laboratory[19]. Quality control procedures were consistent across cycles, and long-term quality control CV were <3 % for 5-methylTHF and <10 % for UMFA and MeFox[18,20–23]. Imputed values (limit of detection (LOD) divided by square root of two) were used if any folate form result was <LOD. Since UMFA measurements in 2011–2014 were biased about 25 % higher due to issues with folic acid calibrator solubility, the UMFA calibration bias has been corrected mathematically in NHANES 2011–2014 prior to data release and UMFA results produced during NHANES 2015–2018 are based on a modified procedure that avoided the calibration bias[24,25].

Ascertainment of kidney function and kidney damage

During NHANES mobile examination centre screenings, serum creatinine was measured using a kinetic rate Jaffe method in NHANES 2011–2016 while using an enzymatic method in NHANES 2017–2018. To appropriately estimate glomerular filtration rate (GFR), regression models were used to correct serum creatinine values in NHANES 2017–2018 as detailed at –https://wwwn.cdc.gov/Nchs/Nhanes/2017–2018/BIOPRO_J.htm. The estimated GFR (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation[26]. Urine albumin concentration was measured in a random, single-voided urine sample using a solid-phase fluorescent immunoassay, and urine creatinine was measured using a Jaffe rate reaction. Urinary ACR was computed and reported in milligrams per gram.

The primary kidney outcome was reduced eGFR, defined as eGFR <60 ml/min/1·73 m^2. The secondary outcome was microalbuminuria, defined as an ACR of 30 mg/g to 299 mg/g, and macroalbuminuria, defined as an ACR of 300 mg/g or higher.

Assessments of covariates

Covariates were selected *a priori* based on biological plausibility and prior empirical evidence associated with folate and kidney function. Detailed information on covariates was available through standardised questionnaires during interviews, including age, sex, race/ethnicity, education level, smoking status, use of folic acid-containing supplements (self-reported use during the 24 h prior to visiting the Mobile Examination Center) and self-reported history of diabetes and hypertension. Baseline body measurement was performed during mobile physical examination. BMI was calculated as weight (kg) divided by the square of height (m^2). Serum total cholesterol were measured enzymatically, and HDL-cholesterol was measured by direct immunoassay. Haemoglobin A1c (HbA1c) was measured using high-pressure liquid chromatography.

Statistical analysis

All statistical analyses accounted for complex survey design factors for NHANES, including sample weights, stratification and clustering, following NHANES analytic and reporting guidelines[17]. Considering potential non-linear relation of folate forms and kidney outcome and unknown cut-offs for folate forms, we divided folate forms into quartiles for the entire study. Characteristics are presented as mean ± standard error (SE) for continuous variables and as proportions for categorical variables. Comparison of characteristics according to quartiles of serum 5-mTHF and UMFA was performed by χ^2 test for categorical variables or ANOVA for continuous variables.

Binomial regression models (i.e. general linear models with the logit link) were used to estimate OR and 95 % CI for kidney outcomes (binary variable, no and yes) according to quartiles of folate forms with the lowest quartile as the reference group.

Variables known as traditional or suspected risk factors for kidney disease[9], or those showed significant differences among different folate levels were selected as covariables. Therefore, model 1 was adjusted for age (years, continuous) and sex (female and male), and model 2 was further adjusted for BMI (continuous), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American and others), education level (less than high school, high school or equivalent and college or above), smoking status (never, past and current), history of diabetes (no and yes) and hypertension (no and yes), total cholesterol (continuous), HDL-cholesterol (continuous) and HbA1c (continuous). We used Bayesian information criterion to determine goodness of fit, and model 2 had lower Bayesian information criterion than crude model and model 1. Furthermore, in order to further improve statistical power, if there were significant relationships between folate forms and outcomes, quartiles were further pooled as post hoc exploratory analyses according to prevalence rate and adjusted OR, to generate binary variables for subsequent subgroup analyses.

To further evaluate whether several folate forms have a confounding effect on one another, stratified analyses by serum 5-mTHF, UMFA and MeFox were performed. As additional exploratory analyses, possible modifications on the relationship of 5-mTHF and UMFA with outcome were assessed for social and demographic variables including age (<55 *v.* ≥ 55 years), sex, BMI (<25 *v.* ≥ 25 kg/m^2) and current smoker (no *v.* yes), which were selected *a priori* based on prior empirical evidence.

A two-tailed $P < 0{\cdot}05$ was considered to be statistically significant in all analyses. Analyses were performed using R 3.6.2 software (http://www.R-project.org/) and R package survey.

Results

Baseline characteristics of the participants

Among the 18 757 adult participants, the prevalence of reduced eGFR, microalbuminuria or macroalbuminuria was 6·5 % (*n* 1438), 8·2 % (*n* 1875) and 1·4 % (*n* 398), respectively. Characteristics of the study participants with weighted estimates and unweighted sample sizes according to quartiles of serum 5-mTHF and UMFA are presented in Table 1. The mean values of serum 5-mTHF, UMFA and MeFox were 40·6 nmol/l, 1·9 nmol/l and 1·9 nmol/l, respectively. Participants with folic acid-containing supplements and those without fasting have higher serum folate levels. Among those without folic acid-containing supplements, female have higher 5-mTHF (average 36·3 nmol/l for female and 32·1 nmol/l for male) and UMFA (average 1·13 nmol/l for female and 0·98 nmol/l for male) levels.

As shown in Table 1, participants with higher level of serum 5-mTHF and UMFA tended to be older, female, non-Hispanic White and more likely to have higher prevalence of self-reported diabetes, as well as have higher level of HbA1c, and MeFox, while less likely to be current smokers. In addition, those with higher level of serum 5-mTHF also trend to have lower BMI, higher HDL-cholesterol, and those with higher level of serum UMFA tended to have higher prevalence of self-reported hypertension, lower level of eGFR and higher level of ACR.

Relationship of folate forms with the risk of Chronic kidney disease outcomes

Overall, there were significant inverse relationships of serum 5-mTHF with reduced eGFR and macroalbuminuria. When serum 5-mTHF was assessed as quartiles, significant lower prevalence of reduced eGFR (OR, 0·71; 95 % CI: 0·57, 0·87) and macroalbuminuria (OR, 0·65; 95 % CI: 0·46, 0·91) were found in participants in quartiles 3–4 (≥ 34·0 nmol/l), compared with those in quartiles 1–2 (<34·0 nmol/l; both $P_{\text{for trend}}$ across quartiles <0·05) (Table 2 and Table 3).

In contrast, there were significant positive relationships of serum UMFA with reduced eGFR and macroalbuminuria. When serum 5-mTHF was assessed as quartiles, significant higher prevalence of reduced eGFR was found in participants in quartiles 2–4 (≥ 0·5 nmol/l; OR, 2·12; 95 % CI: 1·45, 3·12) compared with those in quartile 1 (<0·5 nmol/l; $P_{\text{for trend}}$ across quartiles <0·001), and higher prevalence of macroalbuminuria was found in participants in quartile 4 (≥ 1·0 nmol/l; OR, 1·46; 95 % CI: 1·06, 2·01) compared with those in quartiles 1–3 (<1·0 nmol/l; $P_{\text{for trend}}$ across quartiles = 0·017) (Table 2 and Table 3). However, there were no significant associations of serum 5-mTHF and UMFA with microalbuminuria (Table 4).

More importantly, there were significant positive relationships of serum MeFox with kidney outcomes with significant lower risks of reduced eGFR (OR, 3·34; 95 % CI: 1·60, 6·96), microalbuminuria (OR, 1·25; 95 % CI: 1·06, 1·48) and macroalbuminuria (OR, 2·31; 95 % CI: 1·56, 3·43) in participants in quartile 2–4 (≥ 0·9 nmol/l) compared with those in quartile 1 (<0·9 nmol/l; all $P_{\text{for trend}}$ across quartiles <0·01) (Table 2, Table 3 and Table 4).

Stratified analyses

Stratified analyses were performed (Fig. 1 and Fig. 2) to evaluate the relationships of 5-mTHF and UMFA with the prevalence of reduced eGFR and macroalbuminuria in various subgroups. Overall, we did not find any folate forms that significantly modified the association between other folate forms and different kidney outcomes (all $P_{\text{for interactions}} > 0{\cdot}05$).

Further stratified analyses were conducted to explore other potential interactions (Fig. 1 and Fig. 2), and none of the variables, including age, sex, BMI and smoking status, significantly modified the association between folate forms and different kidney outcomes (all $P_{\text{for interactions}} > 0{\cdot}05$).

Discussion

In a large and nationally representative, randomly selected sample of USA adults, we first demonstrated that high serum 5-mTHF levels were associated with decreased risk of reduced eGFR and macroalbuminuria, while high serum UMFA and MeFox were associated with increased risk of reduced eGFR and macroalbuminuria. Most importantly, none of any folate forms significantly modified the association between other folate forms and renal outcome.

Indeed, synthetic folic acid might have different biological effects than naturally occurring folates. First, there are differences in the bioavailability of 5-mTHF and synthetic folic

Table 1. Baseline characteristics of study participants according to quartiles of 5-methyltetrahydrofolate (5-mTHF) and unmetabolised folic acid (UMFA)*
(Numbers and percentages)

	Quartiles of 5-mTHF, nmol/l									Quartiles of UMFA, nmol/l								
	Q1(<23·4)		Q2(23·4-<34·0)		Q3(34·0–<49·7)		Q4(≥ 49·7)			Q1(<0·5)		Q2(0·5–<0·7)		Q3(0·7–<1·0)		Q4(≥ 1·0)		
	n	%	*n*	%	*n*	%	*n*	%	*P* value	*n*	%	*n*	%	*n*	%	*n*	%	*P* value
n	4676		4660		4715		4706			4642		4691		4677		4747		
Age, years																		
Mean	44·0		43·2		45·3		54·5		<0·001	43·2		44·7		47·6		52·2		<0·001
SE	0·4		0·4		0·4		0·5			0·5		0·4		05		0·4		
Male, *n* (%)	2446	53·3	2371	54·0	2244	49·8	1824	40·0	<0·001	2480	56·2	2216	48·9	2067	45·8	2122	45·4	<0·001
BMI, kg/m^2																		
Mean	30·3		29·5		29·0		28·3		<0·001	29·1		29·1		29·5		29·2		0·023
SE	0·2		0·2		0·2		0·2			0·2		0·1		0·2		0·1		
Ethnicity									<0·001									<0·001
Mexican American	589	9·5	758	11·1	805	10·3	539	5·6		787	12·1	754	10·3	671	8·6	479	5·3	
Non-Hispanic White	1470	57·7	1474	59·4	1726	64·7	2265	75·6		1225	55·6	1707	64·6	1823	65·8	2180	72·4	
Non-Hispanic Black	1504	17·7	1136	12·5	852	8·8	606	5·4		861	10·0	981	10·3	1114	11·6	1142	11·4	
Education									<0·001									0·086
Less than high school	1034	17·2	972	14·6	952	12·5	933	12·6		1031	15·9	982	14	968	13·8	910	12·8	
High school or equivalent	1067	26·6	990	22·9	917	20·6	927	20·5		907	22·6	957	23	1013	23·1	1024	21·4	
College or above	2338	56·2	2378	62·5	2573	66·8	2694	66·9		2453	61·5	2463	63	2434	63·1	2633	65·7	
Current smoking, *n* (%)	1309	26·9	967	21·4	749	15·3	493	11·0	<0·001	931	20·0	949	19·7	839	17·9	799	15·8	0·002
Self-reported diabetes	473	8·5	539	9·2	589	9·3	739	12·1	<0·001	478	8·4	513	9·0	612	9·4	737	12·5	<0·001
Self-reported hypertension	1525	31·8	1478	29·6	1444	28·7	2027	39·3	<0·001	1235	27·0	1419	28·8	1711	33·1	2109	40·7	<0·001
Use of folic acid-containing supplements, *n* (%)	231	5·8	371	9·2	844	23·0	2252	57·7	<0·001	241	6·3	554	14·2	938	27·5	1965	51·1	<0·001
Fasting, *n* (%)	2504	54·7	2365	50·7	2278	48·8	2342	50·4	0·003	2715	59·5	2597	56·7	2453	52·9	1724	35·8	<0·001
	Mean	SE	Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Laboratory results																		
TC, mmol/l	4·9	0·0	4·9	0·0	4·9	0·0	4·9	0·0	0·056	5·0	0·0	4·9	0·0	4·9	0·0	4·9 ±	0·0	<0·001
HDL-C, mmol/l	1·3	0·0	1·4	0·0	1·4	0·0	1·5	0·0	<0·001	1·4	0·0	1·4	0·0	1·4	0·0	1·4 ±	0·0	0·867
HbA1c, %	5·6	0·0	5·6	0·0	5·6	0·0	5·7	0·0	<0·001	5·6	0·0	5·6	0·0	5·6	0·0	5·7 ±	0·0	0·005
eGFR, ml/min/1·73 m^2	99·2	0·7	100·8	0·6	99·3	0·5	89·5	0·5	<0·001	103·9	0·6	99·9	0·5	95·0	0·6	89·5 ±	0·5	<0·001
ACR, mg/g	35·1	4·1	35·8	4·2	26·7	2·4	30·1	3·2	0·225	24·8	2·5	24·8	2·6	32·6	3·6	44·5 ±	4·6	<0·001
5-mTHF, nmol/l	17·3	0·1	28·5	0·1	41·1	0·1	71·3	1·1	<0·001	28·7	0·5	34·8	0·6	42·1	0·6	56·3 ±	1·0	<0·001
UMFA, nmol/l	0·8	0·1	0·9	0·0	1·4	0·1	4·3	0·3	<0·001	0·4	0·0	0·6	0·0	0·8	0·0	5·8 ±	0·3	<0·001
Mefox, nmol/l	1·5	0·0	1·7	0·0	1·9	0·0	2·4	0·1	<0·001	1·3	0·0	1·7	0·0	1·9	0·0	2·6 ±	0·1	<0·001

TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; HbA1c, haemoglobin A1c; eGFR, estimated glomerular filtration rate; ACR, albumin:creatinine ratio.
* All estimates accounted for complex survey designs. Values are presented as mean ± SE for continuous variables and *n* (%) for categorical variables, and comparison of characteristics was performed by χ^2 test for categorical variables or ANOVA for continuous variables.

Table 2. Relationship of folate forms with reduced estimated glomerular filtration rate (eGFR)*
(Numbers and percentages; odd ratio and 95 % confidence intervals)

		Prevalence		Crude Models			Adjusted Models 1			Adjusted Models 2		
	Total	*n*	%	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value
5-mTHF, nmol/l												
Quartiles												
Q1(<23·4)	4676	320	5·6	ref			ref			ref		
Q2(23·4–<34·0)	4660	283	5·3	0·94	0·75, 1·18	0·609	0·90	0·70, 1·16	0·430	0·92	0·70, 1·21	0·564
Q3(34·0–<49·7)	4715	255	4·6	0·81	0·61, 1·06	0·136	0·62	0·45, 0·84	0·004	0·63	0·45, 0·88	0·010
Q4(≥ 49·7)	4706	580	10·3	1·94	1·55, 2·43	<0·001	0·69	0·52, 0·90	0·009	0·71	0·53, 0·95	0·027
$P_{for\ trend}$				<0·001			0·003			0·011		
Category												
Q1–2(<34·0)	9336	603	5·4	ref			ref			ref		
Q3–4(≥ 34·0)	9421	835	7·5	1·41	1·19, 1·66	<0·001	0·70	0·57, 0·85	0·001	0·71	0·57, 0·87	0·003
UMFA, nmol/l												
Quartiles												
Q1(<0·5)	4642	112	2·1	ref			ref			ref		
Q2(0·5–<0·7)	4691	210	4·1	1·99	1·34, 2·96	0·001	1·57	1·03, 2·38	<0·001	1·58	1·02, 2·46	0·047
Q3(0·7–<1·0)	4677	396	7·3	3·66	2·47, 5·41	<0·001	2·21	1·49, 3·29	<0·001	2·19	1·46, 3·29	0·001
Q4(≥ 1·0)	4747	720	12·1	6·36	4·42, 9·15	<0·001	2·64	1·81, 3·84	<0·001	2·46	1·66, 3·65	<0·001
$P_{for\ trend}$				<0·001			<0·001			<0·001		
Category												
Q1(<0·5)	4642	112	2·1	ref			ref			ref		
Q2–4(≥ 0·5)	14 115	1326	7·9	3·94	2·76, 5·64	<0·001	2·2	1·52, 3·18	<0·001	2·12	1·45, 3·12	<0·001
Mefox, nmol/l												
Quartiles												
Q1(<0·9)	4678	46	1·1	ref			ref			ref		
Q2(0·9–<1·4)	4631	147	2·8	2·45	1·21, 4·97	0·016	1·52	0·72, 3·19	0·274	1·49	0·69, 3·23	0·317
Q3(1·4–<2·4)	4744	357	6·4	5·92	3·00, 11·7	<0·001	3·07	1·52, 6·22	0·003	2·87	1·37, 6·00	0·008
Q4(≥ 2·4)	4704	888	15·3	15·58	7·79, 31·16	<0·001	6·6	3·28, 13·29	<0·001	5·81	2·79, 12·08	<0·001
$P_{for\ trend}$				<0·001			<0·001			<0·001		
Category												
Q1(<0·9)	4678	46	1·1	ref			ref			ref		
Q2–4(≥ 0·9)	14 079	1392	8·1	7·61	3·84, 15·07	<0·001	3·75	1·86, 7·57	<0·001	3·34	1·60, 6·96	0·002

5-mTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid.
* All estimates accounted for complex survey designs. Binomial regression models were used to estimate OR and 95 % CI, and model 1 was adjusted for age (continuous), sex; model 2 was adjusted for age (continuous), sex, BMI (continuous), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American and others), education level (less than high school, high school or equivalent and college or above), smoking status (never, past and current), history of diabetes (no and yes) and hypertension (no and yes), total cholesterol (continuous), high-density lipoprotein cholesterol (continuous) and haemoglobin A1c (continuous). Kidney outcomes were defined as binary variable (no and yes). As primary analysis, folate forms were divided into quartiles, and as exploratory analyses, if the results for primary analysis were significant, quartiles were further pooled to binary variables.

acid. Folic acid lacks any coenzyme activity until it is reduced to the metabolically active tetrahydrofolate form, and the reduction process was catalysed by dihydrofolate reductase[27], whose activity varied considerably between individuals and was inhibited by UMFA[13]. In addition, high UMFA level also inhibited methylenetetrahydrofolate reductase (*MTHFR*), the enzyme that produces methyl tetrahydrofolate[28], and thus decrease the bioavailability of 5-mTHF levels. In contrast, the bioavailability of 5-MTHF was higher than that of UMFA, irrespective of the patient's genotype[29]. Second, previous studies have reported that UMFA was associated with neurological and cognitive effects in individuals with lower B_{12} status[30] and with reduced natural killer cell cytotoxicity in some studies *in vivo*[14,15]. In addition, UMFA has a poor antioxidant activity in peroxynitrite scavenging and lipid peroxidation inhibition[31]. On the contrary, 5-mTHF had the most prominent antioxidant activity, especially in peroxynitrite scavenging and superoxide production inhibition[31,32]. Additionally, the structure of 5-mTHF was similar to tetrahydrobiopterin (BH_4), which was necessary to maintain endothelial nitric oxide synthase with a net production balance towards nitric oxide, and thus 5-mTHF can restore nitric oxide-dependent endothelial function[32,33]. As such, it is necessary to examine the potential different health effects of circulating 5-mTHF and UMFA.

Our previous study has observed a U-shaped association of serum 5-mTHF with mortality, while a positive association of UMFA with mortality[34]. However, the association of 5-mTHF and UMFA with renal outcomes remains unclear. To our knowledge, this is the first study to examine the relation of serum 5-mTHF and UMFA levels with renal outcomes, and we found an opposite effect of serum 5-mTHF and UMFA on reduced eGFR and macroalbuminuria. In addition, although there were differences in concentrations of folate forms by fasting status or use of folic acid-containing supplements[18,35], fasting status (yes *v.* no) or use of folic acid-containing supplements (yes *v.* no) did not materially modify the relationship of 5-mTHR or UMFA with study outcomes (online Supplementary Table 2 and Table 3). Of note, in NHANES 2011–2014 (vitamin B_{12} was not available for 2015–2018), the prevalence of metabolic vitamin B_{12} deficiency (<248 pmol/l) was 16·4 %. Since vitamin B_{12} deficiency may lead to methyl folate trap, we categorised data from NHANES 2011–2014 by vitamin B_{12} deficiency and found that the relationships of 5-mTHF with reduced eGFR and macroalbuminuria were more obvious in those without

Table 3. Relationship of folate forms with macroalbuminuria*
(Numbers and percentages; odd ratio and 95 % confidence intervals)

		Prevalence		Crude Models			Adjusted Models 1			Adjusted Models 2		
	Total	*n*	%	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value
5-mTHF, nmol/l												
Quartiles												
Q1(<23·4)	4242	101	1·7	ref			ref			ref		
Q2(23·4–<34·0)	4222	114	2·0	1·23	0·81, 1·86	0·334	1·23	0·81, 1·87	0·328	1·17	0·75, 1·82	0·49
Q3(34·0–<49·7)	4264	92	1·2	0·75	0·50, 1·11	0·154	0·68	0·45, 1·01	0·064	0·72	0·47, 1·11	0·146
Q4(≥ 49·7)	4154	91	1·3	0·80	0·52, 1·23	0·316	0·49	0·31, 0·75	0·002	0·69	0·43, 1·10	0·126
$P_{for\ trend}$				0·116			<0·001			0·044		
Category												
Q1–2(<34·0)	8464	215	1·8									
Q3–4(≥ 34·0)	8418	183	1·3	0·69	0·49, 0·98	0·043	0·51	0·35, 0·73	0·001	0·65	0·46, 0·91	0·017
UMFA, nmol/l												
Quartiles												
Q1(<0·5)	4234	73	1·2	ref			ref			ref		
Q2(0·5–<0·7)	4276	71	1·3	1·05	0·62, 1·78	0·849	0·99	0·59, 1·66	0·967	1·10	0·65, 1·86	0·720
Q3(0·7–<1·0)	4170	101	1·5	1·23	0·82, 1·84	0·316	1·01	0·67, 1·53	0·953	1·21	0·79, 1·86	0·386
Q4(≥ 1·0)	4202	153	2·2	1·82	1·24, 2·68	0·003	1·25	0·84, 1·86	0·268	1·63	1·09, 2·43	0·022
$P_{for\ trend}$				0·004			0·283			0·017		
Category												
Q1–3(<1·0)	12 680	245	1·3	ref			ref			ref		
Q4(≥ 1·0)	4202	153	2·2	1·66	1·19, 2·32	0·004	1·25	0·88, 1·77	0·212	1·46	1·06, 2·01	0·024
Mefox, nmol/l												
Quartiles												
Q1(<0·9)	4323	47	0·6	ref			ref			ref		
Q2(0·9–<1·4)	4221	52	0·8	1·32	0·87, 1·99	0·194	1·12	0·75, 1·67	0·576	1·18	0·75, 1·85	0·475
Q3(1·4–<2·4)	4251	52	0·8	2·47	1·57, 3·90	<0·001	1·95	1·23, 3·08	0·006	1·97	1·21, 3·21	0·010
Q4(≥ 2·4)	4087	210	3·4	6·00	4·01, 8·96	<0·001	4·24	2·84, 6·33	<0·001	3·94	2·62, 5·93	<0·001
$P_{for\ trend}$				<0·001			<0·001			<0·001		
Category												
Q1(<0·9)	4323	47	0·6	ref			ref			ref		
Q2–4(≥ 0·9)	12 559	351	1·9	3·19	2·20, 4·63	<0·001	2·40	1·66, 3·47	<0·001	2·31	1·56, 3·43	<0·001

5-mTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid.
* All estimates accounted for complex survey designs. Binomial regression models were used to estimate OR and 95 % CI, and model 1 was adjusted for age (continuous), sex; model 2 was adjusted for age (continuous), sex, BMI (continuous), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American and others), education level (less than high school, high school or equivalent and college or above), smoking status (never, past and current), history of diabetes (no and yes) and hypertension (no and yes), total cholesterol (continuous), high-density lipoprotein cholesterol (continuous) and haemoglobin A1c (continuous). Kidney outcomes were defined as binary variable (no and yes). As primary analysis, folate forms were divided into quartiles, and as exploratory analyses, if the results for primary analysis were significant, quartiles were further pooled to binary variables.

vitamin B_{12} deficiency, although the $P_{value\ for\ interaction}$ was not significant (online Supplementary Table 2 and Table 3). Along with our previous study[36], these findings suggest the importance of maintaining relatively higher levels of both 5-mTHF and vitamin B_{12} on kidney outcomes.

To date, few interventional studies have explored the effect of folic acid supplementation on CKD progression, and the results remain inconsistent. While the CSPPT Renal Substudy demonstrated statistically significant reductions in the risk of CKD progression with low-dose folic acid supplementation (0·8 mg/d) in a hypertensive population without dietary fortification of folic acid[9], the HOST study reported that treatment with high doses of folic acid (40 mg/d) did not delay the time to initiating dialysis in patients with advanced CKD or end-stage renal disease[11], and, even, the DIVINe trial showed that folic acid (2·5 mg/d) treatment resulted in a greater decrease in eGFR in 238 patients with diabetic nephropathy[10]. A possible explanation was that the latter two trials were conducted in regions with folic acid fortification and used high dose of folic acid, as such, much of the folate in the blood would be UMFA[37], which was associated with increased risk of reduced eGFR and macroalbuminuria as shown in our study. Moreover, it should be noted that CKD individuals may be asked to restrict P or K[38], as well as whole grains or certain fruits and vegetables because of their high P or K content[39,40]. Since food folates occur naturally in richest supply in green leafy vegetables, while folic acid is found in the human diet in folic acid fortified grain products[41], P or K-restricted diet offered to patients with CKD may be associated with declining folate intake. As such, our study may possibly overestimate the benefit of 5mTHF and underestimate the hazard of UMFA. Therefore, our result speculated that UMFA may mask the benefit of 5-mTHF on renal outcomes and suggested that 5-mTHF should replace folic acid in future studies of B vitamin supplementation on CKD.

A notable finding from this study is the null association of 5-mTHF and UMFA with microalbuminuria, whereas a significant opposite association of 5-mTHF and UMFA with macroalbuminuria. Our previous study has found that the presence of CKD at baseline was a significant modifier of the treatment effect of folic acid supplement, with a significant renal protective effect in those with mild-to-moderate CKD, whereas a nominal effect in those without CKD[9]. Since high-grade proteinuria is an independent mediator of progressive kidney damage[42], it was speculated that 5-mTHF and UMFA play a role on promoting

Table 4. Relationship of folate forms with microalbuminuria*
(Numbers and percentages; odd ratio and 95 % confidence intervals)

		Prevalence		Crude Models			Adjusted Models 1			Adjusted Models 2		
	Total	*n*	%	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value
5-mTHF, nmol/l												
Quartiles												
Q1(<23·4)	4575	434	7·5	ref			ref			ref		
Q2(23·4–<34·0)	4546	438	7·5	1·01	0·83, 1·22	0·946	1·02	0·84, 1·25	0·811	1·07	0·85, 1·33	0·580
Q3(34·0-<49·7)	4623	451	8·4	1·14	0·91, 1·42	0·259	1·09	0·87, 1·36	0·476	1·17	0·91, 1·49	0·226
Q4(≥ 49·7)	4615	552	9·6	1·33	1·08, 1·62	0·008	0·95	0·77, 1·18	0·668	1·09	0·86, 1·39	0·490
$P_{for\ trend}$				0·003			0·737			0·387		
UMFA, nmol/l												
Quartiles												
Q1(<0·5)	4569	408	7·7	ref			ref			ref		
Q2(0·5–<0·7)	4620	415	7·5	0·98	0·79, 1·21	0·854	0·92	0·74, 1·13	0·427	0·94	0·75, 1·19	0·622
Q3(0·7–<1·0)	4576	507	8·9	1·18	0·95, 1·46	0·133	1·00	0·81, 1·24	0·997	1·05	0·84, 1·31	0·656
Q4(≥ 1·0)	4594	545	9·1	1·20	0·99, 1·47	0·074	0·9	0·73, 1·10	0·294	0·94	0·75, 1·16	0·551
$P_{for\ trend}$				0·031			0·473			0·790		
Mefox, nmol/l												
Quartiles												
Q1(<0·9)	4631	355	6·1	ref			ref			ref		
Q2(0·9-<1·4)	4579	410	7·6	1·27	1·04, 1·55	0·022	1·11	0·9, 1·37	0·323	1·16	0·94, 1·44	0·168
Q3(1·4-<2·4)	4655	493	8·6	1·45	1·17, 1·79	0·001	1·20	0·97, 1·49	0·104	1·24	1·00, 1·54	0·062
Q4(≥ 2·4)	4494	617	10·8	1·87	1·59, 2·20	<0·001	1·42	1·2, 1·69	<0·001	1·36	1·13, 1·63	0·002
$P_{for\ trend}$				<0·001			<0·001			0·005		
Category												
Q1(<0·9)	4631	355	6·1	ref			ref			ref		
Q2–4(≥ 0·9)	13 728	1520	9·0	1·52	1·31, 1·77	<0·001	1·24	1·06, 1·46	0·011	1·25	1·06, 1·48	0·013

5-mTHF, 5-methyltetrahydrofolate; UMFA, unmetabolised folic acid.
* All estimates accounted for complex survey designs. Binomial regression models were used to estimate OR and 95 % CI, and model 1 was adjusted for age (continuous), sex; model 2 was adjusted for age (continuous), sex, BMI (continuous), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, and others), education level (less than high school, high school or equivalent and college or above), smoking status (never, past and current), history of diabetes (no and yes) and hypertension (no and yes), total cholesterol (continuous), high-density lipoprotein cholesterol (continuous) and haemoglobin A1c (continuous). Kidney outcomes were defined as binary variable (no and yes). As primary analysis, folate forms were divided into quartiles, and as exploratory analyses, if the results for primary analysis were significant, quartiles were further pooled to binary variables.

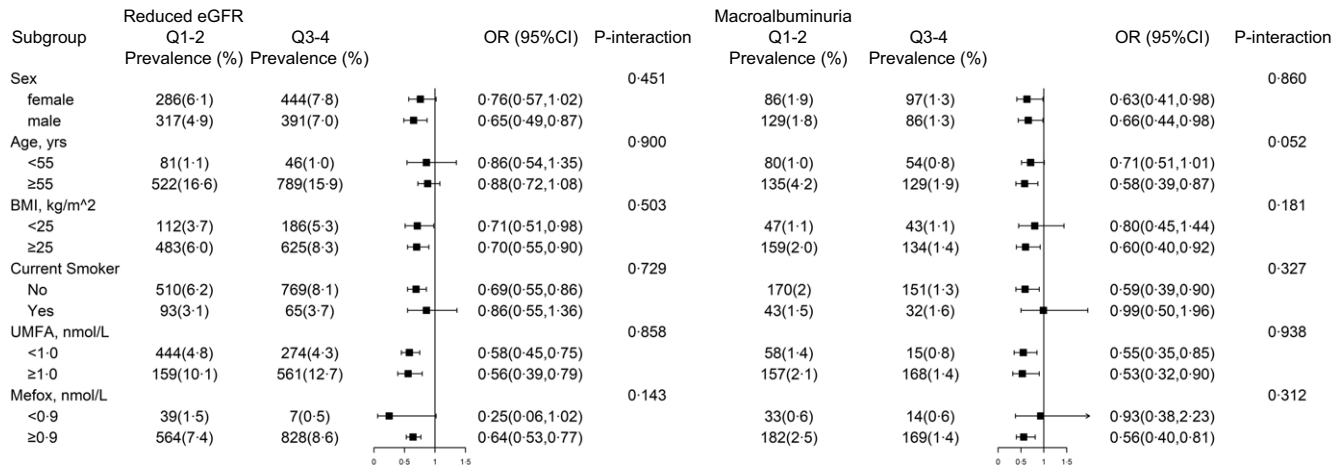

Fig. 1. The association between 5-methyltetrahydrofolate (5-Mthf) and risk of reduced estimated glomerular filtration rate (eGFR) and macroalbuminuria in various subgroups*. *All estimates accounted for complex survey designs. Binomial regression models were used to estimate OR and 95 % CI, and maximum likelihood ratio was used to calculate $P_{value\ for\ interaction}$. Analysis was adjusted for age (continuous), sex, BMI (continuous), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American and others), education level (less than high school, high school or equivalent and college or above), smoking status (never, past and current), history of diabetes (no and yes) and hypertension (no and yes), total cholesterol (continuous), HDL-cholesterol (continuous) and haemoglobin A1c (continuous), if not been stratified. Kidney outcomes were binary variable (no and yes), and folate forms were defined as binary variable according to the results of Tables 2–3.

the progression of already-initiated CKD rather than against CKD initiation. Another interpretation was potential misclassification of microalbuminuria. A subsample of 1241 NHANES III participants was reexamined 2 weeks after the initial examination, and all sampled participants with macroalbuminuria at the first visit had have persistent albuminuria; however, only 63·2 % participants with microalbuminuria at the first visit had micro- or macroalbuminuria at the second visit[43,44]. Therefore, future studies with repeated measurements of albuminuria are needed to verify our results.

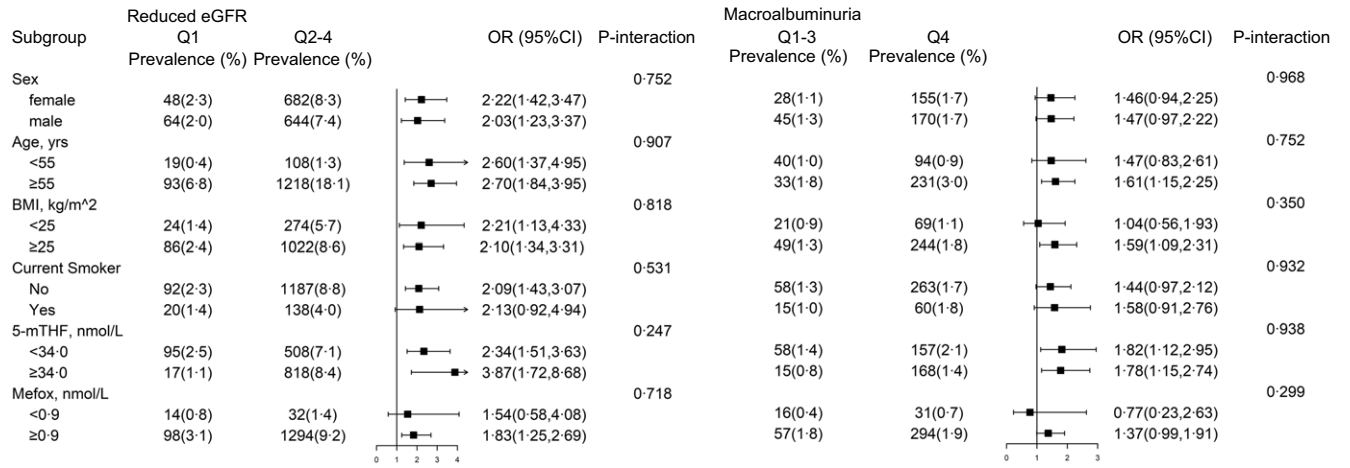

Fig. 2. The association between unmetabolised folic acid (UMFA) and risk of reduced estimated glomerular filtration rate (eGFR) and macroalbuminuria in various subgroups*. *All estimates accounted for complex survey designs. Binomial regression models were used to estimate OR and 95 % CI, and maximum likelihood ratio was used to calculate $P_{\text{value for interaction}}$. Analysis was adjusted for age (continuous), sex, BMI (continuous), race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American and others), education level (less than high school, high school or equivalent and college or above), smoking status (never, past and current), history of diabetes (no and yes) and hypertension (no and yes), total cholesterol (continuous), HDL-cholesterol (continuous) and haemoglobin A1c (continuous), if not been stratified. Kidney outcomes were binary variable (no and yes), and folate forms were defined as binary variable according to the results of Tables 2–3.

Our study additionally sought to evaluate the association of serum MeFox, an oxidation product of 5-mTHF that lacks vitamin biologic activity[45], with renal outcomes. While it has been shown that MeFox can be formed *in vitro* after blood collection[46], some observations raised the possibility that MeFox may be formed *in vivo* rather than solely *in vitro* as part of 5-mTHF oxidation[18,35,47]. Therefore, it was suggested to separately report results for 5-methylTHF and MeFox to provide broader utility, given that MeFox data may provide relevant information regarding the quality of sample handling and insights into folate metabolism. Indeed, more MeFox generation or accumulation in older persons, obese adults and smokers, and MeFox concentrations were positively associated with inflammation, suggesting that MeFox may be an indicator of ageing and negative health factors[18,35]. Consistently, our study found a positive relationship of MeFox with reduced eGFR and albuminuria, and underlying mechanisms is required to further examine.

This study had several strengths, including its large and nationally representative sample, the central lab and standardised measurement methods, the relatively higher population's folate status, the availability of data on several serum folate forms and the comprehensively adjustments for potential confounders. However, our study also has several limitations. First, the cross-sectional design precludes the ability to assess causality. However, although impairment in renal reabsorption of folates in the proximal tubules can lead to a state of folate deficiency[48], it is highly unlikely that reduced eGFR or albumin loss in urine causes lower 5-mTHF, but higher UMFA. Moreover, our previous study did observe that folic acid therapy can significantly delay the progression of CKD among patients with mild-to-moderate CKD[9]. At the same time, our current study further found that albuminuria (ACR < 30 *v.* ≥ 30 mg/g) did not significantly affect the relationship of 5-mTHF with the prevalence of reduced eGFR or macroalbuminuria (online Supplementary Table 2). As such, our findings may possibly indicate that higher 5-MTHF is associated with less risk of kidney damage. However, future cohort studies are necessary to confirm our findings. Second, the folate forms were based on one-time assessment, which may not accurately represent long-term folate levels; however, since folate forms in red blood cell were not available, further studies regarding intracellular folate forms are needed. Third, our study was conducted in USA adults with folic acid fortification. Whether the findings can be extrapolated to other populations will require further investigation. Fourth, we did not have detailed information about MTHFR genotypes, so that we could not assess the possible effect of MTHFR genotypes on the association between folate and kidney outcomes. Fifth, since our study was an exploratory analysis, we did not account for multiple testing. Overall, a more comprehensive picture provided by further studies is required.

Conclusions

In conclusion, using data from a large, nationally representative cohort of USA adults, we found that higher level of 5-mTHF was associated with lower risk of reduced eGFR and macroalbuminuria, whereas higher level of UMFA and MeFox were associated with higher risk of reduced eGFR and macroalbuminuria. Given that folic acid fortification is common in many countries, and there is a high rate of supplement use (approximately 36·6 % among USA adults)[49], our findings, if further confirmed by future studies, emphasised the importance of monitoring the folate forms concentrations and may help counsel future related clinical trials and nutritional guidelines regarding the folate supplement on CKD.

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M. L. and X. Q. designed the research; M. L. and C. Z. analysed the data; M. L. and X. Q. wrote the paper. All authors contributed to data collection and reviewed/edited the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521001665

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