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High folic acid or folate combined with low vitamin B-12 status: potential but inconsistent association with cognitive function in a nationally representative cross-sectional sample of US older adults participating in the NHANES

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ABSTRACT

Background: Potential safety concerns relative to impaired cognitive function may exist when high folic acid exposures are combined with low vitamin B-12 status.

Objectives: We aimed to examine the relation of the coexistence of high folate and low vitamin B-12 status with cognitive function, utilizing various definitions of "high" folate status.

Methods: Cross-sectional data from older adults (≥60 y; $n = 2420$) from the 2011–2014 NHANES were analyzed. High folate status was defined as unmetabolized serum folic acid (UMFA) > 1 nmol/L or serum total folate > 74.1 nmol/L, and low vitamin B-12 status as methylmalonic acid > 271 nmol/L or serum vitamin B-12 < 150 pmol/L. Logistic regression models estimated ORs of scoring low on 1 of 4 cognitive tests: the Digit Symbol Substitution Test (DSST), the Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall (CERAD-DR) and Word Learning tests, and the Animal Fluency test (AF).

Results: A significant interaction was observed relative to scoring low on the DSST (<34; UMFA; *P*-interaction = 0.0071) and AF (serum folate; *P*-interaction = 0.0078) for low vitamin B-12 and high folate status. Among those with low vitamin B-12, high UMFA or high serum total folate was associated with higher risk of scoring low on the DSST (OR: 2.16; 95% CI: 1.05, 4.47) and the AF (OR: 1.93; 95% CI: 1.08, 3.45). Among those with "normal" vitamin B-12, higher UMFA or serum total folate was protective on the CERAD-DR. In noninteraction models, when high folate and normal vitamin B-12 status was the reference group, low vitamin B-12 combined with high UMFA was associated with greater risk based on the DSST (<34, OR: 2.87; 95% CI: 1.85, 4.45; <40, OR: 2.22; 95% CI: 1.31, 3.75) and AF (OR: 1.97; 95% CI: 1.30, 2.97); but low vitamin B-12 and lower UMFA (OR: 1.69; 95% CI: 1.16, 2.47) was also significantly associated for DSST < 40 risk.

Conclusions: Low vitamin B-12 was associated with cognitive impairment both independently and in an interactive manner with high folate for certain cognitive performance tests among older adults. *Am J Clin Nutr* 2020;112:1547–1557.

Keywords: folate, unmetabolized folic acid, vitamin B-12, interaction, cognitive function, older adults, NHANES

Introduction

The DRI for potential excess, the tolerable upper intake level (UL), is set as the highest average daily nutrient intake

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Supplemental Figure 1 and Supplemental Tables 1–10 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Data described in the article, codebook, and analytic code will not be made available unless upon request, given that all data and codebooks are already publicly available and the methods provided in great detail for others to replicate the analysis.

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Abbreviations used: AF, Animal Fluency test; CERAD-DR, Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall test; CERAD-WL, Consortium to Establish a Registry for Alzheimer's Disease Word Learning test; DSST, Digit Symbol Substitution Test; EAR, Estimated Average Requirement; eGFR, estimated glomerular filtration rate; MEC, mobile examination center; MMA, methylmalonic acid; UL, Tolerable Upper Intake Level; UMFA, unmetabolized serum folic acid; 5MeTHF, 5-methyltetrahydrofolate.

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amount that is unlikely to pose risk of adverse health effects in almost all individuals (1). For folic acid, the UL was set based on the observation that high folic acid intakes had the potential for generating neurological damage (2) by "masking" the hematological signs preceding the neurological signs of vitamin B-12 deficiency (3–5). Vitamin B-12 deficiency has been consistently associated with cognitive impairment and neurological disorders (5–7). Vitamin B-12 and folate play essential roles in homocysteine remethylation, a process where the enzyme methionine synthase uses vitamin B-12 as a cofactor to transfer a methyl group from 5-methyltetrahydrofolate (5MeTHF) to homocysteine. Therefore, vitamin B-12 deficiency leads to an elevation of homocysteine, which may be neurotoxic (5, 8). In a meta-analysis of epidemiological data, Beydoun et al. (9) postulated that elevated homocysteine may have a differential effect on specific regions of the brain and thus may manifest in impaired cognitive function, brain atrophy, and dementia. It should be noted that, in populations with folic acid fortification, the primary nutritional determinant of high homocysteine is low vitamin B-12 (10). Moreover, elevated methylmalonic acid (MMA) has been proposed as a more important determinant of age-related cognitive decline than homocysteine (11, 12).

The prevalence of vitamin B-12 deficiency increases with age (13), as well as with a vegetarian diet (14). Low vitamin B-12 status associated with aging is often secondary to reduced gastric acid production, required for digestion and absorption of the protein-bound vitamin B-12 in animal source foods, due to gastric atrophy or long-term use of medications such as proton pump inhibitors, H_2-receptor antagonists, and metformin, and more rarely due to the autoimmune disorder, pernicious anemia (5, 15). Although low folate status has been associated with cognitive decline (16), folate deficiency is rare in the United States today (17, 18) owing to a diverse food supply, fortification (19, 20), and a high prevalence of folic acid–containing dietary supplement use (21), especially among older adults (22). Indeed, considering that most Americans have adequate folate status, concerns lately have shifted toward the potential safety risks of high folate status (23–26), specifically to the folic acid form of the vitamin. Some have postulated that high serum unmetabolized folic acid (UMFA) is indicative of excessive folic acid intake that is above cellular demands (27) and that it may be a marker of high folic acid exposures (28). Nevertheless, it is still unknown whether UMFA is associated with any negative health outcomes, or whether it is even a viable biomarker of high folate exposures (29). Given that UMFA is present among almost all of the US population (26), any purported adverse effects of high folic acid exposures among individuals with low vitamin B-12 status are of potential public health importance (30).

Indeed, the NIH has convened 2 meetings over the past 5 y to address potential safety issues involving high folate status or high folic acid alone (31) and high folate status or high folic acid combined with low vitamin B-12 status specifically (report due in2020). In the first meeting's report, an area of "evidential consistency" was that observational data suggested that high folic acid intake alone (23) or high folic acid intake combined with low vitamin B-12 status (13, 25, 32) was associated with increased risk of cognitive impairment among older adults. A causal interpretation from observational data can never be inferred, but no data are available from randomized controlled trials specifically to address high folic acid in combination with low vitamin B-12 relative to cognitive function. Moreover, existing reports from observational studies are also limited because only 1 or 2 cognitive tests may not adequately reflect the multiple aspects of global and domain-specific cognitive function. In addition, the studies are difficult to compare because there is no agreement on what cutoffs constitute "high" folate status. Therefore, the purpose of this study was to examine whether the coexistence of high UMFA or high serum total folate status combined with low vitamin B-12 status is associated with cognitive performance using multiple biomarkers and multiple cognitive assessment tools among a nationally representative sample of US older adults.

Methods

Data collection

The NHANES is a nationally representative, continuous cross-sectional study of individuals residing in the United States collected by the CDC, National Center for Health Statistics that has a complex, stratified, multistage probability cluster sampling design. NHANES data are collected first when participants are interviewed in their home, with a subsequent visit to a mobile examination center (MEC) where a clinical assessment including the collection of biological specimens for analyte measurements is carried out. Written informed consent was obtained for all participants or proxies; the survey protocol was approved by the Research Ethics Review Board at the National Center for Health Statistics.

Covariates

In the in-home interview, demographic and smoking data were collected using a computer-assisted personal interview and categorized as follows: sex (male and female); age (60 to <70 y, 70 to <80 y, and ≥80 y; in the NHANES, age is top-coded at 85 y to preserve participant confidentiality and thus age groupings were constructed to reduce the influence of many 85-y-olds); race and Hispanic origin (non-Hispanic white, non-Hispanic Black, non-Hispanic Asian, Hispanic, and other); education (less than high school, high school graduate or general equivalency diploma, some college or associates degrees, and bachelor's degree or above); and smoking status (never smoker and former smoker/current smoker). Use of dietary supplements and prescription medications was ascertained in the home with a product inventory combined with the Dietary Supplement and Medication Questionnaire to gain information on the participant's use, over the previous 30 d, of vitamin B-12– or folic acid–containing supplements and medications that may affect vitamin B-12 status (i.e., proton-pump inhibitors, H_2-receptor antagonists, and metformin) (5, 15). For each reported supplement, detailed information about type, consumption frequency, duration, and amount taken was also collected to calculate mean daily intakes.

During the MEC visit, an in-person 24-h dietary recall was collected as part of the USDA's What We Eat in America (33). A second 24-h recall was collected via telephone 3–10 d after the first, with emphasis placed on getting both weekday and weekend reports. Both 24-h recalls were collected using USDA's Automated Multiple-Pass Method and included dietary supplements (34). The frequency of alcohol drinking and the average number of alcohol drinks per occasion were also asked in

the MEC. Heavy drinking was operationalized as ≥14 drinks/wk in males and ≥7 drinks/wk in females (35); 1 drink contains 10 g ethanol and is equivalent to 12 ounces of beer (360 mL), 4 ounces of wine (120 mL), or 1 ounce (30 mL) of distilled spirits.

Cognitive test battery

Four separate tests formed the cognitive battery and were collected in person in the MEC in the requested language of the study participant, but were limited to English, Spanish, Chinese, Korean, or Vietnamese speakers. For those who spoke an Asian language, an interpreter was present during the interview. Participants were asked for consent to audio-record the entire assessments for quality control and scoring purposes.

The Animal Fluency test (AF) assesses verbal fluency and is administered by asking participants to name as many animals as they can in 1 min, with a total score equal to the number of animals mentioned. In NHANES, participants were first asked to name 3 items of clothing, another verbal fluency category, as a practice test. Participants who could not name 3 articles of clothing did not progress to the AF exercise. The AF has an advantage over some other tests because it is not completely dictated by formal educational experiences of a particular culture (36). Test scores have been shown to discriminate persons with normal cognitive function from those who have mild cognitive impairment and more severe forms of cognitive impairment, such as Alzheimer disease (37, 38). No data were available regarding the cutoff scores designated to indicate cognitive impairment in this NHANES study population. A cutoff of <14 was used to define potential cognitive impairment, as was done in a previous study that included a similar US population (39, 40).

The Digit Symbol Substitution Test (DSST) contained in the Wechsler Adult Intelligence Scale is a paper-based tool designed to measure processing speed, sustained attention, and working memory (41, 42). Participants are provided a set of symbols with a matching key and scored on the total number of symbols that are drawn correctly in 120 s. This instrument, although sensitive, is not comprehensive or indicative of all domains of cognitive function. Morris et al. (24) and Bailey et al. (13) have previously used DSST <34 as a cutoff in NHANES to classify potential cognitive impairment, corresponding to the 20th percentile score in 1999–2002; to facilitate comparison of the results, we conducted sensitivity analysis with a cutoff of DSST <34. We also used a DSST score <40 as a cutoff, corresponding to the 25th percentile as suggested by a recent report from the National Center for Health Statistics (42), for the current analysis to reduce the potential impact of the Flynn effect, the phenomenon of intelligence test scores rising over time, on DSST scores (43).

The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) is a comprehensive set of tests used to identify Alzheimer disease by assessing the ability for new learning, delayed recall, and recognition memory (44). The CERAD-Word Learning test (CERAD-WL) consists of 3 consecutive learning trials. For the learning trials, participants are instructed to read aloud 10 unrelated words, 1 at a time, as they are presented. Immediately after the presentation of the words, participants recall as many words as possible. In each of the 3 learning trials, the order of the 10 words is changed. The maximum score possible on each trial is 10. The score from the 3 trials is added to produce a total maximum CERAD-WL score of 30. The CERAD-Delayed Recall test (CERAD-DR) occurred after the other 2 cognitive exercises (AF and DSST) were completed, ~8–10 min from the start of the CERAD-WL trials. For the CERAD-DR, the participant was asked to recall the 10 unrelated words used in the first CERAD-WL trial. Based on prior literature, cutoffs of <17 for CERAD-WL and <5 for CERAD-DR were used to distinguish potential cognitive impairment from healthy cognitive function and lack of cognitive impairment (45).

Biochemical methods

Details on fasting before blood draw were collected from all participants via questionnaire in the MEC before blood draw. Participants who were randomly assigned to a morning session for biospecimen collection, but not those assigned to an afternoon or evening session, were asked to fast for 9 h; therefore, the length of time reported for fasting from food and dietary supplements varied (<3 h, 3 to <8 h, or ≥8 h) (26). Those who took folic acid–containing supplements during the fasting period had high UMFA concentrations (26, 28). Among participants who reported taking any dietary supplements during fasting, those who reported consuming folic acid in the 24-h recall were assumed to have consumed folic acid–containing dietary supplements during fasting. Serum and whole blood samples were collected through venipuncture and analyzed at the CDC's Laboratory for Nutritional Biomarkers; details on specimen processing and laboratory methods have been described elsewhere (46, 47). The microbiological assay was used to estimate RBC folate and serum folate was determined by HPLC–tandem MS. Serum total folate was calculated by adding 5 different serum folate forms: 5MeTHF, UMFA, 5-formyltetrahydrofolate, tetrahydrofolate, and 5,10-methenyltetrahydrofolate. Serum vitamin B-12 was measured by the Roche E-170 vitamin B-12 electrochemiluminescence immunoassay (Roche Diagnostics) and MMA was analyzed by HPLC–tandem MS. Serum creatinine was measured using the Jaffe rate method (kinetic alkaline picrate reaction) and calibrated with an isotope dilution MS reference method (48). Estimated glomerular filtration rate (eGFR; mL · min^{-1} · 1.73 m^{-2}) was calculated for each individual based on serum creatinine concentration, sex, age, and race using the Chronic Kidney Disease Epidemiology Collaboration equation (49). Reduced eGFR was defined as eGFR <60 (49). Urinary albumin was measured using the fluorescent immunoassay and urinary creatinine was analyzed by an Enzymatic Roche Coba 6000 Analyzer (Roche Diagnostics). Albuminuria was defined by a urinary albumin-to-creatinine ratio ≥30 mg/g.

Vitamin B-12 deficiency has historically been classified as <148 pmol/L (50–53); however, it was recently recommended that <150 pmol/L was more appropriate (54, 55). We classified elevated MMA as >271 nmol/L (53, 56–61). Low vitamin B-12 status was operationally defined in this study as having a low serum B-12, an elevated MMA, or both.

Serum total folate represents the sum of the biologically active folate forms. High folate status was classified in various ways for this analysis because no consistent cutoff has yet been developed and used in the literature. The first classification was total folate >45.3 nmol/L (62), which has been used by some (18, 63–65). It was originally proposed "based on the assay's upper-limit capabilities without dilutions, and not on the

biological implications for health" (62). However, it has been criticized owing to lack of verification with clinical outcome data related to this value. For this reason, we also explored other categorizations of high folate, including concentrations above the 75th percentile of the analytical sample for total folate (>74.1 nmol/L), as well as >66 nmol/L, to be consistent with earlier reports on cognitive function relative to serum folate (24). Similarly, we examined 5MeTHF using the 75th percentile cutoffs from the NHANES sample (>69.9 nmol/L), as well as >50 nmol/L, which was the 60th percentile in an earlier report (24), for comparison. We examined UMFA separately. We classified high UMFA using the previously suggested cutoff of >1 nmol/L at roughly the 75th percentile of Americans aged ≥1 y (26). It should be noted that all these cutoffs are based on different distributions; many cutoffs of high folate status have been utilized in the scientific literature to define "high" status and these vary substantially based on the analytical method, outcome of interest, and form of folate being considered, as reported in a systematic review by Colapinto et al. (66).

Analytical sample

A total of 3472 adults aged 60 y and older participated in the NHANES health examination. Among them, 3181 adults completed ≥1 of 4 cognitive assessments. Older adults who did not have data on vitamin B-12 or folate status ($n = 195$), those with reduced eGFR and albuminuria ($n = 234$), those who had a history of stroke ($n = 192$) or self-reported anemia therapy within the past 3 mo ($n = 101$), or those missing folate biomarker data ($n = 11$) were sequentially excluded, resulting in an analytical sample of 2448. For our primary analyses regarding serum total folate and UMFA we further excluded those who consumed folic acid–containing supplements during fasting ($n = 28$), yielding a primary analytical sample of 2420 (**Supplemental Figure 1**).

Statistical models and analysis

Our analytic process had 3 parts: the preliminary analyses for descriptive statistics, the primary analyses to assess the effects of high folate and low vitamin B-12 on risk of cognitive impairment, and sensitivity analyses to examine the impact of several different cutoffs for high serum total folate and 5MeTHF and high RBC folate on cognitive impairment based on 4 different tests, respectively. The primary research question was whether the effect of high folate depends upon vitamin B-12 status; statistically, this translates to a significance test of the interaction between folate and vitamin B-12 in a logistic model that includes the main effects of folate and vitamin B-12 as well as other covariates. All analyses were conducted using SAS-Callable SUDAAN version 11.0.1 (RTI International) and the MEC 4-y combined survey weights to produce estimates reflective of the US community-living older adult population.

Descriptive statistics were estimated using proc descript in SUDAAN and SEs were estimated by Taylor series linearization. Statistical differences were determined by 2-sided Student's t tests using diffvar statements in proc descript. We log transformed all continuous variables before statistical comparison owing to their nonnormal, right-skewed distributions. To reduce multiple comparisons, statistical differences were not determined for demographic characteristics (i.e., age, sex, race, and education) shown in **Table 1**. SUDAAN proc rlogist was used for the logistic regressions that provided ORs and 95% CIs for all explanatory variables. All full models were controlled for age, sex, race/Hispanic origin, education level, smoking, heavy drinking, and fasting hour, all of which have been associated with some serum folate forms (17, 67). Before running the models, we tested to ensure that the basic assumptions for logistic regression were not violated, including observation independence, absence of multicollinearity, sufficient sample size, and absence of strongly influential outliers (68). Logistic regression was based on a smaller-sized sample of participants with complete data on all covariates. A $P < 0.0125$ was considered statistically significant given the exploratory nature of this analysis (corresponding to a traditional P value of 0.05 divided by the 4 cognitive tests); the exact P values were presented to enhance interpretation.

Total usual intake distributions of folate and vitamin B-12 were estimated using an adapted National Cancer Institute method that can incorporate nutrient intake from dietary supplements (69–71): covariates in the usual intake models included day of the week of recalled day (Monday–Thursday compared with Friday–Sunday), sequence of the dietary recall (first compared with second), and dietary supplement use (yes compared with no). This method produced means and SEs, and percentages of those not meeting or exceeding the DRIs (2, 72). The percentage of respondents not meeting the estimated average requirement (EAR) was interpreted as the percentage who were at risk of inadequacy (i.e., the cutoff method). In addition, the percentage with total folic acid intakes above the UL indicated the percentage who were potentially at risk of adverse effects from excess intake; the UL for folate only applies to folic acid, the synthetic form of the vitamin from supplements and fortified foods (2).

Results

Description of the population characteristics

Table 1 presents characteristics of the study participants, divided by low and normal vitamin B-12, and low and high UMFA status. There was a lower prevalence of smoking among older adults with high UMFA, regardless of vitamin B-12 status. No differences in heavy alcohol use were observed for any of the metabolic groups. Older adults with high UMFA and normal vitamin B-12 were more likely than all other groups to use a folic acid– or vitamin B-12–containing dietary supplement, and to exceed the UL for folic acid. Dietary supplement use with vitamin B-12 and folic acid was observed in ∼43% of the low-vitamin-B-12/high-UMFA group. It is notable that no significant differences in vitamin B-12–containing dietary supplement use were found between the low-vitamin B-12/high-UMFA and the normal-vitamin-B-12/lower-UMFA groups. Although low risk of dietary inadequacy was observed for vitamin B-12 intakes (ranging from 1% to 4% in all groups), older adults with higher UMFA had lower prevalence of dietary intakes below the EAR regardless of vitamin B-12 status.

Vitamin B-12–independent effects

As confirmed in previous numerous investigations, the independent role of lower vitamin B-12 status on suboptimal

TABLE 1 Characteristics of participants by vitamin B-12 and UMFA status[1]

Characteristics	Low vitamin B-12 status		Normal vitamin B-12 status	
	UMFA ≤ 1 nmol/L (n = 217)	UMFA > 1 nmol/L (n = 152)	UMFA ≤ 1 nmol/L (n = 1269)	UMFA > 1 nmol/L (n = 782)
Women, %	48.7 ± 4.9	64.9 ± 4.4	50.1 ± 2.2	57.7 ± 2.1
Age, y	69.3 ± 0.6	72.2 ± 0.8	67.7 ± 0.2	69.6 ± 0.5
Race/Hispanic origin, %				
Non-Hispanic white	83.6 ± 3.4	89.1 ± 2.2	77.1 ± 2.3	83.8 ± 2.2
Non-Hispanic Black	5.3 ± 1.6	4.4 ± 1.1	8.5 ± 1.2	8.2 ± 1.6
Non-Hispanic Asian	2.7 ± 0.8	1.4 ± 0.5	4.6 ± 0.7	2.6 ± 0.6
Hispanic	8.4 ± 2.1	5.1 ± 1.5	9.8 ± 1.5	5.4 ± 1.2
Education, %				
<High school	20.7 ± 3.9	17.1 ± 2.7	16.2 ± 1.8	13.1 ± 1.9
High school graduate or general equivalency diploma	23.7 ± 4.5	23.4 ± 4.1	20.7 ± 2.2	21.5 ± 2.0
Some college or associate degree	32.4 ± 4.8	40.0 ± 5.6	29.7 ± 2.2	33.0 ± 2.3
≥Bachelor's degree	23.2 ± 4.0	19.5 ± 3.8	33.4 ± 2.9	32.4 ± 2.8
Smoking,[2] %	17.5 ± 3.3[a]	7.0 ± 2.1[b]	12.9 ± 1.2[a]	7.7 ± 1.2[b]
Heavy drinking,[2] %	15.1 ± 3.7	5.4 ± 2.9	11.5 ± 1.6	8.3 ± 1.2
Folic acid DS use,[2] %	19.4 ± 3.7[a]	43.8 ± 5.3[b]	29.1 ± 1.5[a,b]	60.9 ± 2.4[c]
Vitamin B-12 DS use,[2] %	21.4 ± 4.3[a]	43.0 ± 5.6[b]	33.5 ± 1.7[b]	64.5 ± 2.3[c]
Medication use,[2,3] %	28.2 ± 3.4	37.9 ± 6.1	28.8 ± 1.7	34.8 ± 2.2
Folate intake,[2] DFE/d	633.5 ± 65.6[a]	873.6 ± 43.8[b]	686.7 ± 26.3[a]	1064.6 ± 35.2[c]
<EAR,[2] %	20.0 ± 2.4[a]	5.0 ± 2.2[b]	14.0 ± 1.8[a]	3.0 ± 1.0[b]
>UL,[2] %	0.6 ± 0.5[a]	2.0 ± 0.8[a]	0.8 ± 0.3[a]	6.0 ± 0.9[b]
Vitamin B-12 intake,[2] μg/d	21.1 ± 7.3[a]	36.7 ± 13.6[a]	87.1 ± 9.6[b]	197.4 ± 38.7[c]
<EAR,[2] %	4.0 ± 3.4	2.0 ± 2.0	3.0 ± 1.0	1.0 ± 0.4

[1]Values are means ± SEs. Low serum vitamin B-12 status is defined as either serum vitamin B-12 < 150 pmol/L or methylmalonic acid > 271 nmol/L. The 75th percentile of UMFA from a previous NHANES analysis was used as a cutoff (26). DFE, dietary folate equivalent; DS, dietary supplement; EAR, estimated average requirement; UL, tolerable upper intake level; UMFA, unmetabolized folic acid.

[2]Values in a row with different superscript letters are significantly different based on multiple t tests, $P < 0.0125$. Any nonnormal variables were log transformed before statistical comparison. Demographic data (i.e., sex, age, race/Hispanic origin, and education) were not statistically compared.

[3]Use of metformin, proton inhibitor, or H_2 antagonist that may inhibit vitamin B-12 absorption.

cognitive performance was observed in this study (**Supplemental Table 1**). Although there was a significantly higher prevalence of at-risk scores on the DSST and CERAD (regardless of numerical classification) as well as the AF (data not shown), the fully adjusted risk models were only significant for the DSST, using both numerical classifications, and the AF (Supplemental Table 1). High UMFA was not independently related to cognitive performance on any test in any models.

Interactions of folate and vitamin B-12

Supplemental Tables 2 and **3** show the unadjusted models of the interaction of vitamin B-12 and folate status. In fully adjusted models, there was a significant interaction between vitamin B-12 and folate status as defined by UMFA in relation to the odds of scoring low on the DSST (<34; P-interaction = 0.0071; **Table 2**). The low-vitamin-B-12/high-UMFA group had a significantly higher OR of scoring low on the DSST (<34 but not <40) than did the low-vitamin-B-12/lower-UMFA group (OR: 2.16; 95% CI: 1.05, 4.47). The normal-vitamin-B-12/high-UMFA group had a lower OR of scoring low on the CERAD-DR than did the normal-vitamin-B-12/lower-UMFA group (OR: 0.73, 95% CI: 0.54, 0.98), although the interaction was not significant.

Using serum total folate as the indicator of high folate status (>74.1 nmol/L), a significant interaction between low vitamin B-12 and high folate status was noted only for the AF for serum total folate (P-interaction = 0.0078; **Table 3**) and 5MeTHF, the primary form of the vitamin in serum (P-interaction = 0.0107; **Supplemental Table 4**). Older adults in the low-vitamin-B-12/high-total-folate group had a significantly higher OR of scoring low on the AF (OR: 1.93; 95% CI: 1.08, 3.45) than did the low-vitamin-B-12/low-total-folate group. In relation to the CERAD-DR, the normal-vitamin-B-12/high-total-folate group also had a lower OR of scoring low (OR: 0.68; 95% CI: 0.47, 0.98) than did the normal-vitamin-B-12/low-total-folate group, but no statistical significance was observed for the interaction (P = 0.0815).

When high folate was categorized using the cutoffs applied in previous NHANES analyses, a marginally significant interaction was observed for serum total folate (>66 nmol/L; P-interaction = 0.0495) and 5-meTHF (>50 nmol/L; P-interaction = 0.0159) with the CERAD-DR (**Supplemental Tables 5** and **6**, respectively). No interactions between vitamin B-12 status and folate status were observed when high folate was categorized as serum total folate > 45.3 nmol/L or when RBC folate was at the 75th percentile of our current sample (**Supplemental Tables 7** and **8**, respectively).

Comparing metabolic categories of folate and vitamin B-12

When high folate and normal vitamin B-12 status was the reference group, having low vitamin B-12 status was associated with greater odds of cognitive impairment based on the DSST cognitive test (**Table 4**): (<34, UMFA > 1 nmol/L, OR: 2.87; 95% CI: 1.85, 4.45; <40, UMFA > 1 nmol/L, OR: 2.22; 95%

TABLE 2 Interaction between vitamin B-12 and UMFA status in relation to the odds of a low cognitive score[1]

	Low vitamin B-12 status		Normal vitamin B-12 status		
Cognitive test	UMFA ≤ 1 nmol/L	UMFA > 1 nmol/L	UMFA ≤ 1 nmol/L	UMFA > 1 nmol/L	*P*-interaction
DSST < 34					
Subjects, *n*	204	139	1182	732	
% with outcome (95% CI)	14.2 (9.3, 21.1)	22.1 (15.3, 30.7)	12.1 (9.6, 15.1)	9.4 (7.6, 11.6)	
OR (95% CI)	1 (reference)	*2.16 (1.05, 4.47)*	1 (reference)	0.74 (0.51, 1.08)	0.0071
DSST < 40					
Subjects, *n*	204	139	1182	732	
% with outcome (95% CI)	26.2 (20.2, 33.2)	29.9 (23.0, 38.0)	19.3 (16.3, 22.8)	16.0 (13.2, 19.3)	
OR (95% CI)	1 (reference)	1.31 (0.70, 2.47)	1 (reference)	0.74 (0.54, 1.03)	0.1347
CERAD-WL < 17					
Subjects, *n*	205	143	1210	744	
% with outcome (95% CI)	26.9 (19.6, 35.7)	26.3 (18.5, 36.0)	18.9 (16.2, 22.0)	20.0 (15.6, 25.3)	
OR (95% CI)	1 (reference)	0.79 (0.41, 1.51)	1 (reference)	0.93 (0.60, 1.43)	0.6567
CERAD-DR < 5					
Subjects, *n*	205	143	1210	742	
% with outcome (95% CI)	23.4 (17.2, 31.1)	28.5 (18.6, 41.0)	19.4 (16.2, 23.0)	18.5 (14.9, 22.7)	
OR (95% CI)	1 (reference)	1.01 (0.45, 2.28)	1 (reference)	*0.73 (0.54, 0.98)*	0.4367
AF < 14					
Subjects, *n*	205	141	1210	739	
% with outcome (95% CI)	25.7 (18.1, 35.2)	33.2 (24.4, 43.4)	17.4 (14.8, 20.4)	18.6 (15.3, 22.5)	
OR (95% CI)	1 (reference)	1.33 (0.76, 2.33)	1 (reference)	1.04 (0.74, 1.46)	0.4176

[1]Low serum vitamin B-12 status is defined as either serum vitamin B-12 < 150 pmol/L or MMA > 271 nmol/L. The 75th percentile of UMFA from a previous NHANES analysis was used as a cutoff (26). The multiple logistic regression models were adjusted for age group, sex, race/Hispanic origin, education level, heavy drinking, smoking, and fasting hour. AF, Animal Fluency test; CERAD-DR, Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall test; CERAD-WL, Consortium to Establish a Registry for Alzheimer's Disease Word Learning test; DSST, Digit Symbol Substitution Test; UMFA, unmetabolized folic acid.

CI: 1.31, 3.75 and UMFA ≤ 1 nmol/L, OR: 1.69, 95% CI: 1.16, 2.47). The ORs of scoring low on the DSST (<40) were not statistically different between the low-vitamin-B-12/high-UMFA and the low-vitamin-B-12/lower-UMFA groups ($P = 0.3895$, data not shown). Older adults with low vitamin B-12 combined with high UMFA also had a higher risk of low scores on the AF (<14, OR: 1.97; 95% CI: 1.30, 2.97) than did the referent group. Similarly, both of the low-vitamin-B-12 groups had a higher risk of scoring low on the AF (<14, OR: 3.47; 95% CI: 2.09, 5.76 for high folate; and OR: 1.79; 95% CI: 1.11, 2.89 for lower folate) than the reference group (**Table 5**). The ORs for scoring low on the AF were not statistically different for the low-vitamin-B-12/high-total-folate group compared with the low-vitamin-B-12/lower-total-folate group ($P = 0.0272$; data not shown). In addition, higher risk of cognitive impairment as assessed by the CERAD-DR was observed within the normal-vitamin-B-12 group for both the lower-UMFA (Table 4) (OR: 1.38; 95% CI: 1.02, 1.85) and the lower-total-folate (Table 5) (OR: 1.47; 95% CI: 1.02, 2.13) groups when compared with the reference group. **Supplemental Tables 9** and **10** show the unadjusted models of these comparisons.

Discussion

In this work we sought to examine whether the proposed combination of high folic acid/folate together with low vitamin B-12 was related to cognitive function. First, we confirmed the earlier characterized relation of an interaction of UMFA and vitamin B-12 with cognitive performance as assessed by DSST scores <34 (i.e., the 20th percentile in NHANES 1999–2004) (24). However, we failed to detect a significant interaction when low DSST scores were characterized as <40 [i.e., the 25th percentile in NHANES 2011–2014 (42)]. Next, we examined the potential for an interaction using the same models as we did for UMFA, but expanded the "high" folate definition to various definitions of serum total folate or 5MeTHF, the primary circulating form of folate (67). When high folate status was defined as the 75th percentile in the current sample (serum total folate > 74.1 nmol/L and 5MeTHF > 69.9 nmol/L), a significant interaction of low vitamin B-12 and high folate was observed relative to AF scores; however, when it was defined by the 75th percentile used previously in the NHANES 1999–2002 (serum total folate > 66 nmol/L or 5MeTHF > 50 nmol/L), no significant interactions were observed. No interactions were observed for high serum total folate defined at >45.3 nmol/L, consistent with the Sacramento Area Latino Study on Aging (64) and recent analysis of the Irish Longitudinal Study on Aging (65); but, as previously stated, this cutoff was based on the capability of the analytical method and may be considered arbitrary. Moreover, no relations between RBC folate, a marker of tissue stores and longer-term status (29), and cognitive function were observed, regardless of vitamin B-12 status. No previous research to our knowledge has examined high RBC folate relative to cognitive outcomes (66); thus, future work is needed to examine if high RBC folate is related to cognitive function, utilizing different cutoffs to determine high RBC folate.

The hypothesis that excessive folate exposure aggravates cognitive impairment resulting from low vitamin B-12 status

TABLE 3 Interaction between vitamin B-12 and total folate status in relation to the odds of a low cognitive score[1]

Cognitive test	Low vitamin B-12 status		Normal vitamin B-12 status		*P*-interaction
	Total folate ≤ 74.1 nmol/L	Total folate > 74.1 nmol/L	Total folate ≤ 74.1 nmol/L	Total folate > 74.1 nmol/L	
DSST < 34					
Subjects, *n*	289	54	1501	411	
% with outcome (95% CI)	18.5 (13.8, 24.2)	13.0 (7.1, 22.7)	11.4 (9.4, 13.8)	9.7 (7.4, 12.5)	
OR (95% CI)	1 (reference)	0.68 (0.27, 1.74)	1 (reference)	0.87 (0.58, 1.32)	0.6235
DSST < 40					
Subjects, *n*	289	54	1501	411	
% with outcome (95% CI)	28.3 (23.0, 34.3)	25.5 (16.1, 37.8)	18.4 (15.7, 21.5)	16.7 (12.9, 21.4)	
OR (95% CI)	1 (reference)	0.94 (0.27, 3.26)	1 (reference)	0.95 (0.65, 1.39)	0.9956
CERAD-WL < 17					
Subjects, *n*	291	57	1529	423	
% with outcome (95% CI)	24.3 (19.1, 30.4)	36.7 (25.1, 50.0)	18.9 (15.8, 22.3)	21.0 (17.0, 25.6)	
OR (95% CI)	1 (reference)	1.78 (0.88, 3.59)	1 (reference)	1.04 (0.75, 1.44)	0.1823
CERAD-DR < 5					
Subjects, *n*	291	57	1528	422	
% with outcome (95% CI)	23.3 (18.3, 29.1)	35.4 (20.7, 53.5)	19.6 (16.5, 23.1)	17.5 (13.4, 22.5)	
OR (95% CI)	1 (reference)	1.66 (0.64, 4.30)	1 (reference)	*0.68 (0.47, 0.98)*	0.0815
AF < 14					
Subjects, *n*	289	57	1529	418	
% with outcome (95% CI)	26.2 (20.0, 33.5)	40.6 (28.5, 54.0)	18.5 (15.8, 21.4)	16.2 (12.6, 20.6)	
OR (95% CI)	1 (reference)	*1.93 (1.08, 3.45)*	1 (reference)	0.78 (0.54, 1.13)	0.0078

[1]Low serum vitamin B-12 status is defined as either serum vitamin B-12 < 150 pmol/L or MMA > 271 nmol/L. The 75th percentile of serum total folate concentration was used as a cutoff. The multiple logistic regression models were adjusted for age group, sex, race/Hispanic origin, education level, heavy drinking, smoking, and fasting hour. AF, animal fluency test; CERAD-DR, Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall test; CERAD-WL, Consortium to Establish a Registry for Alzheimer's Disease Word Learning test; DSST, Digit Symbol Substitution Test.

has been proposed by case reports and observational studies, as summarized by Field and Stover (73) and by Berry (74). Although this is an important area of research, very limited data are available to address the knowledge gaps, and the available data are of low causal quality, because they are limited to observational human or experimental animal data. There are also considerable methodological challenges presented by the existing observational data. First, vitamin B-12 deficiency is notoriously difficult to quantify from biospecimens, and prevalence estimates vary by the biomarker used and the cutoffs that are applied (13, 75, 76). Another primary challenge in investigating potential concerns about the adverse health effects of high folate status is the heterogeneity in biomarkers, analytical methods, and cutoffs used to define high folate status (31, 77–79). For example, Morris et al., in a NHANES 1999–2002 report, found that high serum total folate defined as >59 nmol/L was associated with increased odds of cognitive impairment (i.e., DSST < 34, OR: 2.6; 95% CI: 1.1, 6.1) with low vitamin B-12 status (i.e., vitamin B-12 < 148 pmol/L or MMA ≥ 210 nmol/L) and a protective association with normal vitamin B-12 status (OR: 0.4; 95% CI: 0.2, 0.9) (32). However, Miller et al. (64) did not observe any relation of cognitive function scores with low vitamin B-12 (<148 pmol/L) combined with high serum total folate (>45.3 nmol/L) in the Sacramento Area Latino Study on Aging. In a longitudinal analysis of the Framingham Heart Study, Morris et al. (25) observed greater decline in the Mini-Mental State Examination score over 8 y among older adults with low vitamin B-12 status (<258 pmol/L) when serum total folate was >21.75 nmol/L. Thus, we sought to characterize the association of low vitamin B-12 with cognitive impairment relative to different definitions of "high folate." Clearly, the widely used approach of selecting the cutoff based on the distribution may be neither accurate nor reflective of "high" status, it may fluctuate among study samples, and it exhibits secular trends. Thus, it is considered arbitrary (80), and a cutoff based on biological evidence of associations with meaningful health outcomes should be used instead. Such a standard is greatly needed.

Given that low vitamin B-12 is clearly an independent risk factor for cognitive impairment and that folate has been positively associated with cognitive function (16, 81, 82), we assumed that those with normal vitamin B-12 in combination with high UMFA or high total folate would have the "optimal" profile for cognitive health and, therefore, considered this a reference group for further analyses. Our findings support that ensuring optimal concentrations of both vitamin B-12 and folate may be the best public health objective to lessen potential adverse effects (83).

Although an emerging body of literature, including this study, suggests that potential adverse effects may exist with high folate combined with low vitamin B-12 status relative to performance on some cognitive tests, the mechanisms whereby these associations may arise remain unclear and causal inference is weak because the data are observational in nature. Folate and vitamin B-12 interact within one-carbon metabolism, whereas vitamin B-12 deficiency can cause an accumulation of 5MeTHF in cells (i.e., 5MeTHF trap), leading to a functional folate deficiency and impaired DNA biosynthesis (5). Folic acid can partially rescue the effects of vitamin B-12 deficiency on anemia, but is not known to rescue the effects on neurological pathology (73). Possibly, many of those who have high folate status and low vitamin B-12 status would be those who consume dietary supplements but

TABLE 4 Vitamin B-12 and UMFA status in relation to the odds of a low cognitive score[1]

	Low vitamin B-12 status		Normal vitamin B-12 status	
Cognitive test	UMFA ≤ 1 nmol/L	UMFA > 1 nmol/L	UMFA ≤ 1 nmol/L	UMFA > 1 nmol/L
DSST < 34				
Subjects, *n*	204	139	1182	732
% with outcome (95% CI)	14.2 (9.3, 21.1)	22.1 (15.3, 30.7)	12.1 (9.6, 15.1)	9.4 (7.6, 11.6)
OR (95% CI)	1.33 (0.78, 2.24)	*2.87 (1.85, 4.45)*	1.35 (0.93, 1.97)	1 (reference)
DSST < 40				
Subjects, *n*	204	139	1182	732
% with outcome (95% CI)	26.2 (20.2, 33.2)	29.9 (23.0, 38.0)	19.3 (16.3, 22.8)	16.0 (13.2, 19.3)
OR (95% CI)	*1.69 (1.16, 2.47)*	*2.22 (1.31, 3.75)*	1.35 (0.97, 1.86)	1 (reference)
CERAD-WL < 17				
Subjects, *n*	205	143	1210	744
% with outcome (95% CI)	26.9 (19.6, 35.7)	26.3 (18.5, 36.0)	18.9 (16.2, 22.0)	20.0 (15.6, 25.3)
OR (95% CI)	1.37 (0.86, 2.16)	1.08 (0.57, 2.05)	1.08 (0.70, 1.66)	1 (reference)
CERAD-DR < 5				
Subjects, *n*	205	143	1210	742
% with outcome (95% CI)	23.4 (17.2, 31.1)	28.5 (18.6, 41.0)	19.4 (16.2, 23.0)	18.5 (14.9, 22.7)
OR (95% CI)	1.38 (0.80, 2.38)	1.39 (0.73, 2.65)	*1.38 (1.02, 1.85)*	1 (reference)
AF < 14				
Subjects, *n*	205	141	1210	739
% with outcome (95% CI)	25.7 (18.1, 35.2)	33.2 (24.4, 43.4)	17.4 (14.8, 20.4)	18.6 (15.3, 22.5)
OR (95% CI)	1.48 (0.83, 2.64)	*1.97 (1.30, 2.97)*	0.96 (0.68, 1.35)	1 (reference)

[1]Low serum vitamin B-12 status is defined as either serum vitamin B-12 < 150 pmol/L or MMA > 271 nmol/L. The 75th percentile of UMFA from a previous NHANES analysis was used as a cutoff (26). The multiple logistic regression models were adjusted for age group, sex, race/Hispanic origin, education level, heavy drinking, smoking, and fasting hour. AF, animal fluency test; CERAD-DR, Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall test; CERAD-WL, Consortium to Establish a Registry for Alzheimer's Disease Word Learning test; DSST, Digit Symbol Substitution Test; UMFA, unmetabolized folic acid.

cannot absorb vitamin B-12 well and, therefore, low vitamin B-12 status may drive cognitive impairment independently of folate status (73). Even in the population we studied, the prevalence of use of vitamin B-12–containing dietary supplements did not differ between the low-vitamin-B-12/high-UMFA group and the normal-vitamin-B-12/low-UMFA group, suggesting some of the low-vitamin-B-12/high-UMFA group may also have vitamin B-12 malabsorption issues because adequate amounts of dietary and supplemental vitamin B-12 intakes were observed; albeit, the use of medication that may affect vitamin B-12 status did not differ between those with low vitamin B-12 and normal vitamin B-12 status (13). However, this finding does not clearly explain the higher risk in the low-vitamin-B-12/high-UMFA group than in the low-vitamin-B-12/lower-UMFA group. To date, as far as we know no controlled trials testing this hypothesis exist, nor do they seem possible, owing to related ethical issues. These constraints highlight the need for cellular and animal studies, as well as high-quality cohort and observational studies, to further address this research question.

Strengths and limitations

In the 2011–2014 NHANES data, 4 cognitive tests were administered to a nationally representative sample of older adults living in the community. In addition, although the NHANES collected comprehensive information about demographics, lifestyle, and health conditions, which enabled us to account for known key confounders, there is always a possibility of residual confounding. We explored different definitions and cutoffs for folate and vitamin B-12 biomarkers, but additional analyses using different modeling techniques might be more revealing. Future studies may also explore the best way to combine multiple tests to represent specific domains or overall cognitive function. One of the important limitations of our study is that we could not accurately determine malabsorption as a condition and the genetic variants that affect absorption of vitamin B-12 or metabolism of folate (e.g., common polymorphisms in 5,10-methylenetetrahydrofolate reductase); similarly, genetic components associated with cognitive decline and dementias could not be examined in this data set. Owing to the cross-sectional nature of our data, a causal relation cannot be established between the combination of high folate and low vitamin B-12 and cognitive test performance. Finally, note that this exploratory analysis was conservative in that we accounted for multiple comparisons by accounting for the 4 main outcome tests only but not for each statistical test and, in doing so, we reduced the risk of Type II error but also increased the risk of Type I errors; accordingly, all *P* values should be interpreted with this caveat in mind.

Conclusions

In US older adults, performance on cognitive tests was heterogeneously dependent on both vitamin B-12 and folate status in an interactive manner. When vitamin B-12 status was within the normal range, performance on cognitive tests was less likely to be impaired, and higher folate status appeared to be protective. However, when vitamin B-12 status was low,

TABLE 5 Vitamin B-12 and total folate status in relation to the odds of a low cognitive score[1]

Cognitive test	Low vitamin B-12 status		Normal vitamin B-12 status	
	Total folate ≤ 74.1 nmol/L	Total folate > 74.1 nmol/L	Total folate ≤ 74.1 nmol/L	Total folate > 74.1 nmol/L
DSST < 34				
Subjects, *n*	289	54	1501	411
% with outcome (95% CI)	18.5 (13.8, 24.2)	13.0 (7.1, 22.7)	11.4 (9.4, 13.8)	9.7 (7.4, 12.5)
OR (95% CI)	*1.86 (1.27, 2.73)*	1.28 (0.56, 2.91)	1.14 (0.76, 1.73)	1 (reference)
DSST < 40				
Subjects, *n*	289	54	1501	411
% with outcome (95% CI)	28.3 (23.0, 34.3)	25.5 (16.1, 37.8)	18.4 (15.7, 21.5)	16.7 (12.9, 21.4)
OR (95% CI)	*1.69 (1.16, 2.45)*	1.59 (0.46, 5.55)	1.05 (0.72, 1.55)	1 (reference)
CERAD-WL < 17				
Subjects, *n*	291	57	1529	423
% with outcome (95% CI)	24.3 (19.1, 30.4)	36.7 (25.1, 50.0)	18.9 (15.8, 22.3)	21.0 (17.0, 25.6)
OR (95% CI)	1.03 (0.67, 1.58)	1.83 (0.84, 4.00)	0.96 (0.69, 1.33)	1 (reference)
CERAD-DR < 5				
Subjects, *n*	291	57	1528	422
% with outcome (95% CI)	23.3 (18.3, 29.1)	35.4 (20.7, 53.5)	19.6 (16.5, 23.1)	17.5 (13.4, 22.5)
OR (95% CI)	1.38 (0.80, 2.38)	2.29 (0.99, 5.33)	*1.47 (1.02, 2.13)*	1 (reference)
AF < 14				
Subjects, *n*	289	57	1529	418
% with outcome (95% CI)	26.2 (20.0, 33.5)	40.6 (28.5, 54.0)	18.5 (15.8, 21.4)	16.2 (12.6, 20.6)
OR (95% CI)	*1.79 (1.11, 2.89)*	*3.47 (2.09, 5.76)*	1.28 (0.89, 1.86)	1 (reference)

[1]Low serum vitamin B-12 status is defined as either serum vitamin B-12 < 150 pmol/L or MMA > 271 nmol/L. The 75th percentile of serum total folate concentration was used as a cutoff. The multiple logistic regression models were adjusted for age group, sex, race/Hispanic origin, education level, heavy drinking, smoking, and fasting hour. AF, animal fluency test; CERAD-DR, Consortium to Establish a Registry for Alzheimer's Disease Delayed Recall test; CERAD-WL, Consortium to Establish a Registry for Alzheimer's Disease Word Learning test; DSST, Digit Symbol Substitution Test.

high folate was associated with significantly poorer cognitive performance on several tests. The present analysis provides additional evidence that the combination of low vitamin B-12 and high folate status may be associated with poor performance on cognitive function tests in older adults.

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