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# Associations between Genetic Variants and Blood Biomarkers of One-Carbon Metabolism in Postmenopausal Women from the Women's Health Initiative Observational Study

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## ABSTRACT

**Background:** Genetic variation in one-carbon metabolism may affect nutrient concentrations and biological functions. However, data on genetic variants associated with blood biomarkers of one-carbon metabolism in US postmenopausal women are limited, and whether these associations were affected by the nationwide folic acid (FA) fortification program is unclear.

**Objectives:** We investigated associations between genetic variants and biomarkers of one-carbon metabolism using data from the Women's Health Initiative Observational Study.

**Methods:** In 1573 non-Hispanic White (NHW) and 282 Black/African American, American Indian/Alaska Native, Asian/Pacific Islander, and Hispanic/Latino women aged 50–79 y, 288 nonsynonymous and tagging single-nucleotide variants (SNVs) were genotyped. RBC folate, plasma folate, pyridoxal-5′-phosphate (PLP), vitamin B-12, homocysteine, and cysteine concentrations were determined in 12-h fasting blood. Multivariable linear regression tested associations per variant allele and for an aggregated genetic risk score. Effect modifications before, during, and after nationwide FA fortification were examined.

**Results:** After correction for multiple comparisons, among NHW women, 5,10-methylenetetrahydrofolate reductase (*MTHFR*) rs1801133 (677C→T) variant T was associated with lower plasma folate (−13.0%; 95% CI: −17.3%, −8.6%) and higher plasma homocysteine (3.5%; 95% CI: 1.7%, 5.3%) concentrations. Other associations for nonsynonymous SNVs included *DNMT3A* rs11695471 (T→A) with plasma PLP; *EHMT2* rs535586 (G→A), *TCN2* rs1131603 (L349S A→G), and TCN2 rs35838082 (R188W G→A) with plasma vitamin B-12; *CBS* rs2851391 (G→A) with plasma homocysteine; and *MTHFD1* rs2236224 (G→A) and rs2236225 (R653Q G→A) with plasma cysteine. The influence of FA fortification on the associations was limited. Highest compared with lowest quartiles of aggregated genetic risk scores from SNVs in *MTHFR* and *MTRR* were associated with 14.8% to 18.9% lower RBC folate concentrations. Gene–biomarker associations were similar in women of other races/ethnicities.

**Conclusions:** Our findings on genetic variants associated with several one-carbon metabolism biomarkers may help elucidate mechanisms of maintaining B vitamin status in postmenopausal women. *J Nutr* 2022;152:1099–1106.

**Keywords:** one-carbon metabolism, postmenopausal women, single-nucleotide variants, folate, MTHFR

## Introduction

At the molecular level, DNA instability and inflammation are important factors in cancer development (1). DNA instability and poor repair capacity are functions of insufficient nutrients of one-carbon metabolism, including folate, vitamin B-2, vitamin B-6, and vitamin B-12 (1). Because the transport, uptake, and metabolism of one-carbon metabolism nutrients


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require various enzymes, genetic variation can influence the circulating concentrations of these nutrients. One of the most notable genes related to one-carbon metabolism is *MTHFR*, encoding 5,10-methylenetetrahydrofolate reductase, which supplies 5-methyltetrahydrofolate for homocysteine remethylation to methionine (2). Genetic variants in the *MTHFR* 677C→T genotype are associated with reduced plasma folate concentrations, increased plasma homocysteine, and increased risk of colorectal cancer (CRC) (3, 4). In our investigations focusing on postmenopausal women in the United States, homocysteine was positively associated with increased risk of CRC (5), but *MTHFR* 677C→T or blood folate concentrations were not associated with CRC risk (6, 7). The inconsistent findings suggest that further understanding the association between genetic variants and circulating one-carbon metabolism biomarkers is essential in elucidating the role of one-carbon metabolism in the etiology of diseases, such as CRC.

Research investigating the associations of genetic variations with biomarkers of one-carbon metabolism is often constrained by the small number of single-nucleotide variants (SNVs; formerly SNPs) or blood biomarkers being examined. Most previous studies have focused on *MTHFR* variants (3, 8–21), but associations have also been reported for cystathionine β-synthase (*CBS*), folate hydrolase 1 (*FOLH1*), and reduced folate carrier 1 (*RFC1*) variants (8, 10–12, 16, 19, 22). The most common nutrients examined are plasma folate or RBC folate (3, 8–19), followed by homocysteine (3, 11, 12, 14, 16–18, 23), which is an indicator of folate cycle suppression due to the loss of methyl groups from the folate cycle. There are limited data on circulating concentrations of vitamins B-6 and B-12 (11, 18, 19, 23), except for data from genome-wide association studies (GWASs) (24–27). In addition, most of the previous studies were conducted among European populations (3, 8–11, 13, 15, 18, 22, 23, 27), whereas studies among populations in the United States are less common (12, 14, 19, 20, 25, 26). Examining the association between one-carbon metabolism nutrients and SNVs in this latter population is important because folic acid (FA) fortification of enriched cereals and grains began in the United States in 1998. The fortification program has improved folate status (28, 29) and was found in our previous analysis to be associated with changes between one-carbon metabolism nutrients and global DNA methylation concentrations in postmenopausal women (30). However, only a few studies included postfortification blood measurements from participants in the United States (12, 21, 26), and to what extent the fortification program affects the associations between genetic variation and circulating one-carbon metabolism nutrients or biomarkers is unclear.

The primary objective of the present study was to investigate whether any among a large panel of genetic variants in key proteins of one-carbon metabolism were associated with the concentrations of the following one-carbon metabolism biomarkers: RBC folate or plasma folate, vitamin B-6 [indicated by pyridoxal-5′-phosphate (PLP)], vitamin B-12, homocysteine, and cysteine. Our secondary objective was to examine whether any gene–biomarker associations were modified by the initiation of the nationwide FA fortification program.

## Methods

### Study population

Study participants were selected from the Women's Health Initiative Observational Study (WHI-OS), a prospective cohort study that recruited 93,676 postmenopausal women from 40 US clinical centers between 1993 and 1998 (31). The study design and baseline characteristics of this cohort have been described in detail (31). Baseline eligibility requirements included postmenopausal status, age between 50 and 79 y at enrollment, and low likelihood of loss to follow-up within 3 y due to relocation or death resulting from preexisting medical conditions. We analyzed genetic and biomarker data from a nested case-control study of CRC risk within the WHI-OS. In the nested case-control study (5, 7), women were excluded if they had a preexisting intestinal disease, including history of CRC, carcinoma in situ, ulcerative colitis, or Crohn disease. In total, 988 incident cases and 988 controls were matched based on age (±3 y), self-reported race/ethnicity, enrollment date (±1 y), hysterectomy status, and date of blood draw (±6 mo). The cases were restricted to participants who developed CRC 6 months or more after baseline measurements to ensure that the blood samples collected at baseline were unlikely affected by the CRC status. For the current study, analyses were conducted using data from 1859 participants with genetic and biomarker data that passed quality control. Written informed consent was obtained from all participants at the WHI enrollment. The present study was approved by the human subjects institutional review board at the Fred Hutchinson Cancer Research Center and at all relevant institutions and was conducted in accordance with their ethical standards.

### Blood sample processing and analysis

Twelve-hour fasting blood samples were collected from all participants at baseline using EDTA collection tubes. Samples were kept at 4°C for up to 1 h prior to centrifugation at 4°C (10 mins, relative centrifugal force of 1300 × *g*). to obtain plasma and RBCs. Samples were then stored at –70°C until analysis. The mean ± SD interval from the baseline blood draw to a CRC diagnosis was 5.3 ± 3.1 y. Plasma and RBC folate and plasma vitamin B-12 concentrations were determined by radioassays (SimulTRAC; MP Biomedicals), total plasma homocysteine and cysteine concentrations by HPLC with postcolumn fluorescence detection (32), and plasma PLP by HPLC with fluorescence detection (33). The interassay coefficients of variation calculated from 5% blind duplicate samples were as follows: homocysteine, 6.5%; cysteine, 7.1%; plasma folate, 8.6%; RBC folate, 10.2%; vitamin B-12, 6.2%; and PLP, 5.9%.

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Supplemental Tables 1–13 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/jn/.
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Abbreviations used: *ALPL* , alkaline phosphatase, biomineralization associated; *CBS*, cystathionine β-synthase; CEPH, Centre d'Etude du Polymorphisme Humain, or Human Polymorphism Study Center; CRC, colorectal cancer; *CUBN*, cubilin; *DNMT3A*, DNA methyltransferase 3α; *EHMT2*, euchromatic histone lysine methyltransferase 2; FA, folic acid; FDR, false discovery rate; *FOLH1*, folate hydrolase 1; *FUT2*, galactoside 2-α-L-fucosyltransferase 2; GWAS, genome-wide association study; *MTHFD1*, methylenetetrahydrofolate dehydrogenase, cyclohydrolase, and formyltetrahydrofolate synthetase 1; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; *MUT*, methylmalonyl-CoA mutase; *NBPF3*, neuroblastoma breakpoint family member 3; NHW, non-Hispanic white; PLP, pyridoxal-5′-phosphate; *RFC1*, reduced folate carrier 1; SNV, single-nucleotide variant; tagSNV, tagging single-nucleotide variant; *TCN1*, transcobalamin 1; *TCN2*, transcobalamin 2; THF, tetrahydrofolate; WHI-OS, Women's Health Initiative Observational Study.

### Genotyping

Detailed genotyping procedures and information regarding the SNVs have been reported in an earlier study (6). We genotyped 30 one-carbon metabolism genes for both nonsynonymous (i.e., variants with a high likelihood of functional impact according to amino acid changes, genomic location, and laboratory and epidemiologic studies) and tagging SNVs (tagSNVs). TagSNVs were selected based on linkage disequilibrium in the CEPH (Centre d'Etude du Polymorphisme Humain, or Human Polymorphism Study Center) collection of residents of Utah with Western and Northern European ancestry from HapMap Phase II (data release 24 on NCBI B36 assembly). The minor allele frequency cutoff was 5% at an average density of 1 SNV per kB, and $r^2$ was 0.80. On each gene, tagSNVs were selected from 10 kB upstream to 5 kB downstream or from tagSNV selection through the end of the linkage disequilibrium blocks. The Illumina 384-plex BeadXpress GoldenGate Genotyping platform was used to genotype 295 SNVs in the study. For quality control, we included 30 CEPH trios from the HapMap project and 5% blinded duplicates (42 case-control pairs). Exclusion criteria for the SNVs included <95% concordance with blinded or nonblinded duplicates, 95% call rate, deviations from the expected minor allele frequency, or Hardy–Weinberg equilibrium $P$ values < 0.0001. Of 295 SNVs, 288 (98%) passed quality control and entered statistical analyses. The genes and number of SNVs analyzed are given in **Supplemental Table 1**.

### Covariates

Demographic characteristics, including age, race/ethnicity, educational level, annual household income, and health-related characteristics (i.e., personal medical history, use of postmenopausal hormones, medication use, physical activity, and smoking history), were self-reported at baseline using standardized questionnaires (34). Participant height and weight were measured by trained staff using a standard protocol, and BMI was calculated as weight in kilograms divided by height in meters squared. Dietary intake levels of folate and B vitamins were derived from a validated FFQ implemented at baseline (35). Dietary supplement use was determined with an inventory-type questionnaire in which study staff recorded specific nutrients from participants' supplement bottles brought to a clinic visit. Total nutrient intake was summed from dietary and supplemental sources. For FFQs administered on or after August 1, 1997, intakes of both natural folate and synthetic FA added to enriched grains were computed. Accounting for synthetic FA bioavailability, an algorithm was used to calculate dietary folate intake and total folate intake: dietary folate intake = sum of natural folate from food + (1.7 × FA in fortification); total folate intake = sum of dietary folate + (1.7 × FA in supplements) (36).

### Statistical analysis

We investigated the associations between genetic variants and biomarkers of one-carbon metabolism with a priori hypotheses based on enzyme-nutrient function (Supplemental Table 1). For each gene, we examined the associations for the biomarkers that were likely affected directly (e.g., *MTHFR* C677T with plasma and RBC folate and with plasma homocysteine). We conducted multivariable linear regression analyses using genotype of the SNV as an independent variable and the biomarker concentration as the outcome variable, adjusting for age; case-control status; BMI; smoking status (never, former, or current); total intake of folate, vitamin B-6, vitamin B-12, and riboflavin (vitamin B-2) from food and supplements; and FA fortification periods in the models. Alcohol intake was not included in the model because the magnitudes of the associations were similar before and after adjustments for the frequency of alcohol use. The distributions of biomarkers were examined, and logarithmic transformation was performed to improve normality. Genetic variants were modeled as additive models (i.e., 0, 1, or 2 variants as a continuous variable), and the regression coefficient ($\beta$) was converted to percentage change with the formula [exp ($\beta$) − 1] × 100% and interpreted as the estimated percentage difference in the concentration of a biomarker for each additional variant allele. To control for the inflation of false-positive rates from multiple comparisons, false discovery rate (FDR)–adjusted $P$ value with a significance threshold of 0.05 was applied to the associations of tagSNVs based on the total number of SNVs tested for a biomarker. For nonsynonymous SNVs, FDR was performed for each gene–biomarker pair as these SNVs were tested based on strong a priori hypotheses; the threshold was set at 0.05. The above main analysis was conducted using data from non-Hispanic white (NHW) women ($n$ = 1573) to avoid spurious associations resulting from population stratification and to compare our results with other studies that were mainly conducted in white individuals. For the significant gene–biomarkers associations, we replicated them for women of other racial/ethnic groups ($n$ = 282): Black/African American, American Indian/Alaska Native, Asian/Pacific Islander, and Hispanic/Latino. Because several SNVs were associated with RBC folate concentrations, we calculated aggregated genetic risk scores for the association. For each of the significant SNVs, a score of 1 was given for an individual who carried one risk allele (i.e., an allele that was associated with lower RBC folate concentrations); 2, for 2 risk alleles; and 0, for no risk allele. The scores of all the SNVs were summed as a total score, and linear regressions were performed for the percentage differences in RBC folate concentrations among the quartiles and between the 90th and 10th percentiles of the total score.

For regression modeling and evaluating the effect of the modification of FA fortification, we encoded the FA fortification variable as whether blood draws were obtained prefortification (1993–1995), perifortification (1996–1997; i.e., when initial fortification began but was not yet mandated), or postfortification (1998). The effect modification of FA fortification was examined only among SNVs that were statistically significant in the main effect analysis among NHW women to reduce the numbers of comparisons and false-positive findings. Statistical interaction was evaluated by examining the $P$-interaction value of the product term between an SNV and the FA fortification periods; the significance level was controlled using the Bonferroni procedure at 0.0028 (0.05/18 tests). All statistical tests were 2-sided and were conducted with SAS software, version 9.4 (SAS Institute).

## Results

Selected baseline demographic characteristics, dietary intake, and biomarker concentrations of NHW women are summarized in **Table 1**. The mean age was 66.6 y; 24.5%, 54.9%, and 19.6% of women were recruited during the pre-, peri-, and post-FA fortification periods, respectively. Women recruited in the post-FA fortification period had higher folate intake levels as well as higher RBC and plasma folate concentrations compared with women recruited in the pre-FA fortification period. **Table 2** lists top SNVs (nonsynonymous SNVs with nominal $P$ < 0.05 and tagSNVs with FDR-adjusted $P$ < 0.05) in association with the biomarkers of one-carbon metabolism in NHW women. Minor allele frequencies of these SNVs are given in **Supplemental Table 2**; full results for all SNVs are given in **Supplemental Tables 3–8**. Specifically, variant *MTHFR* rs1801133 (677C→T) was associated with lower plasma folate concentrations (−13.0%; 95% CI: −17.3%, −8.6%, per variant allele T) but with higher concentrations of RBC folate (8.7%; 95% CI: 5.7%, 11.8%, per variant allele T). This SNV was also associated with higher plasma concentrations of homocysteine (3.5%; 95% CI: 1.7%, 5.3%, per variant allele T). Another SNV associated with higher RBC folate concentration was the variant gene encoding methionine synthase reductase (*MTRR*) rs1801394 (I49M A→G) (6.6%; 95% CI: 3.7%, 9.6%, per variant allele G). Several variants of tagSNVs in *MTHFR* and *MTRR* were significantly associated with lower RBC folate concentrations (FDR-adjusted $P$ < 0.05); however, they were not associated with plasma folate concentrations.

**TABLE 1** Selected baseline characteristics, dietary intake values, and biomarker concentrations in non-Hispanic white women and by the folic acid fortification status at the time of recruitment[1]

| | | | WHI recruitment period | | | | | |
|---|---|---|---|---|---|---|---|---|
| | All participants | | Pre-FA fortification | | Peri-FA fortification | | Post-FA fortification | |
| Characteristic | *n* | Mean ± SD or % | *n* | Mean ± SD or % | *n* | Mean ± SD or % | *n* | Mean ± SD or % |
| Age, y | 1573 | 67.2 ± 6.8 | 394 | 65.6 ± 6.8 | 871 | 67.5 ± 6.9 | 308 | 68.2 ± 6.0 |
| BMI, kg/m$^2$ | 1573 | 27.3 ± 5.7 | 394 | 27.1 ± 5.9 | 871 | 27.4 ± 5.5 | 308 | 27.4 ± 6.0 |
| Smoking status | | | | | | | | |
| Never smokers | 771 | 49.0 | 190 | 48.2 | 436 | 50.1 | 145 | 47.1 |
| Former smokers | 707 | 45.0 | 182 | 46.2 | 379 | 43.5 | 146 | 47.4 |
| Current smokers | 95 | 6.0 | 22 | 5.6 | 56 | 6.4 | 17 | 5.5 |
| Total intake levels (diet plus supplement) | | | | | | | | |
| Folate, DFE/d | 1573 | 797 ± 477 | 394 | 768 ± 451 | 871 | 784 ± 483 | 308 | 870 ± 484 |
| Vitamin B-6, mg/d | 1573 | 8.6 ± 23.1 | 394 | 9.9 ± 28.0 | 871 | 8.0 ± 21.2 | 308 | 8.5 ± 21.1 |
| Vitamin B-12, μg/d | 1573 | 28.6 ± 100 | 394 | 29.7 ± 125 | 871 | 26.6 ± 90.7 | 308 | 32.9 ± 91.4 |
| Riboflavin, mg/d | 1573 | 6.4 ± 16.4 | 394 | 6.8 ± 17.3 | 871 | 6.2 ± 15.4 | 308 | 6.7 ± 18.1 |
| Biomarkers | | | | | | | | |
| RBC folate, ng/mL | 1573 | 617 ± 261 | 394 | 555 ± 230 | 871 | 614 ± 275 | 308 | 706 ± 232 |
| Plasma folate, ng/mL | 1573 | 20.3 ± 14.7 | 394 | 19.1 ± 14.7 | 871 | 19.1 ± 14.2 | 308 | 25.1 ± 15.2 |
| Plasma PLP, nmol/L | 1544 | 101 ± 99.6 | 392 | 102 ± 105 | 850 | 98.0 ± 93.4 | 302 | 107 ± 109 |
| Plasma vitamin B-12, pg/mL | 1573 | 521 ± 271 | 394 | 548 ± 307 | 871 | 505 ± 260 | 308 | 532 ± 252 |
| Plasma homocysteine, μmol/L | 1573 | 8.6 ± 2.8 | 394 | 8.6 ± 2.7 | 871 | 8.6 ± 2.9 | 308 | 8.5 ± 2.6 |
| Plasma cysteine, μmol/L | 1573 | 286 ± 38.7 | 394 | 286 ± 41.8 | 871 | 286 ± 37.3 | 308 | 286 ± 38.7 |

[1] DFE, daily folate equivalent; FA, folic acid; PLP, pyridoxal-5′-phosphate; WHI, Women's Health Initiative.

The top SNV associated with plasma PLP concentrations was DNA methyltransferase 3α (*DNMT3A*) rs11695471 (T→A) (5.7%; 95% CI: 0.3%, 11.4%, per variant allele A), although the association was not significant after correction for multiple comparisons (FDR-adjusted $P = 0.201$). For plasma vitamin B-12, the variant encoding euchromatic histone lysine methyltransferase 2 (*EHMT2*) rs535586 (G→A) was associated with significantly lower plasma vitamin B-12 concentrations (−5.1%; 95% CI: −8.4%, −1.7%, per variant allele A; FDR-adjusted $P = 0.008$). However, both the variant encoding transcobalamin 2 (*TCN2*) rs1131603 (L349S A→G) (11.5%; 95% CI: 3.4%, 20.3%, per variant allele G; FDR-adjusted $P = 0.084$) and variant *TCN2* rs35838082 (R188W G→A) were associated with higher concentrations of plasma vitamin B-12 (25.5%; 95% CI: 5.4%, 49.4%, per variant allele A; FDR-adjusted $P = 0.093$).

In addition to variant *MTHFR* 677C→T, variant *CBS* rs2851391 (G→A) was associated with higher plasma homocysteine concentrations (2.4%; 95% CI: 0.7%, 4.1%, per variant allele A). For plasma cysteine, methylenetetrahydrofolate dehydrogenase, cyclohydrolase, and formyltetrahydrofolate synthetase 1 (*MTHFD1*) rs2236224 (G→A) and rs2236225 (R653Q G→A) were associated with significantly lower plasma cysteine concentrations (−1.0%; 95% CI: −1.9%, −0.2%, per variant allele A; FDR-adjusted $P = 0.078$).

**Supplemental Table 9** shows the results of effect modification of the FA fortification period on the association of SNVs and the circulating biomarkers. *MTHFR* rs1476413 was significantly associated with RBC folate concentrations during the peri-FA fortification period (−6.68%; 95% CI: −10.58%, −2.60%) but not during the other 2 FA fortification periods ($P$-interaction = 0.0002) after correction for multiple comparisons.

Participants in the highest quartile compared with the lowest quartile of the aggregated genetic risk scores (**Table 3**) for *MTHFR* and *MTRR* genes had 14.79% lower (95% CI: −18.94%, −9.52%, $P < 0.0001$) concentrations of RBC folate. The difference was larger (−23.59%; 95% CI: −28.82%, −17.88%; $P < 0.0001$) when the 90th percentile of the score was compared with the 10th percentile. In a sensitivity analysis, we excluded *MTHFR* 677C→T from the aggregated genetic risk scores because the direction of association was not as expected in the primary analysis; the results did not change materially.

For women of other racial/ethnic groups (**Supplemental Table 10**), the pattern of gene–biomarker associations was similar to those in NHW women. The variant *MTHFR* rs1801133 (677C→T) in association with plasma folate concentrations was significant (−18.9%; 95% CI: −31.6%, −4.9%, per variant allele T; **Supplemental Table 11**). The aggregated genetic risk score from SNVs in *MTHFR* and *MTRR* developed based on NHW women was also associated with RBC folate concentrations in women of other racial/ethnic groups (**Supplemental Table 12**).

## Discussion

In a sample of NHW postmenopausal women enrolled in the WHI-OS, nonsynonymous SNVs in *MTHFR, MTRR, TCN2, CBS, MTHFD1,* and *DNMT3A* were associated with concentrations of blood biomarkers of one-carbon metabolism, including RBC folate, homocysteine, cysteine, plasma folate, plasma B-12, and plasma PLP. There were several significant associations for tagSNVs in *MTHFR* and in *MTRR* with RBC folate concentrations. The results of the aggregated genetic risk score analysis showed a dose–response relation of the genetic variation with RBC folate concentrations.

Our findings on the association of *MTHFR* 677C→T with decreased plasma folate (9, 11–13, 15) and increased homocysteine (10–12) concentrations in women with the TT compared with CC genotypes are largely consistent with the literature, including GWASs (**Supplemental Table 13**). In our study, however, the variant T of *MTHFR* 677C→T was associated with higher concentrations of RBC folate

**TABLE 2** Significant associations of nonsynonymous SNVs and tagSNVs with biomarker concentrations in non-Hispanic white postmenopausal women[1]

| Gene | SNV | Concentration difference per variant allele, % (95% CI) | Nominal *P* value | FDR-adjusted *P* value |
|---|---|---|---|---|
| RBC folate | | | | |
| *MTHFR* | rs1801133 (677C→T, nonsynonymous) | 8.7 (5.7, 11,8) | $9.4 \times 10^{-9}$ | $1.2 \times 10^{-7}$ |
| | rs1476413 (G→A, tag) | −5.8 (−8.7, −2.9) | $1.6 \times 10^{-4}$ | 0.0056 |
| | rs2066471 (G→A, tag) | −9.4 (−12.7, −6.0) | $1.8 \times 10^{-7}$ | $3.9 \times 10^{-5}$ |
| | rs4845881 (A→G, tag) | −5.9 (−8.6, −3.1) | $4.1 \times 10^{-5}$ | 0.0022 |
| | rs4846047 (G→C, tag) | −7.2 (−9.9, −4.4) | $1.1 \times 10^{-6}$ | $1.2 \times 10^{-4}$ |
| | rs7538516 (A→G, tag) | −5.4 (−8.0, −2.7) | $1.4 \times 10^{-4}$ | 0.0056 |
| *MTRR* | rs1801394 (I49M A→G, nonsynonymous) | 6.6 (3.7, 9.6) | $6.0 \times 10^{-6}$ | $1.3 \times 10^{-4}$ |
| | rs162270 (C→A, tag) | 7.3 (3.4, 11.4) | $2.2 \times 10^{-4}$ | 0.0068 |
| | rs326124 (G→A, tag) | 7.8 (4.0, 11.7) | $4.1 \times 10^{-5}$ | 0.0022 |
| Plasma folate | | | | |
| *MTHFR* | rs1801133 (677C→T, nonsynonymous) | −13.0 (−17.3, −8.6) | $5.0 \times 10^{-8}$ | $6.5 \times 10^{-7}$ |
| Plasma PLP | | | | |
| *DNMT3A* | rs11695471 (T→A, nonsynonymous) | 5.7 (0.3, 11.4) | 0.036 | 0.201 |
| Plasma vitamin B-12 | | | | |
| *EHMT2* | rs535586 (G→A, nonsynonymous) | −5.1 (−8.4, −1.7) | 0.0033 | 0.008 |
| *TCN2* | rs1131603 (L349S A→G, nonsynonymous) | 11.5 (3.4, 20.3) | 0.0049 | 0.084 |
| *TCN2* | rs35838082 (R188W G→A, nonsynonymous) | 25.5 (5.4, 49.4) | 0.011 | 0.093 |
| Plasma homocysteine | | | | |
| *CBS* | rs2851391 (G→A, nonsynonymous) | 2.4 (0.7, 4.1) | 0.0046 | 0.0298 |
| *MTHFR* | rs1801133 (677C→T, nonsynonymous) | 3.5 (1.7, 5.3) | $9.4 \times 10^{-5}$ | 0.0012 |
| Plasma cysteine | | | | |
| *MTHFD1* | rs2236224 (G→A, nonsynonymous) | −1.0 (−1.8, −0.2) | 0.021 | 0.078 |
| *MTHFD1* | rs2236225 (R653Q G→A, nonsynonymous) | −1.0 (−1.9, −0.2) | 0.015 | 0.078 |

[1] Multivariable analyses were adjusted for age; colorectal cancer case-control status; BMI (kg/m$^2$); smoking status (never, former, or current); total intake of folate, vitamin B-6, vitamin B-12, and riboflavin (from food and supplements); and folic acid fortification periods. *CBS*, cystathionine $\beta$-synthase; *DNMT3A*, DNA methyltransferase 3$\alpha$; *EHMT2*, euchromatic histone lysine methyltransferase 2; FDR, false discovery rate; *MTHFD1*, methylenetetrahydrofolate dehydrogenase, cyclohydrolase, and formyltetrahydrofolate synthetase 1; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; PLP, pyridoxal-5′-phosphate; SNV, single-nucleotide variant; tagSNV, tagging single-nucleotide variant; *TCN2*, transcobalamin 2.

concentrations, a finding that was opposite to that of plasma folate. The finding of RBC folate was similar among women who were enrolled during pre-, peri-, and post-FA fortification periods (Supplemental Table 9). The reasons for this unexpected finding are not completely clear, but a hypothesis is that competitive protein binding assays, including our assay, may measure RBC folate differentially by the *MTHFR* 677C→T genotype. In RBC, the *MTHFR* polymorphism affects ratios of folate components [e.g., tetrahydrofolate (THF), 5-methyl-THF, 5-formyl-THF, and 10-formyl-THF]. Individuals with the TT genotype have a higher percentage of RBC formyl-THF, but not methyl-THF, than those with the CC genotype (37). However, competitive protein binding assays may differentially under-recover methyl-THF but over-recover formyl-THF, resulting in a pattern of measured RBC folate being higher in TT than CT or CC genotypes (38). A meta-analysis (39) and a large study of young men and women (40) have shown that TT compared with the CT or CC genotype was associated with lower RBC folate concentrations among studies using microbiological assays, which do not have the issue of differential recovery of methyl- and non-methyl-THF forms, but studies using a competitive protein binding assay showed a reversed association (39). Our study is the first to report the results on RBC folate from the assay manufacturer (SimulTRAC), and the result warrants confirmation. Aside from *MTHFR* 677C→T, we found several variants in *MTHFR* and *MTRR* were associated with RBC folate concentrations, and the aggregated genetic risk score derived from these variants was also associated with RBC folate concentrations. Both MTRR and MTHFR are key to 5-methyl-THF utilization, and the *MTRR* I49M (66A→G) G allele is associated with an increase in RBC folate concentrations (21), which is consistent with our finding. TagSNV *MTHFR* rs1537516 was found to be associated with RBC folate concentrations in a GWAS in a young Irish population (Supplemental Table 13) (40). We are unable to rule out the possibility that these associations may be affected by our choice of assay, as there is no report on the differential measurement of competitive protein binding assays or radioassays on RBC folate concentrations by genotypes except for *MTHFR* 677C→T. In addition, we observed more SNVs associated with RBC folate than with plasma folate. The reason is unclear, but a plausible explanation is that because MTHFR and the MTRR reactions are intracellular, the concentrations of folate are more reflective of RBC than plasma. Also, compared with plasma folate, RBC folate is a longer-term measure of folate status, and its associations with genes may be more likely to be detected.

We observed higher concentrations of vitamin B-12 in individuals with *TCN2* variant genotypes, namely, *TCN2* rs1131603 (L349S) and *TCN2* rs35838082 (R188W). *TCN2* rs1131603 (L349S) was also reported by a GWAS of Icelandic and Danish men and women (Supplemental Table 13) (27). *TCN2* encodes a vitamin B-12 protein that binds cobalamin (vitamin B-12) and facilitates the transport of cobalamin into human cells (32). Two GWASs (24, 25) and a candidate gene study (41) have reported several genes that are associated with serum vitamin B-12: *TCN1*, *FUT2* (encoding galactoside 2-$\alpha$-L-fucosyltransferase 2, which promotes vitamin

**TABLE 3** Aggregated genetic risk scores from SNVs in *MTHFR* and *MTRR* in association with RBC folate concentrations in non-Hispanic white postmenopausal women[1]

| Category | RBC folate concentration difference, % (95% CI) | *P* value |
|---|---|---|
| All 9 SNVs[2] | | |
| Q1 | Reference | — |
| Q2 | −8.61 (−13.93, −3.92) | 0.0008 |
| Q3 | −10.42 (−14.79, −5.82) | <0.0001 |
| Q4 | −14.79 (−18.94, −9.52) | <0.0001 |
| ≥90th vs. ≤10th percentile | −23.59 (−28.82, −17.88) | <0.0001 |
| 8 SNVs after excluding *MTHFR* 677C→T[3] | | |
| Q1 | Reference | — |
| Q2 | −13.06 (−17.30, −7.69) | <0.0001 |
| Q3 | −15.63 (−19.75, −10.42) | <0.0001 |
| Q4 | −18.94 (−24.42, −13.93) | <0.0001 |
| ≥90th vs. ≤10th percentile | −20.55 (−29.53, −10.42) | 0.0002 |

[1]Multivariable analyses were adjusted for age, colorectal cancer case-control status, BMI (kg/m$^2$), smoking status (never, former, or current), total intake of folate, vitamin B-6, vitamin B-12, and riboflavin (from food and supplements), and folic acid fortification periods. *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; SNV, single-nucleotide variant.
[2]Risk scores were divided into quartiles (Q) and percentiles based on the distribution: Q1, score = 0 to 6; Q2, score = 7 and 8; Q3, score = 9 to 11; Q4, score = 12 to 18; ≤10th percentile = 0 to 4; ≥90th percentile = 13 to 18.
[3]Risk score were divided into quartiles (Q) and percentiles based on the distribution: Q1, score = 0 to 4; Q2, score = 5 and 6; Q3, score = 7 to 9; Q4, score = 10 to 16; ≤10th percentile = 0 to 2; ≥90th percentile = 11 to 16.

B-12 absorption), *CUBN* (encoding cubilin, or intrinsic factor–cobalamin receptor), and *MUT* (encoding methylmalonyl-CoA mutase). In addition, the G allele of *TCN2* rs1801198 (c.776G→C) was associated with a lower plasma concentration of holotranscobalamin (42), an early marker of changes in cobalamin homeostasis and an active form of vitamin B-12, and a higher concentration of methylmalonic acid (11, 43), a surrogate measure for vitamin B-12 deficiency. We examined the nonsynonymous SNV in relation to plasma B-12 concentrations in our data but did not observe an association. Taking these findings together suggests that the influence on circulating vitamin B-12 or the status of vitamin B-12 could be polygenic.

In addition to the *MTHFR* 677C→T variant, genetic variants in *CBS* can be important determinants for plasma homocysteine concentrations. CBS converts excess homocysteine to cystathionine during transsulfuration. *CBS* rs2851391 identified in our study is an intron SNV, but the variant has been associated with increased risk of spina bifida (44). This SNV has also been linked to plasma homocysteine concentrations in a Canadian population (41) and in 2 GWASs of individuals of European descent and Hispanic individuals (45, 46). Other SNVs in *CBS*, including 833T→C in Chinese and 699C→T in white individuals, are associated with plasma homocysteine concentrations (11, 16).

There are very limited data regarding the association of genetic variants with plasma cysteine concentrations. Our study found an association of *MTHFD1* rs2236224 and rs2236225 (R653Q) variants with lower plasma cysteine concentrations. The effect sizes were small, and the findings will require further confirmation. However, these SNVs may be important because the variants were prevalent (~40% in NHW and 25% in other racial/ethnic women among our study population), and *MTHFD1* rs2236224 is associated with spina bifida (44, 47).

Our findings regarding *DNMT3A* rs11695471 in relation to plasma PLP concentrations have not been reported in the literature. Only a few studies have reported genetic variants in association with plasma PLP concentrations. The *MTHFR* 677C→T TT allele has been associated with a lower concentration of plasma PLP in a Finnish population (11). In one GWAS, variants in alkaline phosphatase, biomineralization associated (*ALPL*) and neuroblastoma breakpoint family member 3 (*NBPF3*) were associated with plasma PLP in US women (25). The ALPL enzyme may influence PLP by regulating its extracellular concentration (48). However, the associations of these SNVs were not confirmed in another GWAS (24).

The influence of FA fortification on the SNV–biomarker associations was limited. In our data, only one association (*MTHFR* rs1476413 and RBC folate) was found to be modified by the FA fortification period, and the magnitude of change in the association was not large. In addition, a suggestive interaction was observed for *MTRR* rs326124, as the variant allele was associated with an increase in RBC folate concentrations in the peri- and post-FA fortification periods (11.8% and 8.4%, respectively) but not in the prefortification period (0.69%). Other notable findings included that the association of *TCN2* rs1131603 (L349S) with plasma vitamin B-12 was attenuated, but the association of *TCN2* rs35838082 (R188W) with plasma vitamin B-12 became stronger (76.1% increase per variant allele; 95% CI: 5.13%, 195%) in women recruited during the post-FA fortification periods compared with women recruited before the period (Supplemental Table 9). The potential of FA fortification leading to increasing one-carbon nutrient concentrations among individuals with these variants requires further investigation.

Our study has several strengths. WHI had extensive recruitment efforts, and participants were enrolled at 40 clinical centers throughout the United States. Several of these centers were specifically minority recruitment centers. The biospecimens and data were collected from healthy individuals before a diagnosis of cancer or other major chronic diseases. The study was able to adjust the results for important confounders, including dietary and supplemental intake of B vitamins. Nevertheless, the limitations should be noted. The generalizability of our study findings may be limited because our study population was mainly self-reported NHW women, in common with most of the literature in this area of research. The interpretation of the results from the other racial/ethnic groups should be cautious because of its small sample size. Our findings in *MTHFR* 677C→T in association with plasma folate concentrations were consistent with data from US Black and Hispanic individuals (20). Another limitation was that the aggregated genetic risk score was not derived from GWAS data and thus limited by the number of SNVs genotyped in our study.

In conclusion, genetic variants in one-carbon metabolism were associated with blood concentrations of one-carbon nutrients in a sample of postmenopausal women. Important associations were revealed in single SNVs of several genes for RBC folate and plasma folate, vitamins B-6 and B-12, homocysteine, and cysteine. Previous investigations of this study population have also provided evidence indicating that vitamins B-6 and B-12 (36), plasma homocysteine and cysteine (5), and vitamin B-6–related SNVs (6) are associated with CRC risk. In light of nutrigenetics and precision prevention of cancer, our

findings will inform future studies exploring the maintenance of B vitamin status and the prevention of CRC and diseases related to one-carbon metabolism.

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