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Common Polymorphisms That Affect Folate Transport or Metabolism Modify the Effect of the *MTHFR* 677C > T Polymorphism on Folate Status[1–3]

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Abstract

Background: Although combinations of biologically relevant polymorphic variants affect folate status, most studies have focused on the effects of individual polymorphisms; however, these effects may be altered by interactions between polymorphisms.

Objective: We investigated the individual and combined effects of polymorphisms that affect folate transport or metabolism on folate status.

Methods: The associations between the methylenetetrahydrofolate reductase (*MTHFR*) 677C > T, methionine transferase reductase (*MTRR*) 66A > G, *MTRR* 524C > T, 5,10-methylenetetrahydrofolate dehydrogenase-5,10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase (*MTHFD1*) 1958G > A, *MTHFD1* -105C > T, dihydrofolate reductase (*DHFR*) 19-bp insertion/deletion, and solute carrier family 19A, member 1 (*SLC19A1*) 80G > A polymorphisms and fasting plasma folate (PF), red cell folate (RCF), and plasma total homocysteine (tHcy) were tested by ANCOVA and Cox regression analysis in 781 Spanish adults.

Results: Folate deficiency (PF <7 nmol/L) was observed in 18.8% of the participants. Geometric mean PF (nmol/L) was lower in *MTHFR* 677TT (10.0; 95% CI: 9.2, 11.9) compared with 677CC (12.4; 95% CI: 11.6, 13.2; $P < 0.001$). RCF (nmol/L) was lower in *MTHFR* 677TT (652; 95% CI: 611, 695) compared with 677CC (889; 95% CI: 851, 929; $P < 0.001$) and in *SLC19A1* 80AA (776; 95% CI: 733, 822) compared with 80GG (861; 95% CI: 815, 910; $P < 0.01$). RCF and tHcy (μmol/L) did not differ in *MTHFR* + *MTRR* 677TT/524TT compared with 677CC/524CC: 780 (95% CI: 647, 941) compared with 853 (95% CI: 795, 915; $P = 0.99$) and 10.2 (95% CI: 8.4, 12.3) compared with 8.9 (95% CI: 8.5, 9.4; $P = 0.99$), respectively. The RR of lowest-tertile RCF (≤680 nmol/L) was 2.1 (95% CI: 1.0, 4.5) for *MTHFR* + *MTRR* 677TT/66GG compared with 677CC/66AA, 2.2 (95% CI: 1.2, 4.1) for *MTHFR* + *MTHFD1* 677TT/1958AA compared with 677CC/1958GG, 2.9 (95% CI: 1.4, 6.0) for *MTHFR* + *MTHFD1* 677TT/-105TT compared with 677CC/-105CC, and 3.5 (95% CI: 1.5, 8.1) for *MTHFR* + *SLC19A1* 677TT/80AA compared with 677CC/80GG. Confining the analysis to the *MTHFR* 677TT genotype, the risk of lowest-tertile RCF was reduced for *MTHFR* + *MTRR* 677TT/66GG compared with 677TT/66AA (RR: 0.5; 95% CI: 0.3, 0.9).

Conclusions: Folate status was lower in the *MTHFR* 677TT and *SLC19A1* 80AA genotypes compared with corresponding reference genotypes. Low folate status risk associated with the *MTHFR* 677TT genotype varied depending on its combination with other polymorphisms. *J Nutr* 2016;146:1–8.

Keywords: population study, plasma folate, red cell folate, homocysteine, *MTHFR* 677C > T, *MTRR* 66A > G, *MTRR* 524C > T, *MTHFD1* 1958G > A, *MTHFD1* -105C > T, *SLC19A1* 80G > A

Introduction

Additive genetic factors are major determinants of folate status (1). Some of the individual or combined effects of rare allelic variants (2) and common polymorphisms that affect cellular folate transport or metabolism are unclear or unknown. High folic acid intake in some populations may contribute to this uncertainty by overcoming the effect of such polymorphisms, as seen by the difference in impact of the methylenetetrahydrofolate reductase

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(*MTHFR*)[11] 677C > T polymorphism in the presence of high or low folate status (3). This polymorphism impairs 5-methyltetrahydrofolate production (4), but to our knowledge there are no genome-wide association studies to date that have found it to be significantly associated with folate status. However, a positive association between the TT genotype and plasma total homocysteine (tHcy) has been reported (5, 6). The *MTHFR* gene has riboflavin as a cofactor, and its effect on tHcy depends on both folate and riboflavin status (7).

5-Methyltetrahydrofolate is a cofactor for homocysteine remethylation to methionine, a reaction catalyzed by the B12-dependent enzyme methionine synthase (MS). Methionine transferase reductase (MTRR), a flavoprotein, plays a role in the reactivation and stabilization of MS. Additional MTRR is required to activate MS in the presence of the variant alleles for the *MTRR* 66A > G and 524C > T polymorphisms (8). The effects of these polymorphisms on tHcy depend on vitamin B-12 (substrate) and riboflavin status (7). Combined *MTHFR* 677TT and *MTRR* 66AG genotypes have been associated with higher tHcy compared with their occurrence alone (9).

The reported effects of polymorphisms of the 5,10 methylenetetrahydrofolate dehydrogenase-5,10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase (*MTHFD1*) gene on folate status vary. The *MTHFD1* 1958AA genotype did not affect plasma folate in a large population-based Norwegian study (10) but was associated with perturbed 1-carbon metabolism in a folate-restricted diet intervention study (11). The *MTHFD1* -105C > T polymorphism did not affect folate status in an Irish study of pregnant women (12). The homozygote variant form of the dihydrofolate reductase (*DHFR*) 19-bp insertion/deletion polymorphism (13) has been associated with lower red cell folate (RCF) when folic acid intake is low and with circulating unmetabolized folic acid when folic acid intake is high (14).

The reduced folate carrier [solute carrier family 19A, member 1 (SLC19A1)] is the principal folate transport mechanism into the cell. The *SLC19A1* 80G > A polymorphism (15, 16) affects folate binding and uptake (16), but its reported effects on folate status are inconsistent (10, 15, 17–19).

We hypothesized that the effects of the polymorphisms on folate status may be altered by folic acid intake from supplements or from fortified foods. Therefore, we investigated individual and combined effects of the different polymorphisms with *MTHFR* 677C > T on folate status in a representative sample of an adult population that had not been exposed to mandatory folic acid fortification or vitamin B supplement use.

Methods

Subjects. This cross-sectional study, carried out from 1998 to 2002, has been described previously (20). Adults aged 18–75 y, stratified by age and sex, were randomly selected from population registers from 3 villages in Tarragona, Spain. Collectively, 789 people participated in the study (65% of those randomly selected). Exclusion criteria were as follows: family settlement in the community for <2 generations, illness or medication that affected folate metabolism, vitamin B supplement use, pregnancy, parturition during the last 6 mo, or lactation.

Of the 789 participants, 5 with altered renal function (plasma creatinine >97 μmol/L for women and >124 μmol/L for men) and 3 who received cobalamin injections were excluded. A further 58 participants were excluded from tHcy analysis because their samples were processed 2–4 h after sample collection.

The study was approved by the Sant Joan University Hospital, Reus and Jordi Gol Gorina Foundation Ethics committees, and all participants provided signed informed consent. Participants provided a fasting blood sample, had a medical checkup in which data on lifestyle and habits were collected, and completed dietary records for 3 d (2 during the workweek and 1 on a holiday) of food consumed.

Blood samples. Blood was collected in EDTA-K_3 vacutainers and kept at 4°C until processed (within 2 h). For RCF determinations, whole blood was diluted 1:10 with 1% ascorbic acid solution and left at room temperature for 30 min before storage. Plasma was separated and washed, and erythrocyte hemolysates were immediately prepared on ice. Leukocytes were separated from the remaining blood cells. Aliquots of all of the processed samples were stored at −80°C in the Pere Virgili Institute for Health Research biobank. DNA was extracted from the leukocytes using the PureGene DNA extraction kit (Gentra Systems).

Plasma tHcy was determined by fluorescence polarization immunoassay with an IMx autoanalyzer (Abbott Laboratories). Plasma creatinine was determined by Jaffé reaction (Química Clínica Aplicada), and erythrocyte glutathione reductase and aspartate aminotransferase activation coefficients in erythrocyte hemolysates were determined on a COBAS MIRA autoanalyzer (Roche Diagnostics) as previously described (7). PF and RCF were determined by microbiological assays with *Lactobacillus casei* (21) and plasma cobalamin by *L. leichmannii* (22). Folate deficiency was defined as PF <7 nmol/L (23). The *MTHFR* 677C > T (rs1801133), *MTRR* 66A > G (rs1801394), *MTRR* 524C > T (rs1532268), *MTHFD1* 1958G > A (rs2236225), *MTHFD1* -105C > T (rs1076991), *DHFR* 19-bp insertion/deletion (rs70991108), and *SLC19A1* 80G > A (rs1051266) polymorphisms were determined by matrix-assisted laser desorption/ionization/time-of-flight MS (24).

Statistical methods. Variables with skewed distributions were natural log transformed for the application of parametric tests. Hardy-Weinberg equilibrium of the observed allele frequencies and linkage disequilibrium between alleles at different loci were determined by CubeX (25). Differences in the frequency of low folate status according to age group or genotype were assessed using a chi-square test.

We tested the effects of the polymorphisms on PF, RCF, and tHcy in separate multiple linear regression analysis (MLRA) models. For each polymorphism, heterozygotes compared with normal and variant homozygotes compared with normal homozygote genotypes were included in the PF and RCF models. These comparisons were adjusted for age, sex, BMI, education level, serum creatinine, energy-adjusted folate intake, plasma cobalamin, plasma betaine, erythrocyte glutathione reductase, erythrocyte aspartate aminotransferase activation, alcohol intake (g/d), and smoking (cigarettes per day). Interactions between smoking and each polymorphism and between the different polymorphisms were tested by including their products in the model (e.g., smoking X polymorphism). PF was included in the tHcy model instead of folate intake. Individual polymorphisms that were associated with PF or RCF or tHcy ($P < 0.1$) were identified, and their combined effects with *MTHFR* 677C > T genotypes on folate and tHcy status were determined.

Mean PF, RCF, and tHcy were compared between different genotypes for each polymorphism and between combined genotypes for the selected polymorphism combinations by ANCOVA. Analyses were

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[3] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[11] Abbreviations used: *DHFR*, dihydrofolate reductase; MLRA, multiple linear regression analysis; MS, methionine synthase; *MTHFD*, 5,10 methylenetetrahydrofolate dehydrogenase-5,10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase; *MTHFR*, methylenetetrahydrofolate reductase; MTRR, methionine transferase reductase; PF, plasma folate; RCF, red cell folate; *SLC19A1*, solute carrier family 19A, member 1; tHcy, plasma total homocysteine.

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adjusted for the same covariates listed for MLRA but also included heterozygote compared with homozygote normal and homozygote variant compared with normal homozygote genotypes for the *MTRR* 66A > G, *MTRR* 524C > T, *MTHFD1* 1958G > A, *MTHFD1* -105C > T, and *SLC19A1* 80G > A polymorphisms (excluding the polymorphism that was being studied) as relevant. The unadjusted geometric means are reported. Type I error was maintained at 5% by applying Bonferroni correction for multiple comparisons.

The risks of lowest tertile RCF for the combinations of each polymorphism with the *MTHFR* 677 C > T polymorphism compared to the double homozygote common genotype (reference) for each combination were investigated in multiple Cox regression models including the same covariates listed for the ANCOVA models. The heterozygote compared with homozygote normal and homozygote variant compared with homozygote normal genotypes of all of the polymorphisms that did not form part of the genotype combination being studied were also included as covariates in the models. Model goodness of fit was assessed by chi-square. Only polymorphism combinations that significantly affected the risks are reported. Data were analyzed using SPSS version 22.0 (IBM). The level of significance was set at $P < 0.05$ for 2-tailed contrasts.

Results

The general characteristics of the population are summarized in **Table 1**. Folate deficiency was observed in 18.8% of participants and in 24.2% of women of fertile age (18–44 y). The percentage of participants with folate deficiency decreased with age. The reported energy-adjusted folate intake in 83.5% of the population and 86.7% of women of fertile age was less than the recommended intake of 400 μg/d (26).

Individual polymorphism effects. All of the studied genotypes were in Hardy-Weinberg equilibrium, and we did not observe a significant linkage disequilibrium between any polymorphisms. The genotype and allele frequencies and the proportion of carriers of homozygote variant genotypes with low folate status are reported in **Table 2**. The homozygote variant genotype was most frequent in the lowest PF and RCF tertiles ($P < 0.05$ and 0.001, respectively) for the *MTHFR* 677C > T polymorphism only.

MLRA showed no effect of the *DHFR* 19-bp insertion/deletion polymorphism on folate status and thus was not analyzed further (**Supplemental Table 1**). Numerous associations between genotypes and folate or tHcy status at a significance level of $P < 0.1$ were identified by MLRA. The *MTHFR* 677CT, *MTHFR* 677TT, and *MTRR* 66GG genotypes were inversely associated with PF. The *MTHFR* 677CT, *MTHFR* 677TT, *SLC19A1* 80AA, and *MTHFD1* -105TT genotypes were inversely associated with RCF, whereas the *MTRR* 66AG and *MTHFD1* 1958AA genotypes were positively associated with RCF. The *MTHFR* 677TT and *MTRR* 66AG genotypes were positively associated with tHcy, whereas the *MTRR* 524TT genotype was inversely associated with tHcy (Supplemental Table 1). PF, RCF, and tHcy for these polymorphisms are reported in **Table 3**. After analysis by ANCOVA, adjusting for lifestyle and other variables and correcting for multiple comparisons, lower PF and RCF and higher tHcy were observed in the *MTHFR* 677TT compared with 677CC and 677CT genotypes. Lower RCF was observed in the *SLC19A1* 80AA (variant) compared with the 80GG genotype and a tendency for lower PF in the *SLC19A1* 80AA compared with the 80GA genotype ($P = 0.07$) was also observed.

Interactions between polymorphisms. PF, RCF, and tHcy are reported in **Table 4** for the *MTHFR* 677C > T genotypes combined with each of the other polymorphisms. Using MLRA, we observed a significant interaction between the *MTHFR* 677C > T and *MTRR* 66A > G genotypes on RCF ($P < 0.001$ for the interaction term). Stratified analysis by the *MTHFR* 677C > T genotype showed a higher RCF in the presence of the *MTRR* 66GG (variant) compared with 66AA genotype in participants with the *MTHFR* 677TT genotype. RCF was lower in all participants with the *MTHFR* 677TT genotype compared with the 677CC genotype except in the case of those that had the *MTHFR* + *MTRR* 677TT/524TT, double-homozygote variant combination. No significant interaction with the *MTHFR* 677C > T polymorphism was observed for either of the *MTHFD1* polymorphisms. In these cases, the associations of the variant genotypes with lower folate and higher tHcy status compared with the homozygote common genotypes were driven by the *MTHFR* 677TT genotype. PF tended to be lower in the *MTHFR* + *SLC19A1* 677TT/80AA compared with the 677CC/80GG combination ($P = 0.06$), and RCF was lower in all *SLC19A1* 80AA genotypes compared with 80GG genotypes regardless of the *MTHFR* 677C > T genotype with which it was combined. The risks of lowest-tertile RCF (≤680 nmol/L) associated with double-homozygote variant allele combinations compared with double-homozygote common allele combinations for *MTHFR* 677C > T combined with *MTRR* 66A > G, *MTRR* 524C > T, *MTHFD1* 1958G > A, *MTHFD1* -105C > T, and *SLC19A1* 80G > A polymorphisms are reported in **Figure 1**. All models were significant ($P < 0.001$). Each double-homozygote variant combination except for *MTHFR* + *MTRR* 677TT/524TT increased the risk of lowest-tertile RCF compared with the double-homozygote common allele combinations.

TABLE 1 General characteristics of the adult men and women studied[1]

Characteristics	*n*	Values
Age, y	781	42.9 (41.8, 43.9)
Older than 60 y, %	130	16.6 (14.0, 19.3)
Women, %	406	52.0 (48.5, 55.5)
Fertile women (18–44 y), %	236	30.2 (27.0, 33.4)
BMI, kg/m^2	766	27.0 (26.7, 27.4)
Smokers, %	779	34.5 (31.2, 37.9)
At-risk alcohol consumers,[2] %	764	12.8 (10.5, 15.2)
ITS consumers, %	763	2.7 (1.7, 4.2)
Secondary education or higher, %	781	46.6 (43.1, 50.1)
Folate intake < 400 μg/d, %	739	83.5 (80.8, 86.2)
Plasma folate,[3] nmol/L	781	11.5 (11.0, 11.9)
Folate-deficient (plasma folate <7 nmol/L), %	781	18.8 (16.1, 21.6)
18–29 y	184	33.2 (26.8, 40.2)***
30–44 y	210	27.6 (22.0, 34.0)
45–59 y	199	9.0 (5.8, 13.8)
60–75 y	130	7.8 (4.2, 13.6)
Fertile women (18–44 y)	236	24.2 (18.7, 29.6)
Red cell folate,[3] nmol/L	781	810 (789, 832)
Plasma cobalamin,[3] pmol/L	779	346 (338, 355)
Plasma betaine,[3] μmol/L	775	37.9 (37.1, 38.8)
tHcy,[3] μmol/L	723	9.4 (9.2, 9.6)

[1] Values are arithmetic means or percentages (95% CIs) unless otherwise indicated. The sample size for the entire study population varied between 723 and 781 because of missing data and the exclusion of blood samples that were not processed within 2 h of collection in the case of tHcy. The chi-square test was used to compare the frequency of folate deficiency between the age groups: ***P-trend <0.001. ITS, illegal toxic substances; tHcy, fasting plasma total homocysteine.
[2] Intake >24g/d in men and >16g/d in women.
[3] Values are geometric means (95% CIs).

TABLE 2 Genotype and allele frequencies of the polymorphisms and their association with low folate status in adult men and women[1]

Polymorphism	*n*	Genotype frequency			Allele frequency		V/V in lowest PF tertile (≤9.1 nmol/L)	P^2	V/V in lowest RCF tertile (≤680 nmol/L)	P^2
		C/C	C/V	V/V	C	V				
MTHFR 677C > T	775	35.6	46.3	18.1	59.7	40.3	43.6	<0.05	58.6	<0.001
MTRR 66A > G	771	26.1	50.2	23.7	51.1	48.9	32.7	0.34	32.8	0.15
MTRR 524C > T	771	39.6	47.2	13.2	62.9	37.1	25.5	0.34	22.5	0.17
MTHFD1 1958G > A	770	32.8	47.0	20.2	57.3	42.7	31.2	0.63	34.4	0.31
MTHFD1 -105C > T	775	23.6	47.9	28.5	46.7	53.3	33.3	0.61	33.8	0.10
DHFR 19-bp in/del	770	35.7	44.9	19.4	59.7	40.3	33.6	0.39	32.2	0.57
SLC19A1 80G > A	773	25.5	51.0	23.5	50.4	49.6	35.7	0.80	37.4	0.40

[1] Values are percentages. C, common allele; *DHFR*, dihydrofolate reductase; in/del, insertion/deletion; *MTHFD1*, methylenetetrahydrofolate dehydrogenase-5,10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine transferase reductase; PF, plasma folate; RCF, red cell folate; *SLC19A1*, solute carrier family 19 (folate transporter), member 1; v, variant allele.
[2] *P* values are from the chi-square test that was used to compare the frequencies of the genotypes (C/C, C/V, and V/V) for each polymorphism (within each line of the table) between the plasma and red cell folate tertiles.

The variant alleles in the *MTRR* polymorphisms opposed rather than enhanced the effect of the *MTHFR* 677TT genotype. The RR (95% CI) of lowest-tertile RCF was reduced for the *MTHFR* + *MTRR* 677TT/66GG combination compared with the 677TT/66AA combination [0.52 (95% CI: 0.28, 0.98; $P < 0.05$)]. The concentration of tHcy (μmol/L) was lower in the

TABLE 3 Folate and total homocysteine status for individual polymorphisms in adult men and women[1]

Polymorphism	*n*	Plasma folate, nmol/L	*n*	Red cell folate, nmol/L	*n*	tHcy, μmol/L
MTHFR 677C > T						
CC	276	12.4 (11.6, 13.2)[a]	276	889 (851–929)[a]	255	8.9 (8.6, 9.2)[a]
CT	359	11.3 (10.6, 11.9)[a]	359	821 (791, 852)[a]	335	9.2 (9.0, 9.5)[a]
TT	140	10.0 (9.2, 11.9)[b]	140	652 (611, 695)[b]	128	11.2 (10.5, 11.9)[b]
MTRR 66A > G						
AA	201	11.1 (10.3, 12.0)	201	764 (724, 805)	187	9.3 (8.9, 9.7)
AG	387	11.9 (11.3, 12.6)	387	833 (801, 866)	358	9.5 (9.2, 9.8)
GG	183	10.9 (10.1, 11.8)	183	820 (778, 865)	169	9.5 (9.0, 9.9)
MTRR 524C > T						
CC	305	11.0 (10.4, 11.7)	305	788 (755, 822)	287	9.5 (9.2, 9.8)
CT	364	11.6 (10.9, 12.3)	364	818 (785, 852)	335	9.5 (9.2, 9.8)
TT	102	12.2 (11.1, 13.4)	102	860 (811, 911)	92	8.9 (8.4, 9.4)
MTHFD1 1958G > A						
GG	254	11.0 (10.3, 11.8)	254	780 (745, 816)	237	9.5 (9.2, 9.9)
GA	364	11.5 (10.9, 12.2)	364	830 (798, 864)	339	9.3 (9.0, 9.6)
AA	157	11.9 (10.9, 12.9)	157	814 (766, 865)	142	9.6 (9.2, 10.1)
MTHFD1 -105C > T						
CC	182	12.1 (11.2, 13.1)	182	833 (790, 878)	167	9.4 (9.0, 9.7)
CT	369	11.2 (10.7, 12.0)	369	819 (786, 854)	341	9.3 (9.1, 9.6)
TT	219	10.9 (10.2, 11.7)	219	775 (740, 813)	206	9.6 (9.2, 10.0)
SLC19A1 80G > A						
GG	197	11.9 (11.0, 12.9)	197	861 (815, 910)[a]	183	9.6 (9.3, 10.0)
GA	394	11.4 (10.9, 12.0)	394	804 (775, 833)[a,b]	362	9.4 (9.1, 9.7)
AA	182	10.9 (10.0, 11.9)[†]	182	776 (733, 822)[b]	171	9.2 (8.8, 9.6)

[1] Values are geometric means (95% CIs). The sample size was lower in the case of tHcy because of the exclusion of blood samples that were not processed within 2 h of collection. ANCOVA with post hoc Bonferroni correction for multiple comparisons was used to compare plasma variable concentrations between genotypes for each polymorphism. The models were adjusted for age, sex, BMI, education level, serum creatinine, energy-adjusted folate intake, plasma cobalamin, plasma betaine, erythrocyte glutathionine reductase activation coefficient, erythrocyte aspartate aminotransferase activation coefficient, alcohol intake (g/d), smoking (cigarettes/d), heterozygote compared to homozygote normal genotypes and homozygote variant compared to homozygote normal genotypes for the *MTRR* 66A > G, *MTRR* 524C > T, *MTHFD1* 1958G > A, *MTHFD1* -105C > T, and *SLC19A1* 80G > A polymorphisms, excluding the polymorphism being studied from this list, as relevant. Labeled means in a column (within the same polymorphism group) without a common letter differ, $P < 0.05$. [†]Different from the *SLC19A1* 80GA genotype in the same column, $P = 0.07$. *MTHFD1*, methylenetetrahydrofolate dehydrogenase-5, 10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine transferase reductase; *SLC19A1*, solute carrier family 19A (folate transporter), member 1; tHcy, fasting plasma total homocysteine.

TABLE 4 Folate and homocysteine status for the genotype combinations in adult men and women[1]

Combination	*n*	Plasma folate, nmol/L	Red cell folate, nmol/L	tHcy, μmol/L
MTHFR 677C > T + *MTRR* 66A > G				
CC-AA	69	12.7 (11.1, 14.5)	912 (837, 993)	8.5 (7.9, 9.1)
CC-AG	137	12.7 (11.5, 14.0)	881 (825, 940)	9.2 (8.8, 9.6)
CC-GG	69	11.7 (10.3, 13.2)	887 (816, 964)	8.9 (8.4, 9.4)
CT-AA	88	10.6 (9.5, 11.8)	774 (725, 827)	9.1 (8.6, 9.6)
CT-AG	193	12.0 (11.1, 12.9)	854 (811, 898)	9.3 (9.0, 9.7)
CT-GG	76	10.4 (9.1, 11.8)*	803 (735, 876)	9.1 (8.6, 9.7)
TT-AA	43	9.7 (8.2, 11.6)*	562 (500, 630)***	11.2 (10.1, 12.5)***
TT-AG	57	10.0 (8.8, 11.3)***	670 (603, 744)***	11.1 (10.2, 12.0)***
TT-GG	38	10.5 (8.8, 12.6)	743 (666, 828)*,†	11.4 (9.9, 13.1)***
MTHFR 677C > T + *MTRR* 524C > T				
CC-CC	100	12.0 (10.8, 13.2)	853 (795, 915)	8.9 (8.5, 9.4)
CC-CT	130	13.2 (11.9, 14.6)	930 (868, 995)	9.0 (8.6, 9.4)
CC-TT	45	11.4 (9.8, 13.2)	862 (785, 947)	8.7 (7.9, 9.6)
CT-CC	146	10.9 (9.9, 12.0)	817 (769, 868)	9.4 (9.0, 9.7)
CT-CT	170	11.1 (10.2, 11.9)	812 (769, 858)	9.3 (8.9, 9.7)
CT-TT	41	13.6 (11.8, 15.7)	890 (822, 964)	8.6 (7.9, 9.3)
TT-CC	59	9.9 (8.7, 11.2)**	629 (570, 693)***	11.2 (10.2, 12.3)***
TT-CT	63	9.9 (8.6, 11.5)	645 (584, 713)***	11.5 (10.4, 12.6)***
TT-TT	16	11.1 (8.7, 14.2)	780 (647, 941)	10.2 (8.4, 12.3)
MTHFR 677C > T + *MTHFD1* 1958G > A				
CC-GG	98	12.2 (10.9, 13.5)	848 (791, 910)	8.9 (8.5, 9.3)
CC-GA	126	12.2 (11.1, 13.4)	913 (856, 973)	8.8 (8.3, 9.3)
CC-AA	52	13.5 (11.4, 15.9)	911 (811, 1022)	9.4 (8.8, 10.0)
CT-GG	116	10.7 (9.7, 11.7)	772 (724, 824)	9.5 (9.1, 10.0)
CT-GA	174	11.4 (10.5, 12.3)	836 (792, 883)	9.1 (8.7, 9.4)
CT-AA	69	12.0 (10.6, 13.7)	869 (805, 937)	9.0 (8.5, 9.6)
TT-GG	40	9.6 (8.0, 11.4)	653 (573, 745)***	11.3 (10.2, 12.6)***
TT-GA	64	10.5 (9.3, 12.0)	675 (611, 746)***	11.1 (10.2, 12.0)***
TT-AA	36	9.6 (8.1, 11.4)	611 (547, 684)***	11.3 (9.8, 13.0)***
MTHFR 677C > T + *MTHFD1* -105C > T				
CC-CC	70	13.1 (11.4,15.0)	889 (824, 958)	8.9 (8.3, 9.4)
CC-CT	139	12.0 (11.0, 13.2)	921 (860, 986)	8.9 (8.5, 9.3)
CC-TT	66	12.6 (10.8, 14.3)	830 (766, 901)	9.0 (8.5, 9.6)
CT-CC	85	12.1 (10.8, 13.6)	862 (797, 933)	9.2 (8.7, 9.8)
CT-CT	166	11.2 (10.3, 12.2)	796 (752, 843)	9.2 (8.8, 9.5)
CT-TT	107	10.7 (9.7,11.8)	827 (778, 879)	9.3 (8.8, 9.8)
TT-CC	27	10.0 (8.5, 11.8)	631 (549, 725)***	11.1 (9.7, 12.7)*
TT-CT	64	10.1 (8.9, 11.6)	684 (618, 757)***	11.1 (10.3, 12.0)***
TT-TT	46	9.4 (8.0, 11.0)	606 (546, 673)***	11.4 (10.0, 12.9)***
MTHFR 677C > T + *SLC19A1* 80G > A				
CC-GG	72	13.6 (12.0, 15.5)	998 (918, 1086)	9.3 (8.7, 9.9)
CC-GA	141	12.5 (11.5, 13.6)	870 (821, 921)	8.9 (8.5, 9.3)
CC-AA	63	11.0 (9.5, 12.8)	818 (739, 906)*	8.7 (8.1, 9.3)
CT-GG	79	11.4 (10.2, 12.8)	891 (826, 962)	9.2 (8.7, 9.7)
CT-GA	184	11.2 (10.4, 12.1)	812 (770, 856)	9.2 (8.9, 9.6)
CT-AA	95	11.2 (9.9, 12.6)	784 (731, 841)***	9.2 (8.7, 9.8)
TT-GG	46	10.2 (8.7, 12.0)	643 (575, 720)***	11.1 (10.0, 12.3)**
TT-GA	69	10.0 (8.9, 11.2)	665 (613, 721)***	11.3 (10.4, 12.4)***
TT-AA	24	9.6 (7.4, 12.5)[§]	651 (525, 807)***	11.1 (9.5, 12.9)

[1] Values are geometric means (95% CIs). The sample size was lower in the case of tHcy because of the exclusion of blood samples that were not processed within 2 h of collection. The models were adjusted for age, serum creatinine erythrocyte aspartate aminotransferase activation coefficient, erythrocyte glutathionine reductase activation coefficient, plasma betaine and vitamin B-12, energy-adjusted folate intake, current smoking, daily alcohol consumption, BMI, education level, and heterozygote compared with homozygote normal genotypes and homozygote variant compared with homozygote normal genotypes for the *MTRR* 66A > G, *MTRR* 524C > T, *MTHFD1* 1958G > A, *MTHFD1* -105C > T and *SLC19A1* 80G > A polymorphisms (excluding those included in the studied combination as relevant). ANCOVA with post hoc Bonferroni correction for multiple comparisons was used to compare plasma variable concentrations between genotype combinations within each polymorphism combination. Different from double homozygote normal genotypes within the same polymorphism combination: *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, [§]$P = 0.06$. [†]Different from TT-AA within the same polymorphism combination, $P < 0.01$. *MTHFD1*, methylenetetrahydrofolate dehydrogenase-5,10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine transferase reductase; *SLC19A1*, solute carrier family 19 (folate transporter), member 1; tHcy, fasting plasma total homocysteine.

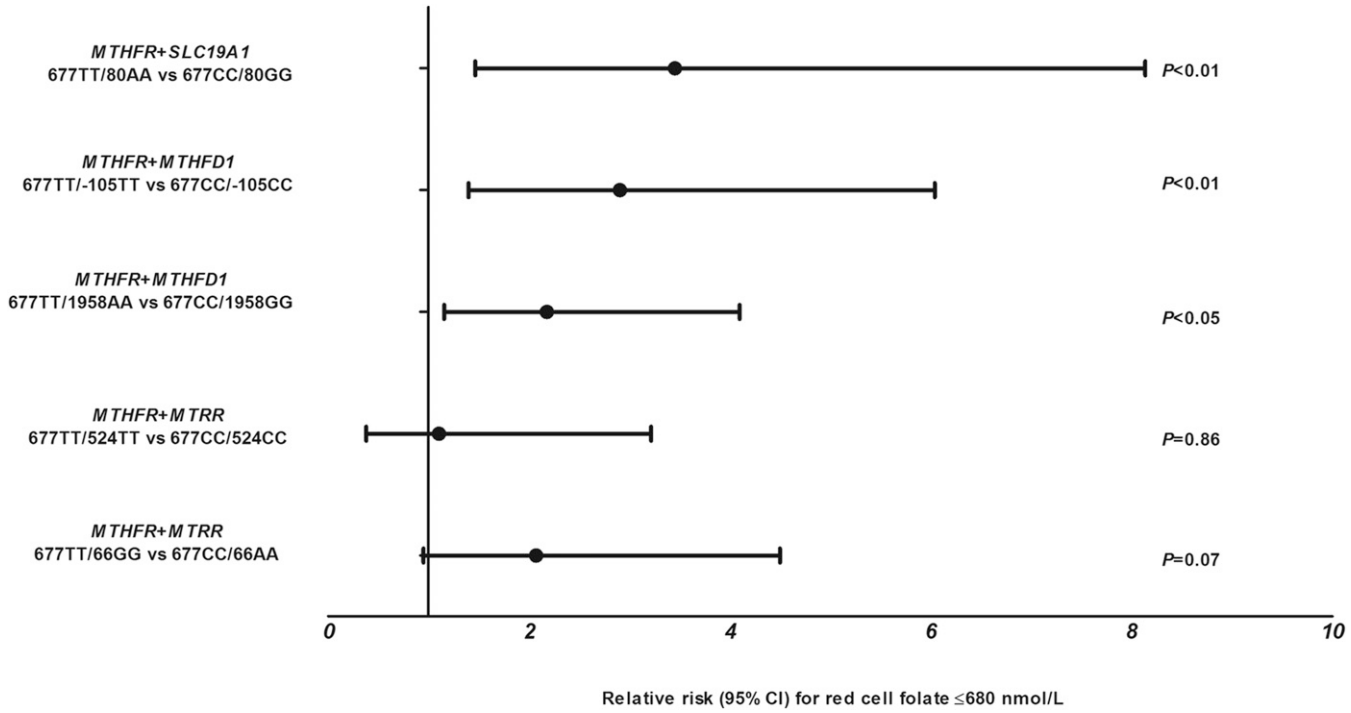

FIGURE 1 RRs (95% CIs) of lowest-tertile red cell folate for the double-homozygote variant genotypes compared with double-homozygote common genotypes in adult men and women (n = 673). RRs (95% CIs) were estimated by multiple Cox regression adjusting for age, sex, BMI, education level, serum creatinine, energy-adjusted folate intake, plasma cobalamin, plasma betaine, erythrocyte aspartate aminotransferase activation coefficient, erythrocyte glutathionine reductase activation coefficient, alcohol intake (g/d) and smoking (cigarettes/d), heterozygote compared with homozygote normal genotypes and homozygote variant compared with homozygote normal genotypes for the *MTHFR* 677C > T, *MTRR* 66A > G, *MTRR* 524C > T, *MTHFD1* 1958G > A, *MTHFD1* -105C > T, and *SLC19A1* 80G > A polymorphisms when not included in the studied combination. Complete data for all of the covariates included in the models were available for 673 participants. The x axis intersects with the y axis at the reference category for each genotype combination. *MTHFD1*, 5,10 methylenetetrahydrofolate dehydrogenase-5,10-methylenetetrahydrofolate cyclohydrolase-10-formyltetrahydrofolate synthetase; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine transferase reductase; *SLC19A1*, solute carrier family 19 (folate transporter), member 1.

reference *MTHFR* + *MTRR* 677CC/524CC combination than in the 677TT/524CC and 677TT/524CT combinations (Table 4). However, there was no difference in tHcy between the *MTHFR* + *MTRR* 677TT/524TT and the 677CC/524CC combinations (P = 0.99) (Table 4). The *MTHFR* + *MTRR* 677TT/524CC and 677TT/524CT combinations were also associated with an increased risk of lowest-tertile RCF compared with the reference 677CC/524CC combination 2.4 (95% CI: 1.4, 4.1) and 1.8 (95% CI: 1.0, 3.1), respectively. However, this was not the case for the *MTHFR* + *MTRR* 677TT/524TT combination in which the risk of lowest-tertile RCF was not increased compared with the reference combination (Figure 1).

Discussion

Principal findings and biological relevance. Despite the established effect of the *MTHFR* 677C > T polymorphism on folate status, the evidence regarding its biological relevance in terms of clinical outcomes is conflicting, and this has often been attributed to the varying impact of folate status. We report that homozygote variant genotypes for other polymorphisms that affect folate status and transport are present in up to 33% of individuals with the *MTHFR* 677TT genotype. The *SLC19A1* 80AA genotype was associated with lower RCF status compared with the double-homozygote common allele reference genotype combination (*MTHFR* + *SLC19A1* 677CC/80GG) regardless of which *MTHFR* 677C > T genotype with which it was combined. We also report that polymorphisms in *MTRR* modified the effect of the *MTHFR* 677TT genotype on folate status. These findings, in a population with relatively low folate status, advance our understanding of how the *MTHFR* 677C > T polymorphism interacts with other polymorphisms. Such interactions may not be detected in studies that include participants exposed to folic acid from supplements or mandatory fortification of cereal products. Furthermore, these interactions may be part of the reason for variations in the metabolic and clinical effects of the *MTHFR* 677C > T polymorphism reported in previous studies (27). Genotype frequencies similar to previous studies were observed for the *MTHFR* 677C > T (10), *MTRR* 66A > G (28), *MTRR* 524C > T (29), *MTHFD1* 1958G > A (10), *MTHFD1* -105C > T (12), *DHFR* 19-bp insertion/deletion (13), and *SLC19A1* 80G > A (15) polymorphisms.

Polymorphisms with no individual effect on folate status. As previously reported, PF, RCF, and tHcy did not differ in the presence of the *MTRR* 66A > G (30) and *MTHFD1* 1958G > A (10, 31) polymorphisms. We observed no associations between the *MTHFD1* -105C > T or *DHFR* 19-bp insertion/deletion polymorphisms and folate status.

Polymorphisms with individual effects on folate status. We confirmed lower PF (2, 32, 33), RCF (32), and higher tHcy (33) in participants with the *MTHFR* 677TT compared with the 677CC genotype. RCF was lower in the *SLC19A1* 80AA compared with the 80GG genotype. These results support the argument that the *SLC19A1* 80A allele is the variant allele and that the polymorphism

impairs folate transport by the reduced folate carrier. Norwegian (10) and United Kingdom studies reported no effect of the *SLC19A1* 80AA genotype on PF or tHcy (17). The effect of the genotype on PF did not reach significance in our study ($P = 0.07$), and there was no effect on tHcy either, but there was a clear effect on RCF. Folate transport into the cell depends on the reduced folate carrier, which would explain why RCF is affected if the polymorphism affects folate substrate binding and uptake by this transporter (16). Its effect may be missed in studies that only consider PF. Alternative folate transport routes such as the proton-coupled folate transporter (34) and folate receptors affect plasma folate and would not be directly affected by the polymorphism. It is also possible that we detected the effect in our study because of the relatively low folate status of our population. Although the models point toward lower folate status being associated with the A allele, we cannot rule out the possibility of a higher folate status scenario in the presence of the G allele.

Variant allele combinations associated with lower folate status compared with common allele combinations. The *MTHFR* + *SLC191A1* 677TT/80AA genotype combination was observed in 17.2% of participants with the *MTHFR* 677TT genotype. This combination was associated with a 3.5 times greater risk of low-tertile RCF compared with the *MTHFR* + *SLC19A1* 677CC/80GG genotype combination. The *SLC19A1* 80G > A polymorphism may result in reduced 5-methyltetrahydrofolate entering into the cell, further compromising folate status in the presence of the *MTHFR* 677C > T polymorphism.

The *MTHFD1* polymorphisms may affect 5-methyltetrahydrofolate production because of the lower half-life of *MTHFD1* in the presence of the variant 1958A allele (35) and because of lower 5,10-methylenetetrahydrofolate production caused by lower *MTHFD1* promotor activity in the case of the variant -105T allele (12). However, the effects of these polymorphisms on folate status were only observed when combined with the *MTHFR* 677TT genotype.

Variant allele combinations associated with higher folate status than common alleles. Despite lower RCF in the *MTHFR* + *MTRR* 677TT/66GG combination compared with the 677CC/66AA combination, it was higher compared with the 677TT/66AA combination. However, tHcy did not differ between the *MTHFR* + *MTRR* 677TT/66GG and 677TT/66AA combinations despite their differences in RCF. We hypothesize that the higher RCF concentration in the *MTHFR* 677TT + *MTRR* 66GG combination results from less 5-methyltetrahydrofolate utilization for homocysteine remethylation as the result of reduced *MTRR* activity in the presence of the 66GG genotype. In a study of participants with relatively high folate status (9), the *MTHFR* 677TT and *MTRR* 66AG genotype combination was previously associated with elevated tHcy, whereas folate status was unaffected. The *MTRR* 524TT genotype counteracted the effect of the *MTHFR* 677TT genotype, as seen by the lack of difference in folate status and in tHcy for the *MTHFR* + *MTRR* 677TT/524TT compared with the 677CC/524CC combination, and in contrast to all of the other *MTHFR* 677TT combinations, there was no greater risk of low RCF status for the *MTHFR* + *MTRR* 677TT/524TT compared with the 677TT/524CC combination. This finding requires further investigation because only 16 participants in our study had this combination.

Strengths and limitations. Statistical power may have been lacking for some of the less frequent double-homozygote combinations, such as *MTHFR* + *MTRR* 677TT/524TT. A larger sample size would be required to study the effects of combinations of >2 polymorphisms. High folate status may override the effect of some of the polymorphisms studied on folate status, but this did not occur in our study given that vitamin supplement users were excluded and that mandatory fortification with folic acid is absent in Spain. This aspect of our study can be corroborated by the relatively low plasma folate concentrations observed compared with other studies that included supplement users and in which participants were exposed to mandatory fortification. We conclude that the effect of the *MTHFR* 677C > T polymorphism on folate status is altered by other common polymorphisms that affect folate metabolism or transport.

Acknowledgments

OB, JDF-B, SC, and MMM developed the hypothesis, designed the study, analyzed the data, and wrote the paper; AMM, CJG-M, PMU, and KM participated in sample analysis, data interpretation, and contributed to the writing of the paper. SC and LR oversaw the fieldwork of the study with input from JDF-B and MMM; JDF-B and MMM were primarily responsible for the final content. All authors read and approved the final manuscript.

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