The 677C→T variant of *MTHFR* is the major genetic modifier of biomarkers of folate status in a young, healthy Irish population

Barry Shane,[1] Faith Pangilinan,[2] James L Mills,[3] Ruzong Fan,[4] Tingting Gong,[4] Cheryl D Cropp,[5] Yoonhee Kim,[5] Per M Ueland,[6] Joan E Bailey-Wilson,[5] Alexander F Wilson,[5] Lawrence C Brody,[2] and Anne M Molloy[7]

[1]Department of Nutritional Sciences and Toxicology, University of California, Berkeley, CA; [2]Medical Genomics and Metabolic Genetics Branch, National Human Genome Research Institute, Bethesda, MD; [3]Division of Intramural Population Health Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, Bethesda, MD; [4]Department of Biostatistics, Bioinformatics, and Biomathematics, Georgetown University Medical Center (GUMC), Washington, DC; [5]Computational and Statistical Genomics Branch, National Human Genome Research Institute, Bethesda, MD; [6]Department of Clinical Science, University of Bergen and Haukeland University Hospital, Bergen, Norway; and [7]Department of Clinical Medicine, School of Medicine, Trinity College Dublin, Dublin, Ireland

ABSTRACT

Background: Genetic polymorphisms can explain some of the population- and individual-based variations in nutritional status biomarkers.

Objective: We sought to screen the entire human genome for common genetic polymorphisms that influence folate-status biomarkers in healthy individuals.

Design: We carried out candidate gene analyses and genome-wide association scans in 2232 young, healthy Irish subjects to evaluate which common genetic polymorphisms influence red blood cell folate, serum folate, and plasma total homocysteine.

Results: The 5,10-methylenetetrahydrofolate reductase (*MTHFR*) 677C→T (rs1801133) variant was the major genetic modifier of all 3 folate-related biomarkers in this Irish population and reached genome-wide significance for red blood cell folate ($P = 1.37 \times 10^{-17}$), serum folate ($P = 2.82 \times 10^{-11}$), and plasma total homocysteine ($P = 1.26 \times 10^{-19}$) concentrations. A second polymorphism in the *MTHFR* gene (rs3753584, $P = 1.09 \times 10^{-11}$) was the only additional *MTHFR* variant to exhibit any significant independent effect on red blood cell folate. Other *MTHFR* variants, including the 1298A→C variant (rs1801131), appeared to reach genome-wide significance, but these variants shared linkage disequilibrium with *MTHFR* 677C→T and were not significant when analyzed in *MTHFR* 677CC homozygotes. No additional non-*MTHFR* modifiers of red blood cell or plasma folate were detected. Two additional genome-wide significant modifiers of plasma homocysteine were found in the region of the dipeptidase 1 *(DPEP1)* gene on chromosome 16 and the Twist neighbor B (*TWISTNB*) gene on chromosome 7.

Conclusions: The *MTHFR* 677C→T variant is the predominant genetic modifier of folate status biomarkers in this healthy Irish population. It is not necessary to determine *MTHFR* 677C→T genotype to evaluate folate status because its effect is reflected in concentrations of standard folate biomarkers. The *MTHFR* 1298A→C variant had no independent effect on folate status biomarkers. To our knowledge, this is the first genome-wide association study report on red blood cell folate and the first report of an association between homocysteine and *TWISTNB*. *Am J Clin Nutr* 2018;108:1334–1341.

Keywords: biomarkers, folate, homocysteine, vitamin B-12, methylenetetrahydrofolate reductase, 1-carbon metabolism, Trinity Student Study

INTRODUCTION

Serum (or plasma) and red blood cell folate concentrations are the most commonly used biomarkers for assessing folate status (1). Serum folate concentrations are influenced by recent folate intake as well as by fasting status. Red blood cell folate concentrations are considered a better indicator of long-term folate status because red blood cell folate stores are established during erythropoiesis and the lifetime of the mature circulating red blood cell is ∼120 d (2). Plasma total homocysteine is used as a functional biomarker of folate status, although

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Address correspondence to BS (e-mail: bandie@berkeley.edu).

Abbreviations used: *CHMP1A*, chromatin modifying protein 1A isoform; *DPEP1*, dipeptidase 1; *FOLR,* folate receptor; *FPGS*, folylpolyglutamate synthetase; GWAS, genome-wide association study; LD, linkage disequilibrium; MTHFR, 5,10-methylenetetrahydrofolate reductase; *SLC*, solute carrier; SNP, single nucleotide polymorphism; *TWISTNB*, Twist neighbor B.

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plasma homocysteine concentrations are also influenced by other vitamins (vitamin B-12 and vitamin B-6), by methionine intake, and by renal function (3).

A number of studies have shown that folate status indicators can be influenced by common genetic polymorphisms and such polymorphisms are likely to be partly responsible for the variation in biomarker concentrations in individuals (4–9). For example, subjects homozygous for the 677T allele variant (rs1801133) of the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene have reduced serum and red blood cell folate concentrations whereas plasma homocysteine concentrations are increased in these individuals, at least in those with poorer folate or riboflavin status (4, 6, 10).

Previous genome-wide association studies (GWASs) have reported significant associations between several genetic variants, including the *MTHFR* 677C→T variant, and plasma homocysteine concentrations (11–18). One deep-sequencing project identified the *MTHFR* 677C→T variant as a genome-wide determinant of serum folate (19) whereas other GWAS efforts were unable to detect any genome-wide signals for serum folate (11, 12, 20). A GWAS of red blood cell folate concentrations has, to our knowledge, not previously been reported. In this study, we investigated genetic influences on commonly used indicators of folate status in a healthy, young population.

METHODS

Subjects

The Trinity Student Study enrolled students attending the University of Dublin, Trinity College between February 2003 and 2004. In total, 2524 subjects met the eligibility criteria which included age between 18 and 28 y, no current serious medical condition, and Irish ethnicity based on grandparents' origins. Ethical approval was obtained from the Dublin Federated Hospitals Research Ethics Committee, which is affiliated with Trinity College Dublin, and subjects gave written informed consent. The study was also reviewed by the Office of Human Subjects Research at the US NIH. Further details relating to the Trinity Student Study have been published elsewhere (21–24). Fifteen subjects with no questionnaire data and 1 duplicate sample were excluded, leaving 2508 valid participants whose samples were assigned to analysis. Participants were not required to fast. Each participant gave 30 mL of blood for preparation of EDTA-plasma or serum and red blood cell lysates. All samples were processed within 3 h of collection and aliquots were stored at −80°C. Blood samples and questionnaire data were coded and made anonymous before analysis. A study population flow chart is shown in **Supplemental Figure 1**.

Biochemical analyses

Serum folate and red blood cell folate were measured by microbiological assays as previously described (25). The between-assay CVs were <11.0%. Plasma total homocysteine was assayed by Bevital (www.bevital.no) via an automated isotope-dilution gas chromatography–mass spectrometry method (26). Details of methodologies for the measurement of other metabolites noted in **Table 1** have been described previously (22, 27). The between-day CVs were ≤8.1%.

Genetic approach and analysis

In the GWAS analyses, genotyping and quality control of genotype data were performed as previously described (27). Briefly, DNA for each sample was extracted from peripheral blood. Eighteen subjects were excluded because of insufficient DNA on extraction, and genome-wide single nucleotide polymorphism (SNP) genotyping was carried out on 2490 subjects with the use of Illumina 1M HumanOmni1-Quad_v1-0_B chips. These arrays include 758,443 genotyped SNPs.

Because of concerns that correction factors required in the GWAS might exclude the possibility of finding genome-wide physiologically relevant markers, we decided to initially carry out a candidate gene analysis through the use of a cleaned-up subset of the GWAS data. We selected 76 SNPs to tag common variation in 9 genes which have been shown, or might be expected, to influence folate status and/or metabolism. The genes selected are involved in folate transport: the intestinal folate transporter [*PCFT*/solute carrier family 46 member 1 *(SLC46A1)*], the reduced folate carrier (*RFC/SLC19A1*), the mitochondrial folate transporter (*MFTC/SLC25A32*), and the folate receptor (*FOLR1*); tissue folate accumulation and retention: folylpolyglutamate synthetase (*FPGS*) and folate hydrolase (*FOLH1*); and folate interconversion, metabolism, and catabolism: *MTHFR*, methenyltetrahydrofolate synthetase (*MTHFS*), and dihydrofolate reductase (*DHFR*) (**Supplemental Table 1**). Because plasma total homocysteine concentrations are influenced by folate status, the same candidate gene variants were used to analyze homocysteine concentrations, although these variants would only represent a subset of genes that might be expected to influence homocysteine. We also evaluated the GWAS data to investigate whether additional gene variants that were not part of our candidate gene study influenced folate status biomarkers. In all analyses, SNPs were tested for association with biomarkers via the simple linear regression model executed in PLINK version 1.7 under the assumption of an additive genetic model.

Red blood cell folate, serum folate, and plasma total homocysteine concentrations had nonnormal distributions. In the candidate gene analyses, inverse normal rank transformation was applied to make them satisfy the normality assumption before examination of associations with metabolite variables through linear regression analysis. The inverse normal rank−transformed metabolic data were regressed on each SNP for association analysis with adjustment for gender, serum vitamin B-12 concentrations, and use of folic acid supplements. Bonferroni corrections for these tests were based on the number of SNPs tested for all candidate genes (significance set at $P = 0.05/76$).

In the total GWAS data analyses, SNPs were again tested for association with red blood cell and serum folate and plasma total homocysteine via the simple linear regression model under the assumption of an additive genetic model. In this case log-transformed data adjusted for age and gender were used. The GWAS genome-wide significance level was set at $P < 5 \times 10^{-8}$.

We also performed metabolite statistical analyses using SAS software (version 9.4; SAS Institute Inc., Cary, NC). Independent Student's *t* tests were used to determine statistically significant differences between groups. Pearson correlation coefficients were used to test associations between metabolites. Kruskal-Wallace and Mann-Whitney analyses were used for data that exhibited nonnormal distributions.

TABLE 1
Characteristics of the Trinity Student Study population (2232 subjects)

	Men			Women			
	n	Mean	SD	*n*	Mean	SD	*P*[1]
Age, y	922	22.6	1.7	1310	22.3	1.6	<0.0001
BMI, kg/m^2	903	23.4	2.9	1306	22.7	3.1	<0.0001
Alcohol intake, g/d	921	33.3	24.8	1309	18.5	15.6	<0.0001
Creatinine, μmol/L	921	75.4	11.7	1307	59.6	10.0	<0.0001
C-reactive protein, mg/L	804	1.40	3.60	1237	1.58	2.58	0.2189
Hemoglobin, g/dL	922	15.4	1.3	1310	13.4	1.6	<0.0001
Serum ferritin, μg/L	865	66.5	40.3	1282	26.8	22.1	<0.0001
Serum folate, nmol/L	921	32.4	17.2	1310	35.1	19.5	0.0006
Red blood cell folate, nmol/L	922	1099	416	1309	1058	437	0.0274
Serum vitamin B-12, pmol/L	922	350	138	1310	319	148	<0.0001
Serum holotranscobalamin II, pmol/L	913	64.2	29.2	1307	56.1	31.4	<0.0001
Plasma homocysteine, μmol/L	922	9.42	3.38	1310	8.12	2.58	<0.0001
Plasma methylmalonic acid, μmol/L	908	0.18	0.07	1302	0.19	0.09	0.0851
Folic acid users in past week	180 of 922 (19.5%)			352 of 1310 (26.9%)			0.0001[2]
Folic acid intake among users, μg/d	180	200[3]	100–300[4]	352	200[3]	100–286[4]	0.378[5]

[1]Gender differences. An independent *t* test was used for all gender comparisons except folic acid supplements per week and folic acid users in the past week, as noted.
[2]Chi-square test.
[3]Median.
[4]IQR intake among folic acid users.
[5]Mann-Whitney *U* test.

RESULTS

Characteristics of the selected population

High-quality genotyping data were obtained for 2438 of the 2490 samples genotyped. Two hundred and six subjects were excluded for various reasons including sex discrepancies, abnormal sex chromosomes, siblings, cousins, and other detected relatedness, low call rate, no phenotype, or outlier. The characteristics of the final study sample (2232 subjects) are shown in Table 1. Folic acid supplements were consumed by 532 participants in the week before the study (23.3%). No subjects had medical conditions that would affect folate absorption at the time of the study.

Red blood cell and serum folate were significantly correlated (Pearson's $r = 0.61$; $P < 0.0001$). Both serum and red blood cell folate were significantly associated with plasma homocysteine ($r = -0.38$; $P < 0.0001$ and $r = -0.33$; $P < 0.0001$, respectively). Red blood cell folate and plasma homocysteine concentrations were significantly lower in women than in men ($P = 0.027$ and $P < 0.0001$, respectively) whereas serum folate was ~8% higher in women ($P = 0.0006$). Plasma total vitamin B-12 concentrations were significantly correlated with red blood cell folate ($r = 0.21$; $P < 0.0001$) and serum folate ($r = 0.18$; $P < 0.0001$) and negatively correlated with plasma homocysteine ($r = -0.28$; $P < 0.0001$).

Candidate gene analysis of effects of genetic polymorphism on folate status indicators

In the candidate gene analyses 76 SNPs from the GWAS chips were analyzed in 2232 subjects. The results were adjusted for gender, serum vitamin B-12 concentrations, and for whether or not subjects received folic acid supplements in the week before blood collection. In order to remove the effect of the *MTHFR* 677C→T variant (rs1801133), the analyses were repeated in 989 subjects who were homozygous for the C allele.

Red blood cell folate

For each SNP analyzed individually, red blood cell folate was significantly positively correlated with serum vitamin B-12 ($P = 1.5–4.2 \times 10^{-23}$, depending on SNP), with whether or not subjects consumed folate supplements ($P = 0.3–1.6 \times 10^{-11}$), and with gender ($P = 0.005–0.008$, lower in women). **Table 2** shows the SNPs that significantly influenced red blood cell folate concentrations after adjustment for multiple analyses. The most significant SNP was the rs1801133 677C→T variant of *MTHFR* ($P = 3.15 \times 10^{-17}$) with lower red blood cell folate in subjects with the minor T allele. Nine other SNPs, all in the *MTHFR* gene, also remained significant after Bonferroni adjustment, including the rs1801131 1298A→C variant, which has been reported to exhibit biological effects distinct from the *MTHFR* 677 variant (28, 29), but their *P* values were ≥5 orders of magnitude higher than for rs1801133. Biological effects of the *MTHFR* 1298 variant are somewhat controversial because the *MTHFR* 677 and 1298 variants share significant linkage disequilibrium (LD, D' = 0.997, $r^2 = 0.218$) and may be reporting on the same association signal. Consistent with this, the minor *MTHFR* 1298C allele was associated with higher red blood cell folate, i.e., the opposite effect to the *MTHFR* 677T allele. The other significant SNPs were located in the *MTHFR* gene region which exhibits high LD (**Supplemental Figure 2**). The significant association of these SNPs with red blood cell folate most likely represents a single association signal driven largely by *MTHFR* rs1801133.

TABLE 2
Candidate gene SNPs that significantly influenced red blood cell folate concentrations after adjusting for multiple analyses[1]

SNP	Gene	*P* SNP[2]	*P* SNP in *MTHFR* 677CC subjects[3] ($n = 989$)
rs1801133	*MTHFR*	3.15×10^{-17}	
rs3753584	*MTHFR*	5.88×10^{-12}	9.87×10^{-4}
rs4846052	*MTHFR*	2.16×10^{-9}	0.332
rs3737966	*MTHFR*	2.92×10^{-9}	0.342
rs4846054	*MTHFR*	3.95×10^{-9}	0.287
rs1537516	*MTHFR*	6.18×10^{-7}	0.020
rs1801131	*MTHFR*	1.14×10^{-6}	0.217
rs17037397	*MTHFR*	1.96×10^{-6}	0.010
rs13306567	*MTHFR*	1.21×10^{-4}	0.081
rs1476413	*MTHFR*	1.43×10^{-4}	0.464
SNP pairs[4]			
rs1801133	*MTHFR*	3.93×10^{-11}	
rs3753584	*MTHFR*	1.03×10^{-5}	
rs1801133	*MTHFR*	2.74×10^{-12}	
rs1801131	*MTHFR*	0.32	

[1]Data from 2232 subjects were adjusted for gender, serum vitamin B-12 concentration, and folic acid supplements. SNPs were tested with the use of a simple linear regression model executed in PLINK version 1.7 under the assumption of an additive genetic model. *MTHFR*, 5,10-methylenetetrahydrofolate reductase; SNP, single nucleotide polymorphism.

[2]Bonferroni threshold for SNPs: $P = 6.6 \times 10^{-4}$ (0.05/76).

[3]Bonferroni threshold for SNPs in *MTHFR* 677CC subjects: $P = 6.7 \times 10^{-4}$ (0.05/75).

[4]Bonferroni threshold adjustment for SNP pairs: $P = 1.75 \times 10^{-5}$ (0.05/(76 × 75/2)).

The analysis of the *MTHFR* SNPs was repeated as SNP pairs with rs1801133 (*MTHFR* 677) to attempt to adjust for the possible effect of LD with rs1801133. When we did this, only rs3753584 remained significant with a *P* value of 1.03×10^{-5}, as opposed to a *P* value of 3.93×10^{-11} for rs1801133 in the same analysis (Table 2). Finally, to eliminate any possibility that significant association was due to LD with rs1801133, we repeated the individual single SNP analyses in 989 subjects who were homozygous for the *MTHFR* 677C allele (Table 2). None of the 75 SNPs analyzed in these subjects was significant after Bonferroni adjustment and most were nonsignificant even without adjustment (i.e., $P > 0.05$). However, a strong signal was still seen for rs3753584 and its *P* value was only slightly above that required for significance. It may not have reached significance in *MTHFR* 677CC subjects because of the smaller number of subjects. Subjects expressing the minor G allele of rs3753584 had significantly increased red blood cell folate concentrations (**Table 3**). However, this variant shares significant LD with rs1801133 and the rs3753584 G variant is only seen on a *MTHFR* 677C background (Table 3, Supplemental Figure 2). Part of the increased red blood cell folate noted in rs3753584 G subjects was due to the absence of *MTHFR* 677T alleles. However, a significant increase in red blood cell folate was also observed with the rs3753584 G allele in *MTHFR* 677CC subjects (Table 3) and the rs3753584 variant does appear to be a second independent modifier of folate concentrations.

Serum folate

For each SNP analyzed individually, serum folate was significantly positively correlated with serum vitamin B-12 ($P = 1.0$–2.5×10^{-20}, depending on the SNP), with whether or not subjects consumed folate supplements ($P = 0.9$–1.7×10^{-15}), and with gender ($P = 0.003$–0.004, higher in women).

Table 4 shows the SNPs that significantly influenced serum folate concentrations after adjustment for multiple analyses. The most significant SNP was the rs1801133 677C→T variant of *MTHFR* ($P = 3.01 \times 10^{-11}$) with lower serum folate in subjects with the minor T allele. Four other SNPs, all in *MTHFR* and including rs3753584, remained significant after Bonferroni adjustment, although their *P* values were 6 orders of magnitude higher than for rs1801133. When analyzed in subjects homozygous for the C allele of rs1801133, none of these SNPs was statistically significant (Table 4). Two other *MTHFR* SNPs did achieve significance when analyzed in *MTHFR* 677CC subjects (rs1476413 and rs2066471, see Table 4) but neither was significant when initially analyzed in the total population.

Although serum folate was highly correlated with red blood cell folate, fewer significant SNPs were detected in the serum folate analysis than in the red blood cell folate analysis, and *P* values were higher. This probably reflected the greater variation in serum folate and also the fact that the serum samples collected in this study were nonfasting.

Plasma total homocysteine

Table 5 shows the SNPs that significantly influenced plasma homocysteine concentrations after adjustment for multiple analyses. Again, the most significant SNP was the rs1801133 677C→T variant of *MTHFR* ($P = 1.64 \times 10^{-16}$) with higher plasma homocysteine in subjects with the minor T allele. Two other SNPs in the *MTHFR* gene also remained significant after Bonferroni adjustment, but with much higher *P* values than for rs1801133. Neither of these SNPs was significant when analyzed in *MTHFR* 677CC subjects. The *MTHFR* 1298 variant (rs1801131) had no significant effect on homocysteine concentrations when analyzed in the total population or in *MTHFR* 677CC subjects. Of the 75 SNPs analyzed in *MTHFR* 677CC subjects, an SNP in *FPGS* had the lowest *P* value, although this did not reach statistical significance after Bonferroni adjustment (Table 5), and this SNP was not significant when analyzed in the total population.

GWAS analyses of folate status indicators

The results from the GWAS data analyses are shown in **Supplemental Tables 2–4**. The data shown were analyzed with the use of log-transformed data adjusted for age and gender, but essentially identical results were found with the use of inverse rank-ordered data, as were used in the candidate gene analyses.

Red blood cell and serum folate

Supplemental Table 2 shows the 32 SNPs affecting red blood cell folate that reached genome-wide significance ($P < 5 \times 10^{-08}$). All 32 SNPs were on chromosome 1 in the region of the *MTHFR* gene. rs1801133 was the most significant ($P = 1.37 \times 10^{-17}$) and accounted for ~7% of the variation

TABLE 3
Effect of *MTHFR* rs3753584 variants on red blood cell folate concentrations in *MTHFR* 677 C→T variants (rs1801133)[1]

		Red blood cell folate (nmol/L), mean ± SD *MTHFR* rs3753584			
		AA	AG	GG	*P* value
Total	—	1040 ± 422 ($n = 1558$)	1145 ± 430 ($n = 599$)	1255 ± 467 ($n = 73$)	<0.0001[2]
MTHFR 677	CC	1096 ± 425 ($n = 554$)	1151 ± 436 ($n = 362$)	1255 ± 467 ($n = 73$)	0.0043[3]
MTHFR 677	CT	1048 ± 407 ($n = 745$)	1136 ± 422 ($n = 237$)	NA ($n = 0$)	0.0072[4]
MTHFR 677	TT	897 ± 425 ($n = 142$)	NA ($n = 0$)	NA ($n = 0$)	

[1] *MTHFR*, 5,10-methylenetetrahydrofolate reductase.
[2] Kruskal-Wallis test.
[3] Kruskal-Wallis test. In pairwise post-test comparisons that used Dunn's multiple comparison tests, only AA and GG differed significantly.
[4] Mann-Whitney U test.

in red blood cell folate concentrations (H^2a, Supplemental Table 2). The next-most significant SNP had a *P* value >4 orders of magnitude higher. None of these SNPs reached genome-wide significance when analyzed in *MTHFR* 677CC subjects. Supplemental Table 3 shows similar data for serum folate. The highest-ranked SNP was rs1801133 ($P = 2.82 \times 10^{-11}$) and the 4 other lower-ranked SNPs that reached genome-wide significance were not significant when analyzed in *MTHFR* 677CC subjects. As was found in the candidate gene analysis, *P* values for SNPs were higher for serum folate compared with red blood cell folate.

Plasma total homocysteine

Supplemental Table 4 shows the 29 SNPs affecting plasma homocysteine that reached genome-wide significance ($P < 5 \times 10^{-8}$). Again, the most significant SNP was rs1801133 ($P = 1.26 \times 10^{-19}$), which accounted for ~4% of the variation in plasma homocysteine concentrations. An additional 22 chromosome-1 SNPs in the vicinity of the *MTHFR* gene had *P* values ≥8 orders of magnitude higher than rs1801131 but still reached genome-wide significance. None of these SNPs had any significant effect on homocysteine concentrations in *MTHFR* 677CC subjects.

Additional genome-wide significant SNPs were detected on chromosome 7 in the vicinity of the Twist neighbor B (*TWISTNB*) gene and on chromosome 16 in the region of the chromatin modifying protein 1A isoform (*CHMP1A*) gene (Supplemental Table 4). A strong signal was retained in the chromosome 16 region in *MTHFR* 677CC subjects, although the *P* value increased to above that representing genome-wide significance, possibly reflecting the reduced size of the *MTHFR* 677CC population. The chromosome 7 signal around the *TWISTNB* gene was considerably muted in *MTHFR* 677CC subjects.

The reduced *TWISTNB* signal in 677CC subjects was somewhat surprising. Because one possible explanation could be an interaction between the *MTHFR* gene on chromosome 1 and the *TWISTNB* gene on chromosome 7, we further analyzed the effects of the *TWISTNB* (chromosome 7) and *CHMP1A*

TABLE 4
Candidate gene SNPs that significantly influenced serum folate concentrations after adjusting for multiple analyses[1]

SNP	Gene	*P* SNP[2]	*P* SNP in *MTHFR* 677CC subjects[3]
rs1801133	*MTHFR*	3.01×10^{-11}	—
rs9651118	*MTHFR*	3.45×10^{-5}	0.0455
rs13306567	*MTHFR*	1.55×10^{-4}	0.0547
rs17037397	*MTHFR*	3.25×10^{-4}	0.146
rs3753584	*MTHFR*	6.61×10^{-4}	0.521
rs1476413	*MTHFR*	0.471	1.39×10^{-4}
rs2066471	*MTHFR*	0.128	5.58×10^{-4}

[1] Data were adjusted for gender, serum vitamin B-12 concentration, and folic acid supplements. SNPs were tested via a simple linear regression model executed in PLINK version 1.7 under the assumption of an additive genetic model. Bonferroni adjustment for SNP pairs ($P = 1.75 \times 10^{-5}$ [0.05/(76 × 75/2)]) was not satisfied for any SNP after adjusting for the effect of the *MTHFR* rs1801133 variant. *MTHFR*, 5,10-methylenetetrahydrofolate reductase; SNP, single nucleotide polymorphism.
[2] Bonferroni threshold for SNPs: $P = 6.6 \times 10^{-4}$ (0.05/76).
[3] Bonferroni threshold for SNPs in *MTHFR* 677CC subjects: $P = 6.7 \times 10^{-4}$ (0.05/75). 989 subjects.

TABLE 5
Candidate gene SNPs that significantly influenced plasma total homocysteine concentrations after adjusting for multiple analyses[1]

SNP	Gene	*P* SNP[2]	*P* SNP in *MTHFR* 677CC subjects[3]
rs1801133	*MTHFR*	1.64×10^{-16}	
rs9651118	*MTHFR*	7.58×10^{-7}	0.100
rs17037397	*MTHFR*	3.87×10^{-6}	0.061
rs1801131	*MTHFR*	0.074	0.036
rs10760503	*FPGS*	0.052	0.005

[1] Data were adjusted for gender, serum vitamin B-12 concentration, and folic acid supplements. SNPs were tested via a simple linear regression model executed in PLINK version 1.7 under the assumption of an additive genetic model. *FPGS*, folylpolyglutamate synthetase; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; SNP, single nucleotide polymorphism.
[2] Bonferroni threshold for SNPs: $P = 6.6 \times 10^{-4}$ (0.05/76).
[3] Bonferroni threshold for SNPs in *MTHFR* 677CC subjects: $P = 6.7 \times 10^{-4}$ (0.05/75). 989 subjects.

TABLE 6
Effect of *TWISTNB* (rs17354370) and *CHMP1A* (rs164746) variants on homocysteine concentrations in each *MTHFR* 677 C→T genotype group (rs1801133)[1]

		TWISTNB				*CHMP1A*			
		Homocysteine (μmol/L), mean ± SD				Homocysteine (μmol/L), mean ± SD			
		AA	AG	GG	*P* value[2]	CC	CT	TT	*P* value[2]
MTHFR	CC	8.4 ± 2.0 (n = 469)	8.1 ± 2.1 (n = 422)	7.8 ± 1.8 (n = 98)	0.0047	7.9 ± 1.8 (n = 319)	8.2 ± 2.1 (n = 474)	8.7 ± 2.2 (n = 196)	5.76×10^{-5}
MTHFR	CT	8.9 ± 2.9 (n = 486)	8.3 ± 2.3 (n = 415)	8.0 ± 1.8 (n = 82)	2.35×10^{-5}	8.4 ± 3.2 (n = 313)	8.5 ± 2.3 (n = 455)	8.9 ± 2.2 (n = 213)	5.67×10^{-5}
MTHFR	TT	11.0 ± 5.7 (n = 142)	10.3 ± 5.8 (n = 99)	9.2 ± 3.2 (n = 18)	0.35	9.8 ± 3.6 (n = 72)	11.1 ± 6.3 (n = 138)	10.4 ± 5.7 (n = 49)	>0.05

[1] *CHMP1A*, chromatin modifying protein 1A isoform; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *TWISTNB*, Twist neighbor B.
[2] The *P* values are from the *F* test of ANOVA.

(chromosome 16) variants on homocysteine concentrations in subjects with each of the different *MTHFR* 677 variants (**Table 6**). The minor allele (G) of the *TWISTNB* (rs17354370) variant decreased homocysteine concentrations in all *MTHFR* 677 genotypes. This effect, which appeared to be quantitatively smaller than the change in homocysteine caused by the minor 677T allele of *MTHFR*, was most significant in *MTHFR* 677CT individuals. The lack of a significant effect in *MTHFR* 677TT individuals appeared to be due to the reduced number of subjects and the greater variation of homocysteine concentrations in these subjects. Consequently, these data do not support an interaction between the *TWISTNB* and *MTHFR* SNPs, but do not exclude the possibility. The minor T allele of the *CHMP1A* (rs164746) variant was associated with increased plasma homocysteine concentrations in all 3 *MTHFR* 677 genotypes. This effect was not statistically significant in *MTHFR* 67TT subjects, possibly due to the smaller number of subjects and increased variation in this group (Table 6).

DISCUSSION

In this study we explored genetic modifiers of 3 folate status biomarkers in a young, healthy Irish population. Previous small-scale studies have shown that a common polymorphism in the *MTHFR* gene (rs1801133, 677C→T) lowers serum (plasma) and red blood cell folate concentrations. However, multiple GWAS analyses, with the exception of a large-scale study on the Icelandic population (19), have failed to find genome-wide significant markers that influence plasma folate concentrations and, in some cases, have failed to find any suggestion of an effect of *MTHFR* gene variants. To our knowledge, a red blood cell folate GWAS analysis has not previously been reported.

In our candidate gene analysis, 10 SNPs in the *MTHFR* gene significantly affected red blood cell folate after adjustment for multiple analyses, with the *MTHFR* 677C→T (rs1801133) variant giving the strongest signal by far (Table 2). After adjusting for LD of the additional 9 positive *MTHFR* SNPs with rs1801133 by doing SNP pair analyses, rs3753584 was the only additional variant that remained significant. Of note, the signal for the *MTHFR* 1298A→C variant (rs1801131) completely disappeared.

Standard analyses that use a simple linear regression model and assume an additive genetic effect to separate the effects of 2 SNPs through the use of pair analysis may not be very accurate when comparing some variants such as *MTHFR* 677C→T. The T allele changes an alanine (A) in the *MTHFR* protein to a valine (V), which decreases the affinity of its FAD cofactor. Loss of the flavin cofactor causes conversion of the active dimeric enzyme to a less stable monomeric form, and increased turnover of the protein. Binding of the folate substrate stabilizes the bound FAD on the enzyme. Consequently, the increased loss of enzyme activity observed in subjects homozygous for the 677T variant, and also metabolic effects such as lowered serum and red blood cell folate and increased plasma homocysteine, are seen only in individuals with poorer folate and riboflavin status. Changes are seen in subjects heterozygous for the *MTHFR* 677C→T variant, but the extent of these changes is less than half that observed in 677T homozygotes (10). Therefore, the additive genetic model, where 1 T allele would have half the effect of 2 T alleles, does not model the actual situation that well. Because the active *MTHFR* enzyme is a dimer, one-quarter of enzyme in heterozygous subjects would be A/A dimers (as in 677C homozygotes), half would be A/V dimers, and one-quarter V/V dimers (as in 677T homozygotes). If the A/V dimeric form is as stable as the A/A dimer, the effects of homozygosity for 677T would be 4 times that of the heterozygote. Because of this, we repeated the analysis using only subjects homozygous for the *MTHFR* 677C variant, because this eliminates any effect of the T allele. The intronic rs3753584 variant was the only additional candidate *MTHFR* SNP to exhibit a significant effect on red blood cell folate when studied in *MTHFR* 677C homozygotes. The increase in red blood cell folate associated with the minor rs3753584 G allele could be explained by increased MTHFR expression, but we have no direct evidence for this. Over 30 SNPs with genome-wide significance were found in the GWAS analysis of red blood cell folate. All were in the region of the *MTHFR* gene and the *MTHFR* 677C→T (rs1801133) variant exhibited the strongest signal (Supplemental Table 2). None of the SNPs was close to exhibiting genome-wide significance in *MTHFR* 677C homozygotes. Thus, rs1801133 is the major genetic modifier of red blood cell folate concentrations in this population.

Similar results were seen in the candidate gene and GWAS analyses for serum folate, although a more limited number of significant SNPs, all in the *MTHFR* gene region, were found. Again, the *MTHFR* 677C→T (rs1801133) variant gave the strongest signal and none of the other SNPs was close to being

significant in *MTHFR* 677C homozygotes. It is likely that the smaller number of initial hits in the serum folate analysis than in the red blood cell folate analysis reflects the fact that the serum samples were nonfasting, but it may also reflect the fact that redblood cell folate is a more stable biomarker of folate status than serum or plasma folate.

Although we are unaware of any previous GWAS analysis of red blood cell folate, several analyses of plasma folate genetic modifiers have been published (11, 12, 19, 20). The only study to report genome-wide significant SNPs found 2 significant modifiers: rs1801133 (*MTHFR* 677C→T) and rs652197, a SNP in the *FOLR3* gene, encoding 1 of the folate receptors on chromosome 11 (19). We were unable to detect a significant signal for the folate receptor *FOLR1*, which is in close proximity to *FOLR3*, in our candidate gene analysis or any signal on chromosome 11 in our GWAS analysis. Other GWASs did not find any significant signal for plasma folate but did find a modest association in the fidgetin (*FIGN)* gene region on chromosome 2 (11) or in the myelin transcription factor 1 like (*MYT1L)* gene region on chromosome 2 (20), neither of which we were able to confirm. Modest signals for various *MTHFR* SNPs, including rs3737965 (11) and rs1999594 (12), have been reported. We did find a genome-wide significant association for rs1999594 in our serum folate GWAS (Supplemental Table 3) but it was not significant in subjects homozygous for the *MTHFR* 677C variant. It is likely that the reason why only 2 GWASs found genome-wide significance for rs1801133 as a modifier of serum or plasma folate is that the populations studied [Icelandic (19) and Irish (current study)] were genetically more homogeneous than in the other studies.

The *MTHFR* 677C→T (rs1801133) variant was the most significant signal in the candidate gene analysis of plasma homocysteine concentrations. Two other SNPs in *MTHFR* also reached significance but their signals were completely lost when analyzed in *MTHFR* 677C homozygotes. Twenty-nine SNPs reached genome-wide significance in our homocysteine GWAS analysis. Twenty-three were in the *MTHFR* region including rs1801133, which gave the strongest signal. None of the chromosome 1 *MTHFR* SNPs were significant in *MTHFR* 677C homozygotes, indicating they were reporting on rs1801133. No effect of the *MTHFR* 1298A→C variant (rs1801131) on homocysteine concentrations was detected.

rs1801133 has previously been reported to be a genome-wide significant modifier of plasma homocysteine in a number of GWASs (11, 12, 14, 18). Hazra et al. (11) also reported that 2 other SNPs close to *MTHFR* (rs12085006 and rs1999594) had stronger associations with homocysteine than rs1801133, and these associations remained nominally statistically significant after mutual adjustment for rs1801133 effects in a multivariate regression model. We also found genome-wide associations for rs12085006 and rs1999594 in our homocysteine GWAS (Supplemental Table 4), but these associations were completely absent in subjects homozygous for the *MTHFR* 677C variant.

Significant signals for homocysteine were also found in our study on chromosome 16 in the region of the *CHMP1A* gene (2 SNPs) and on chromosome 7 in the region of the *TWISTNB* gene (4 SNPs) (Supplemental Table 4, Table 6). *CHMP1A* rs164746 is essentially a perfect proxy with dipeptidase 1 *(DPEP1)* rs154657 ($D' = 1$; $r^2 = 0.998$ in the Trinity Student population). *DPEP1* has been reported to be a GWAS hit for homocysteine (14, 18). *DPEP1* (EC 3.4.13.11) is a kidney membrane enzyme that hydrolyzes a variety of dipeptides and is implicated in renal metabolism of glutathione and its conjugates (30). *DPEP1* is also a genome-wide signal for plasma total cysteine in the Trinity Student Study with the minor allele associated with higher cysteine, as was found for total homocysteine (unpublished data).

The 4 SNPs detected in the *TWISTNB* region of chromosome 7 (7p21.1) have not previously been reported as modifiers of plasma homocysteine concentrations. *TWISTNB* encodes a DNA-dependent RNA polymerase that is a component of RNA polymerase I which synthesizes ribosomal RNA precursors. van Meurs et al. (18) did report a significant chromosome 7 signal but this was in the GTP-binding protein 10 (*GTPBP10*, 7q21.13) region, which is distant from *TWISTNB*, and we did not observe any signal in this region.

Our studies clearly indicate that the *MTHFR* 677C→T (rs1801133) variant is the major genetic modifier of folate status biomarkers and may be the only significant modifier of serum (plasma) and red blood cell folate concentrations, at least in young, healthy adults in the Irish population. The question arises of whether this confounds the use of these biomarkers in assessing folate status, and whether determining an individual's rs1801133 genotype would be of benefit in interpreting folate status when using these biomarkers. It has previously been established (4, 6, 10), and has been shown here, that the T allele of *MTHFR* 677 causes decreases in both serum and red blood cell folate, the latter being a proxy for tissue folate, which would indicate an impairment in folate status. Plasma homocysteine is also increased, indicative of poorer folate function in tissues. Because the poorer folate status caused by this variant is reflected in the changes in the concentrations of these biomarkers for folate status, there does not appear to be any added benefit in genotyping subjects for this variant when interpreting folate status through the use of these biomarkers. Carriers of the T allele are also responsive to folate intake (31), which indicates that a T allele–dependent low folate status is responsive to treatment, also making genetic testing unnecessary.

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The authors' responsibilities were as follows: BS, AMM, LCB, and JLM: designed the research; AMM, JLM, FP, and PMU: collected and analyzed the data; RF, TG, CDC, YK, JEB-W, AFW, and AMM: performed statistical analysis; BS and AMM: wrote the initial draft of the manuscript; FP, JLM, BS, and LCB: critically revised the manuscript; and all authors: had responsibility for accuracy of the final content, and read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

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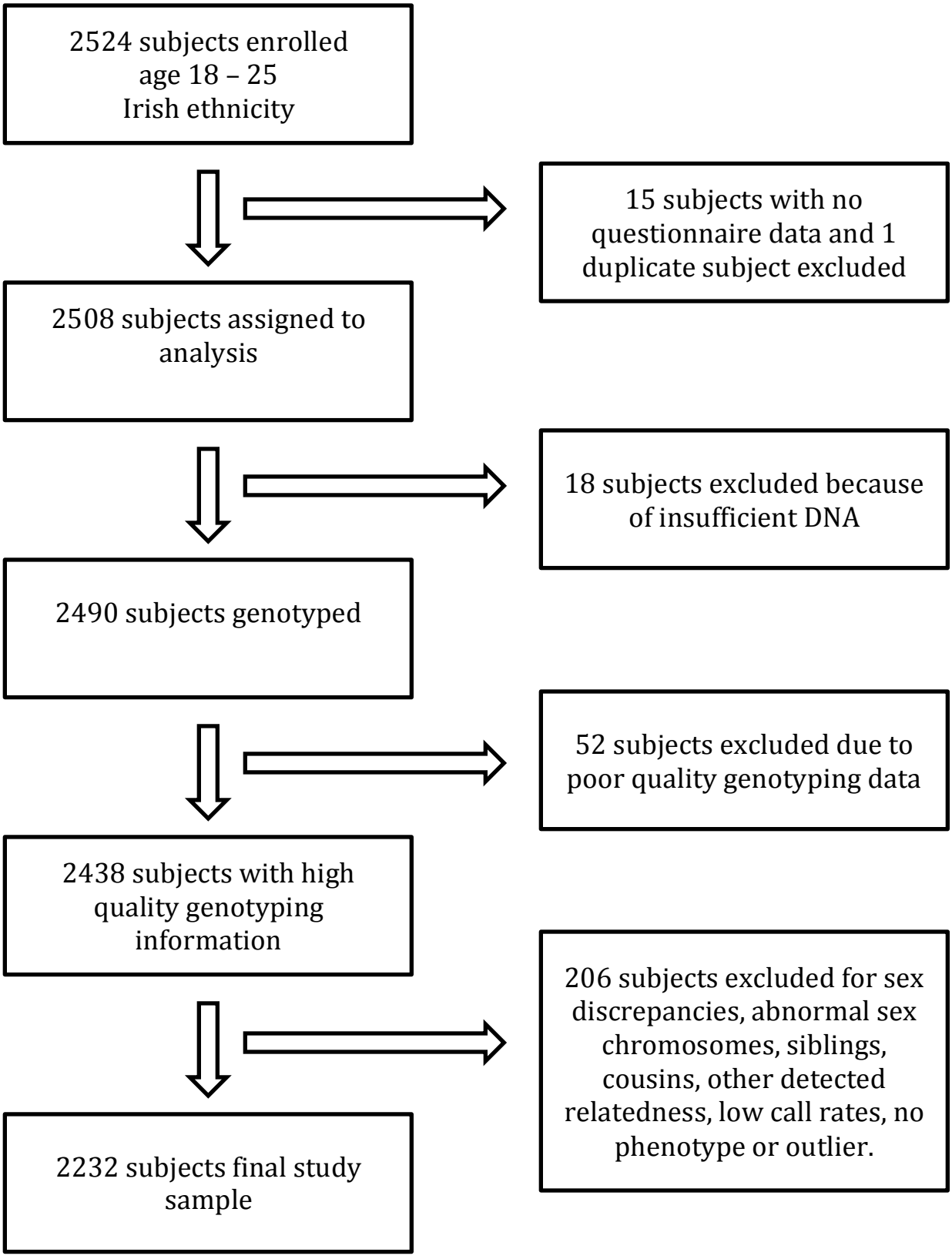
Supplemental Figure 1. Flow Chart for Study Population



Supplemental Table 1. 76 SNPs used in Candidate Gene Analyses

Gene	SNP	chromosome	position
MTHFR	rs4846048	1	11768839
MTHFR	rs2184226	1	11770023
MTHFR	rs3737966	1	11770346
MTHFR	rs1537516	1	11770448
MTHFR	rs1476413	1	11774887
MTHFR	rs1801131	1	11777063
MTHFR	rs1801133	1	11778965
MTHFR	rs1572151	1	11780298
MTHFR	rs4846052	1	11780538
MTHFR	rs2066471	1	11783045
MTHFR	rs13306567	1	11783052
MTHFR	rs17037397	1	11784750
MTHFR	rs9651118	1	11784801
MTHFR	rs3753584	1	11787173
MTHFR	rs3737964	1	11789631
MTHFR	rs4846054	1	11791817
DHFR	rs1650723	5	79957786
DHFR	rs11951910	5	79975227
DHFR	rs863215	5	79983761
DHFR	rs1650697	5	79986537
DHFR	rs6151599	5	79986788
DHFR	rs380691	5	79987790

MFTC/SLC25A32	rs3098259	8	104479596
MFTC/SLC25A32	rs1061196	8	104480104
MFTC/SLC25A32	rs3133810	8	104484294
MFTC/SLC25A32	rs17803441	8	104486221
MFTC/SLC25A32	rs3134295	8	104496535
MFTC/SLC25A32	rs750606	8	104497706
FPGS	rs1544105	9	129602546
FPGS	rs7856096	9	129606360
FPGS	rs10760503	9	129618225
FOLH1	rs6485964	11	49122813
FOLH1	rs16906158	11	49127350
FOLH1	rs34033751	11	49132803
FOLH1	rs7113251	11	49143860
FOLH1	rs3974729	11	49153523
FOLH1	rs588458	11	49170624
FOLH1	rs202700	11	49173275
FOLR1	rs2071010	11	71578612
MTHFS	rs685487	15	77923184
MTHFS	rs8923	15	77924615
MTHFS	rs2733103	15	77925626
MTHFS	rs17284990	15	77931252
MTHFS	rs16971450	15	77941626
MTHFS	rs6495446	15	77942037

MTHFS	rs7177659	15	77945097
MTHFS	rs6495449	15	77945214
MTHFS	rs17285431	15	77949320
MTHFS	rs6495451	15	77951108
MTHFS	rs2562744	15	77961443
MTHFS	rs2733106	15	77964742
MTHFS	rs12438477	15	77965338
MTHFS	rs2586182	15	77970821
MTHFS	rs2733088	15	77971712
MTHFS	rs12440798	15	77973395
MTHFS	rs2115540	15	77977363
MTHFS	rs2163005	15	77981535
PCFT/SLC46A1	rs4795434	17	23741044
PCFT/SLC46A1	rs11656835	17	23741176
PCFT/SLC46A1	rs2239910	17	23747793
PCFT/SLC46A1	rs739439	17	23747949
PCFT/SLC46A1	rs2239907	17	23749871
PCFT/SLC46A1	rs17719944	17	23753580
RFC/SLC19A1	rs2838952	21	45754591
RFC/SLC19A1	rs12483377	21	45755537
RFC/SLC19A1	rs2236484	21	45756112
RFC/SLC19A1	rs7499	21	45756756
RFC/SLC19A1	rs17004785	21	45757046
RFC/SLC19A1	rs7867	21	45757080
RFC/SLC19A1	rs2838956	21	45769452

RFC/SLC19A1	rs2838958	21	45772995
RFC/SLC19A1	rs4818789	21	45773255
RFC/SLC19A1	rs12659	21	45775984
RFC/SLC19A1	rs1051269	21	45776434
RFC/SLC19A1	rs3788205	21	45788806
RFC/SLC19A1	rs1023159	21	45790608

Supplemental Table 2. SNPs that reached genome-wide significance in association with log-transformed red cell folate in 2232 Irish participants ($p < 5 \times 10^{-08}$). Data adjusted for age and gender.

gene	SNP	CHR	BP	N	BETA	SE	R^2	[1]H[2]a	P value	[2]P value 677CC
MTHFR	RS1801133	1	11778965	2229	-0.0463	0.005379	0.03221	0.0680	1.37×10^{-17}	-
MTHFR	RS13306561	1	11788391	2228	0.04649	0.006782	0.02067	0.0202	9.22×10^{-12}	8.93×10^{-4}
MTHFR	RS3753584	1	11787173	2230	0.04631	0.006781	0.02051	0.0200	1.09×10^{-11}	1.01×10^{-3}
MTHFR	RS17367504	1	11785365	2229	0.04631	0.006784	0.02049	0.0200	1.12×10^{-11}	7.36×10^{-4}
MTHFR	RS17037390	1	11783430	2227	0.04542	0.006754	0.01992	0.0193	2.22×10^{-11}	8.74×10^{-4}
CLCN6	RS2050265	1	11802286	2230	0.04538	0.006789	0.01966	0.0191	2.93×10^{-11}	1.86×10^{-3}
CLCN6	RS198358	1	11826663	2229	0.0392	0.005873	0.01961	0.0196	3.12×10^{-11}	.014
CLCN6	RS7537765	1	11809890	2230	0.04522	0.006788	0.01953	0.0190	3.39×10^{-11}	2.06×10^{-3}
CLCN6	RS17037452	1	11818262	2228	0.0452	0.006792	0.0195	0.0190	3.57×10^{-11}	2.02×10^{-3}
CLCN6	RS12567136	1	11806318	2228	0.04501	0.006799	0.01931	0.0189	4.48×10^{-11}	2.48×10^{-3}
CLCN6	RS503040	1	11813282	2229	0.03372	0.005154	0.01886	0.0184	7.49×10^{-11}	> 0.05
CLCN6	RS198401	1	11810971	2227	0.03364	0.005149	0.01882	0.0183	7.94×10^{-11}	> 0.05
CLCN6	RS535107	1	11812055	2230	0.03369	0.005157	0.0188	0.0183	7.95×10^{-11}	> 0.05
CLCN6	RS198406	1	11820179	2230	0.03337	0.005133	0.01862	0.0179	9.77×10^{-11}	> 0.05
CLCN6	RS198408	1	11820717	2224	0.03319	0.005138	0.01843	0.0177	1.28×10^{-10}	> 0.05
CLCN6	RS198391	1	11799004	2228	0.03312	0.005165	0.01814	0.0177	1.74×10^{-10}	> 0.05
CLCN6	RS198393	1	11802272	2230	0.03293	0.005136	0.01811	0.0175	1.76×10^{-10}	> 0.05
CLCN6	RS12406667	1	11796438	2230	0.03297	0.005157	0.01802	0.0176	1.97×10^{-10}	> 0.05
CLCN6	RS198392	1	11801749	2229	0.03291	0.005148	0.01802	0.0174	1.99×10^{-10}	> 0.05

MTHFR	RS6541003	1	11778454	2230	0.03278	0.005164	0.01777	0.0172	2.63×10^{-10}	> 0.05
MTHFR	RS3737966	1	11770346	2229	0.03296	0.005221	0.01759	0.0175	3.28×10^{-10}	> 0.05
NPPB	RS198389	1	11841858	2229	0.0327	0.005204	0.01742	0.0173	3.96×10^{-10}	> 0.05
CLCN6	RS17037425	1	11792970	2230	0.044	0.007033	0.01726	0.0168	4.73×10^{-10}	2.77×10^{-3}
MTHFR	RS4846052	1	11780538	2229	0.03212	0.00515	0.01717	0.0166	5.31×10^{-10}	> 0.05
CLCN6	RS4846054	1	11791817	2229	0.03166	0.005171	0.01656	0.0160	1.08×10^{-9}	> 0.05
NPPA	RS632793	1	11833264	2229	0.03198	0.005249	0.01639	0.0162	1.31×10^{-9}	> 0.05
NPPB	RS198388	1	11839927	2229	0.03166	0.005224	0.01622	0.0162	1.60×10^{-9}	> 0.05
NPPB	RS198375	1	11836344	2230	0.03161	0.005269	0.01589	0.0157	2.32×10^{-9}	> 0.05
NPPB	RS1318408	1	11848368	2229	0.04467	0.007682	0.01495	0.0146	6.95×10^{-9}	8.14×10^{-4}
NPPB	RS12562952	1	11849643	2230	0.0464	0.008024	0.01479	0.0147	8.39×10^{-9}	2.35×10^{-3}
KIAA2013	RS2639453	1	11905131	2229	-0.03421	0.006251	0.01327	0.0276	4.93×10^{-8}	> 0.05
MTHFR	RS4846049	1	11772952	2229	0.02969	0.005436	0.01322	0.0125	5.22×10^{-8}	> 0.05

SNPs were tested using a simple linear regression model executed in PLINK v.1.7 under the assumption of an additive genetic model.

[1] H^2a is locus specific heritability (variance of additive model / variance of phenotype)

[2] P-value for SNP association in MTHFR 677CC homozygous subjects (n = 989).

Supplemental Table 3. SNPs that reached genome-wide significance in association with log-transformed serum folate in 2232 Irish participants ($p < 5 \times 10^{-08}$). Data adjusted for age and gender.

gene	SNP	CHR	BP	N	BETA	SE	R^2	P value	[1]P value 677CC
MTHFR	RS1801133	1	11778965	2230	-0.04932	0.007373	0.01969	2.82×10^{-11}	-
LOC390997	RS7545014	1	11857240	2229	-0.04013	0.006977	0.01464	1.01×10^{-8}	1.35×10^{-4}
LOC390997	RS7554327	1	11859991	2230	-0.03998	0.006987	0.01448	1.20×10^{-8}	1.07×10^{-4}
RNU5E	RS1999594	1	11881803	2230	0.03981	0.006996	0.01433	1.43×10^{-8}	7.74×10^{-3}
RNU5E	RS12085006	1	11881310	2229	0.03964	0.007004	0.01418	1.71×10^{-8}	7.52×10^{-3}

SNPs were tested using a simple linear regression model executed in PLINK v.1.7 under the assumption of an additive genetic model.

[1] P-value for SNP association in MTHFR 677CC homozygous subjects (n = 989).

Supplemental Table 4. SNPs that reached genome-wide significance in association with log-transformed plasma total homocysteine in 2232 Irish participants ($p < 5 \times 10^{-08}$). Data adjusted for age and gender.

gene	SNP	CHR	BP	N	BETA	SE	R^2	[1]H2a	P value	[2]P value 677CC
MTHFR	RS1801133	1	11778965	2229	0.03087	0.003374	0.03623	0.0353	1.26×10^{-19}	-
RNU5E	RS1999594	1	11881803	2229	-0.02195	0.003217	0.02048	0.0173	1.14×10^{-11}	> 0.05
RNU5E	RS12085006	1	11881310	2228	-0.02186	0.003221	0.02028	0.0172	1.45×10^{-11}	> 0.05
RNU5E	RS12724129	1	11880226	2229	-0.0197	0.003292	0.01583	0.0114	2.51×10^{-9}	> 0.05
MFN2	RS4240897	1	11965342	2229	-0.01945	0.003287	0.01548	0.0124	3.76×10^{-9}	> 0.05
TWISTNB	RS17354370	7	19566392	2230	-0.02096	0.003546	0.01544	0.0134	3.93×10^{-9}	.0047
LOC390997	RS2050267	1	11869160	2227	-0.01981	0.003356	0.01541	0.0077	4.14×10^{-9}	> 0.05
LOC390997	RS4846069	1	11871618	2220	-0.01973	0.00336	0.01531	0.0082	4.95×10^{-9}	> 0.05
LOC390997	RS2050270	1	11869043	2230	-0.01968	0.003354	0.01522	0.0076	5.08×10^{-9}	> 0.05
TWISTNB	RS2024353	7	19545401	2230	-0.02142	0.003651	0.01522	0.0102	5.09×10^{-9}	.011
LOC390997	RS2050268	1	11869131	2230	-0.01968	0.003355	0.01521	0.0076	5.14×10^{-9}	> 0.05
MFN2	RS730123	1	11992879	2230	0.01915	0.003298	0.0149	0.0148	7.32×10^{-9}	> 0.05
LOC390997	RS2336377	1	11872557	2222	0.0187	0.003251	0.01469	0.0142	1.00×10^{-8}	> 0.05
LOC390997	RS1321072	1	11873905	2229	-0.0193	0.003357	0.01463	0.0083	1.02×10^{-8}	> 0.05
TWISTNB	RS4721780	7	19515433	2229	-0.02012	0.00351	0.01453	0.0101	1.13×10^{-8}	.029
LOC390997	RS12749498	1	11875214	2230	-0.01913	0.003354	0.01438	0.0080	1.34×10^{-8}	> 0.05
LOC390997	RS1321073	1	11874213	2230	-0.01913	0.003354	0.01438	0.0080	1.34×10^{-8}	> 0.05
LOC390997	RS17037520	1	11874993	2230	-0.01913	0.003354	0.01438	0.0080	1.34×10^{-8}	> 0.05
LOC390997	RS2065611	1	11876113	2230	-0.01913	0.003354	0.01438	0.0080	1.34×10^{-8}	> 0.05

LOC390997	RS4845886	1	11871247	2230	-0.01913	0.003354	0.01438	0.0080	1.34×10^{-8}	> 0.05
LOC390997	RS12036266	1	11875901	2229	-0.0191	0.003355	0.01434	0.0080	1.41×10^{-8}	> 0.05
RNU5E	RS12756891	1	11879071	2226	-0.01911	0.003358	0.01435	0.0080	1.44×10^{-8}	> 0.05
RNU5E	RS4846076	1	11879352	2219	-0.01916	0.003368	0.01439	0.0080	1.45×10^{-8}	> 0.05
MFN2	RS3753579	1	11961178	2229	-0.01895	0.003386	0.01387	0.0073	2.45×10^{-8}	> 0.05
TWISTNB	RS10245919	7	19516175	2224	-0.01919	0.003448	0.01375	0.0102	2.93×10^{-8}	.030
LOC390997	RS4314892	1	11872424	2186	-0.01866	0.00338	0.01376	0.0075	3.81×10^{-8}	> 0.05
CHMP1A	RS164746	16	88236520	2228	0.01776	0.003219	0.01349	0.0131	3.85×10^{-8}	5.76×10^{-5}
CHMP1A/DPEP1	RS154657	16	88235597	2230	0.01774	0.003217	0.01346	0.0131	3.91×10^{-8}	5.49×10^{-5}
RNU5E	RS10779767	1	11886826	2202	-0.01948	0.003546	0.01354	0.0059	4.38×10^{-8}	> 0.05

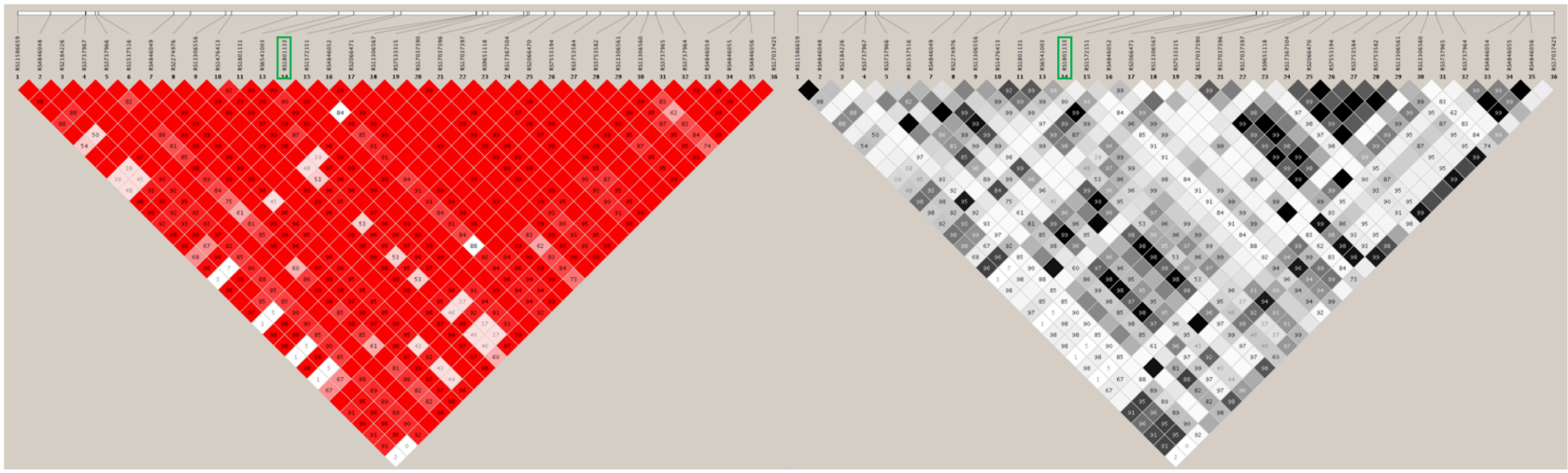
SNPs were tested using a simple linear regression model executed in PLINK v.1.7 under the assumption of an additive genetic model.

[1] H^2a is locus specific heritability (variance of additive model / variance of phenotype)

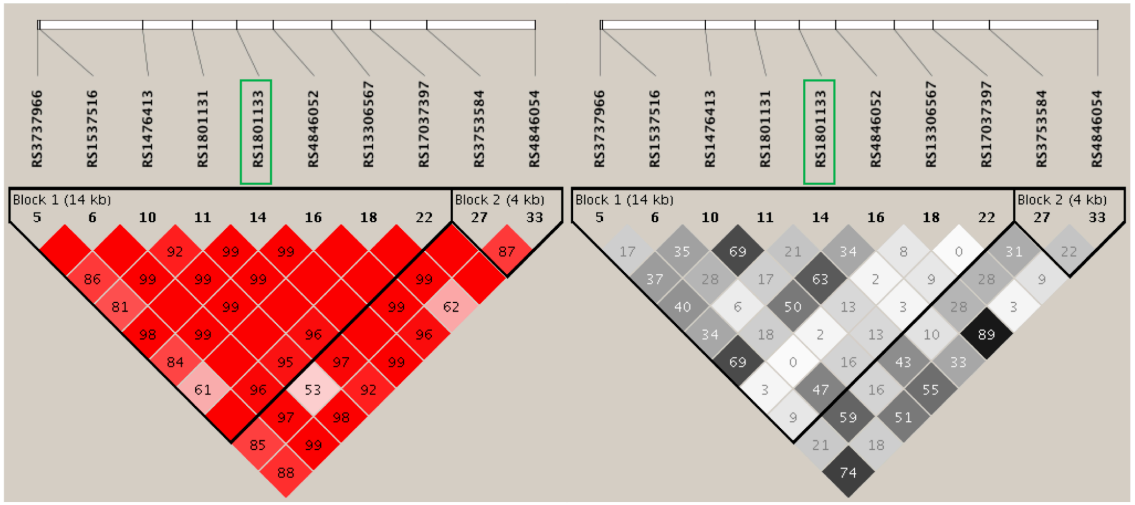
[2] P-value for SNP association in MTHFR 677CC homozygous subjects (n = 989).

Online Supporting Material

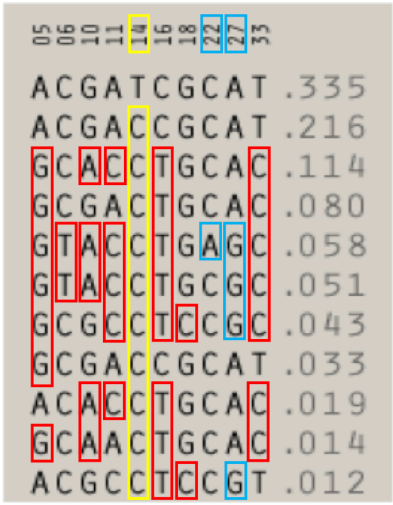
A



B



C



Supplemental Figure 2. Linkage disequilibrium (LD) in the *MTHFR* gene region. **A**) D' (left) and r^2 (right) plots of pairwise LD measures are shown for 35 SNPs in *MTHFR* and its 5kb flanks. SNPs are numbered sequentially from left to right, with the SNP IDs shown above. *MTHFR* 677C>T (rs1801133) is boxed in green in each plot. A subset of 16 tagSNPs were selected for association analyses. **B**) D' (left) and r^2 (right) plots of pairwise LD measures are shown for 10 SNPs in *MTHFR* that are associated with red cell folate (RCF) after correction for multiple tests (Table 2). SNPs numbering is preserved from (A), and the SNP IDs are shown above. The *MTHFR* 677C>T rs1801133 label is boxed in green in each plot. Adjacent to it is MTHFR 1298 A>C (rs1801131, SNP 11). **C**) Haplotypes and their frequencies (right column) are shown for the 10 SNPs in (B) (numbering and SNP IDs are preserved). Alleles that are associated with higher levels of RCF are boxed in color: yellow, *MTHFR* 677C>T (rs1801133); red, SNP alleles that are not associated with changes in RCF in *MTHFR* 677CC homozygotes; blue, SNP alleles that are associated with increased RCF in *MTHFR* 677CC homozygotes.