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The reduced folate carrier (*SLC19A1*) c.80G>A polymorphism is associated with red cell folate concentrations among women

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Summary

Low folate status may be a consequence of suboptimal intake, transport or cellular utilization of folate and, together with elevated homocysteine, is a recognized risk factor/marker for several human pathologies. As folate transport across cell membranes is mediated in part by the reduced folate carrier (RFC1), variants within this gene may influence disease risk via an effect on folate and/or homocysteine levels. The present study was undertaken to assess the association between the *SLC19A1* (*RFC1*) c.80G>A polymorphism and folate/homocysteine concentrations in healthy young adults from Northern Ireland.

The *SLC19A1* c.80G>A polymorphism was not strongly associated with either serum folate or homocysteine concentrations in either men or women. However, in women, but not in men, this polymorphism explained 5% of the variation in red blood cell (RBC) folate levels (P=0.02). Relative to women with the *SLC19A1* c.80GG genotype, women with the GA and AA genotypes had higher RBC folate concentrations. Consequently, compared to women with the *SLC19A1* c.80AA and GA genotypes, women who are homozygous for the 80G allele may be at increased risk of having a child affected with a neural tube defect and of developing pathologies that have been associated with folate insufficiency, such as cardiovascular disease.

Keywords

Reduced folate carrier; folate; homocysteine; *RFC1*; *SLC19A1*; SNP

Introduction

Folate/homocysteine metabolism supports several important biological processes including nucleic acid synthesis and the methylation of a variety of substrates (eg. proteins, DNA and lipids). A low folate, high homocysteine phenotype is a risk factor for, or a marker of, many

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human pathologies including spina bifida and cardiovascular disease (Lucock, 2000). Any impairment in folate bioavailability as a consequence of intake, transport or cellular utilization might contribute to the pathogenesis of diseases that have been associated with low folate status.

Folate transport is facilitated by the reduced folate carrier (MIM:600424; solute carrier family 19, gene names: *SLC19A1, RFC1*), organic anion carriers, folate receptors alpha and beta, and multidrug resistance-associated proteins (Matherly & Goldman, 2003). RFC1 is a high capacity, bi-directional transporter of 5-methyl-tetrahydrofolate (5-methylTHF) (Figure 1) and can also transport folic acid, albeit with lower capacity; it also facilitates transport of the antifolate drug methotrexate (MTX) (Matherly & Goldman, 2003). The *SLC19A1* gene is polymorphic inhumans (Chango *et al.*, 2000; Whetstine *et al.*, 2002; http://snp500cancer.nci.nih.gov). The most extensively studied variant of the *SLC19A1* gene is a single nucleotide polymorphism 80G>A in the coding region (*SLC19A1* c.80G>A; rs61510559; often referred to as *RFC1* 80A>G), which results in the substitution of an arginine with a histidine at residue 27 in the amino acid sequence.

The relationship between the *SLC19A1* c.80G>A polymorphism and folate/homocysteine metabolism is unclear. Several groups have reported a lack of association with red blood cell (RBC) folate (Vesela *et al.*, 2005; Chango *et al.*, 2000), plasma folate (Winkelmayer *et al.*, 2003; Chango *et al.*, 2000; Devlin *et al.*, 2006) and homocysteine (Winkelmayer *et al.*, 2003; Yates & Lucock, 2005; Devlin *et al.*, 2006). However, some studies have shown non-significant trends between the *SLC19A1* c.80G>A polymorphism and RBC folate levels (Morin *et al.*, 2003; Yates & Lucock, 2005), and among patients with thrombotic vascular disease increased plasma folate concentrations were observed in *SLC19A1* c.80AA homozygotes (Yates & Lucock, 2005). In contrast, the largest study to date (N>10 000) identified a borderline significant decrease in serum folate levels in individuals with genotypes that included the *SLC19A1* c.80A alleles; however, the relationship between the *SLC19A1* genotype and RBC folate levels was not reported for that study (Fredriksen *et al.*, 2007). Interestingly, two studies have provided evidence that the effect of the *SLC19A1* c.80G>A variant may be influenced by the *MTHFR* g.677C>T genotype (*MTHFR* c.665C>T; rs1801133; often referred to as *MTHFR* 677C>T). Specifically, when *SLC19A1* c.80G>A genotype was considered in the context of *MTHFR* g.677C>T genotype, doubly homozygous *MTHFR* g.677TT - *SLC19A1* c.80GG individuals had elevated homocysteine (Chango *et al.*, 2000; Devlin *et al.*, 2006), relative to those with other *MTHFR*/*SLC19A1* genotype combinations.

Taken together, the above reports suggest that further investigation of the relationship between the *SLC19A1* c.80G>A polymorphism and folate/homocysteine phenotype is warranted. The aim of this study was to investigate the relationship between the *SLC19A1* c.80G>A genotype and RBC folate, serum folate and homocysteine concentrations in young, reproductive age adults.

Materials and Methods

Study population

The Young Hearts Project (YH) is an ongoing longitudinal study designed to monitor cardiovascular disease risk factors in children and young adults living in Northern Ireland (Boreham *et al.*, 1993). Briefly, a sample of 12 year old (n=509) and 15 year old (n=506) boys and girls were enrolled from post-primary schools in Northern Ireland between 1989 and 1990 (YH1). Between October 1997 and October 1999, all YH subjects were invited to participate in YH3, a hospital-based screening evaluation (Gallagher *et al.*, 2002). The participation rate for YH3, which was conducted when the subjects were between 20 and 26 years of age, was 48.2% (n=489). Compared to non-respondents, YH3 subjects tended to be from families with

higher socioeconomic status and to have had lower body mass indices at the baseline YH1 examination. In addition, male YH3 subjects were leaner and reported lower saturated fat intake at baseline relative to the male non-participants (Boreham *et al.*, 2004). Ethical approval for each phase of the study was granted by the Research Ethics Committee of Queen's University Belfast. The current paper is based on self-reported data on smoking status, use of alcohol and multivitamin supplements, and fasting blood samples collected as part of YH3.

Laboratory Methods

Blood samples were collected from fasted subjects for the determination of biochemical parameters and for DNA extraction (Miller *et al.*, 1988). Homocysteine concentrations were measured by an established high performance liquid chromatography method (Ubbink *et al.*, 1991). Serum folate concentrations were determined by time-resolved immunofluorescence on an AutoDelfia analyzer (Wallac, UK), and RBC folate concentrations were determined by a microbiological assay as previously described (Molloy & Scott, 1997), and are expressed as nanomoles per liter of packed RBCs.

SLC19A1 c.80A>G genotypes were determined by a modification of a published method (Skibola *et al.*, 2004) using TaqMan 5' Nuclease Real-Time PCR assay on a PTC-200 DNA Engine (Bio-Rad, Hercules, CA) with fluorescence detection by a Chromo4 Real-Time PCR Detector (Bio-Rad). Individual PCR amplification reactions (20 µl) were composed of 2 µl sample DNA, 1x TaqMan Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA), 0.5 µM forward primer (5'-GGCCTGACCCCGAGCT-3') and 0.5 µM reverse primer (5'-AGCCGTAGAAGCAAAGGTAGCA-3'), 100 nM "G"-specific probe (VIC-CACGAGGCGCCGC), and 50 nM "A"-specific probe (6FAM-CGAGGTGCCGCCAG). The probes were synthesized by Applied Biosystems. PCR was performed with an initial incubation at 95°C for 10 min followed by 60 cycles of denaturation at 95°C for 30 sec and extension/5' nuclease step at 63°C for 1 min. Dual fluorescence was detected after each completed 70 sec cycle. Genotypes were assigned using Opticon Monitor 3 analysis software (Bio-Rad). *MTHFR* g.677C>T genotypes have previously been reported (Kluijtmans *et al.*, 2003).

Statistical methods

Descriptive analyses of the study variables were conducted using data from all study subjects, and included medians and percentiles for continuous variables (i.e. RBC folate, serum folate and homocysteine) and proportions for categorical variables (i.e. smoking status, use of alcohol and multivitamin supplements, and genotypes). Deviations from Hardy-Weinberg equilibrium for the *SLC19A1* c.80G>A and *MTHFR* g.677C>T genotypes were assessed by χ^2 analysis.

Simple and multiple linear regression analyses were conducted using log-transformed RBC folate, serum folate or homocysteine values as the main outcome measures. These analyses were restricted to the subset of YH3 study participants for whom there was complete data for all of the variables in any set of regression analyses. Simple linear regression models were fitted to the data and the coefficient of determination (R^2) estimated from these models was used to assess the proportion of variation in the outcome variable that was explained by each predictor variable. *SLC19A1* c.80G>A genotypes were coded using two dummy variables, one reflecting the comparison of the GG and GA genotypes and the other the comparison of the GG and AA genotypes. Each potential behavioral risk factor (i.e. use of multivitamins, cigarettes or alcohol) was coded as a dichotomous (i.e. yes/no) variable. Multiple regression models included predictor variables as well as two-way interaction terms. Interactions were coded as the product of a dichotomous behavioral variable and the dummy variables defining genotype. Hence, two interaction terms were included in each of the models that allowed for interactions. Nested models were compared to determine the change in the proportion of

variation in the outcome variable explained by the addition of a single predictor variable or interaction term to the model. Specifically, the difference in the adjusted R^2 values for a model with and a model without a given variable was calculated. The significance of individual predictor variables within a model was assessed using the t-statistic, and P-values<0.05 were considered to be statistically significant. All analyses were conducted using SAS version 9.1.

Results

The characteristics of YH3 study subjects are summarized, for the whole population and separately by sex, in Table 1. *SLC19A1* c.80G>A and *MTHFR* g.677C>T genotypes in the population as a whole, and in the male and female subsets, were in Hardy-Weinberg equilibrium. Data from two study subjects, each of whom had an extreme outlying value for one of the biochemical measurements made in the YH3 samples (i.e. serum B_{12} = 1230 pmol/l and serum folate = 213 nmol/l), were excluded from all analyses.

In the full study sample, *SLC19A1* c.80G>A genotype was not a strong predictor of either serum folate (R^2=0.004) or homocysteine concentrations and (R^2=0.003), and was only modestly associated with RBC folate concentrations (R^2=0.02, *P*=0.04) (Table 2). The median RBC folate concentrations of those with the *SLC19A1* c.80AA, GA and GG genotypes were 699.8, 671.9 and 594.9 nmol/l RBCs, respectively.

As males and females differ with respect to several of the biochemical and lifestyle variables (Table 1) of interest in this study, further analyses were performed separately for males and females. Median values of the biochemical variables, by *SLC19A1* c.80G>A genotype and sex, are provided in Table 3. The *SLC19A1* c.80G>A genotype was not strongly associated with serum folate or homocysteine concentrations in either males or females (Table 2). Among females, RBC folate levels increased with the number of *SLC19A1* c.80A alleles (Table 3), and the *SLC19A1* c.80G>A genotype explained approximately 5% of the variation in RBC folate (*P*=0.02) (Table 2), Although RBC folate levels in males also increased with the number of *SLC19A1* c.80A alleles (Table 3), *SLC19A1* c.80G>A genotype explained a relatively small proportion of the variation (~1%) in RBC folates among males (Table 2).

Multivariate regression analyses were performed to estimate the proportion of RBC folate variation attributable to *SLC19A1* c.80G>A genotype in the context of *MTHFR* g.677C>T genotype, an established determinant of folate/homocysteine phenotype (Jacques *et al.*, 1996; Harmon *et al.*, 1996; Kluijtmans *et al.*, 2003). Analyses to assess potential interactions between the *SLC19A1* c.80G>A and *MTHFR* g.677C>T genotypes were not undertaken due to the small number of subjects in several of the combined genotype categories. Accounting for the effects of the *MTHFR* g.677C>T variant, the *SLC19A1* c.80G>A genotype accounted for approximately 3% of the variation in RBC folate levels in females, but less than 1% of the variation in RBC folate levels in males (Table 4). Further, among females, the *SLC19A1* c.80G>A genotype accounted for a higher proportion of the variation in RBC folate than did the *MTHFR* g.677C>T variant (i.e. *MTHFR* g.677C>T genotype accounted for ~1% of the variation in RBC folate after accounting for the effects of the *SLC19A1* c.80G>A genotype). No interactions between the *SLC19A1* c.80G>A genotype and smoking, alcohol, or multivitamin use was observed in either males or females (data not presented), although it should be noted that firm conclusions are precluded by the small number of observations in several of the categories.

Discussion

The data presented here indicate that in healthy young Northern Irish women the *SLC19A1* c.80G>A polymorphism is significantly associated with RBC folate concentrations, with higher

levels observed in women with genotypes including the A allele. While a similar trend was observed in men, this variant explained a smaller proportion of the variation in RBC folate in males as compared to females. The observed association between the *SLC19A1* c.80A allele and relatively high RBC folate levels in women is consistent with the trend observed by Morin *et al.* (2003).

Neither in our study nor in those reported by others is there any apparent impact of the *SLC19A1* c.80G>A polymorphism on homocysteine (Winkelmayer *et al.*, 2003; Yates & Lucock, 2005; Devlin *et al.*, 2006). Furthermore, our study provided no evidence that this polymorphism significantly influences variation in serum folate concentrations and in this respect it is similar to several other studies (Winkelmayer *et al.*, 2003; Devlin *et al.*, 2006; Chango *et al.*, 2000), a notable exception being the very large (N= 10 601) study of Fredriksen *et al.* (2007) in which there was a borderline significant effect on serum folate.

Folic acid supplements prevent up to 70% of NTDs (MRC Vitamin Research Group, 1991; Czeizel & Dudas, 1992) and women carrying fetuses with spina bifida have low folate and high homocysteine concentrations (Mills *et al.*, 1995; Kirke *et al.*, 2004). The important role of RFC1 in folate transport has prompted several groups to evaluate the *SLC19A1* c.80G>A polymorphism as a risk factor. Some groups have reported that the *SLC19A1* c.80GG genotype is a risk factor for spina bifida (Shaw *et al.*, 2002; De Marco *et al.*, 2003; Pei *et al.*, 2005; Morin *et al.*, 2003), whilst others have found no evidence to support any such association (Relton *et al.*, 2004; O'Leary V *et al.*, 2006; Vieira *et al.*, 2005). A single report has suggested that the *SLC19A1* 12 c.80AA genotype is a maternal risk factor for anencephaly, but not for spina bifida (Relton *et al.*, 2003). Our observations are biologically consistent with the former reports.

Our findings may also have relevance to therapies involving the anti-folate drug methotrexate, which is transported to cells by RFC1. Others have reported that methotrexate concentrations (Laverdiere *et al.*, 2002; Dervieux *et al.*, 2004) are higher in patients with the *SLC19A1* c.80AA genotype, suggesting that it might be possible to individually tailor dosing strategies by taking *SLC19A1* c.80G>A genotype and sex into consideration.

In conclusion, we have demonstrated that the *SLC19A1* c.80GG genotype is associated with relatively low folate concentrations in Northern Irish women. As a maternal low folate/high homocysteine phenotype is associated with increased risk of neural tube defects (NTDs) in offspring (Mills *et al.*, 1995; Kirke *et al.*, 2004), women with the *SLC19A1* c.80GG genotype may have an increased risk of having a child affected by an NTD relative to those with the GA and AA genotypes. In addition, *SLC19A1* c.80GG homozygous women may be at increased risk of a range of other major pathologies, including cardiovascular disease, in which a low folate/high homocysteine phenotype is a predisposing feature.

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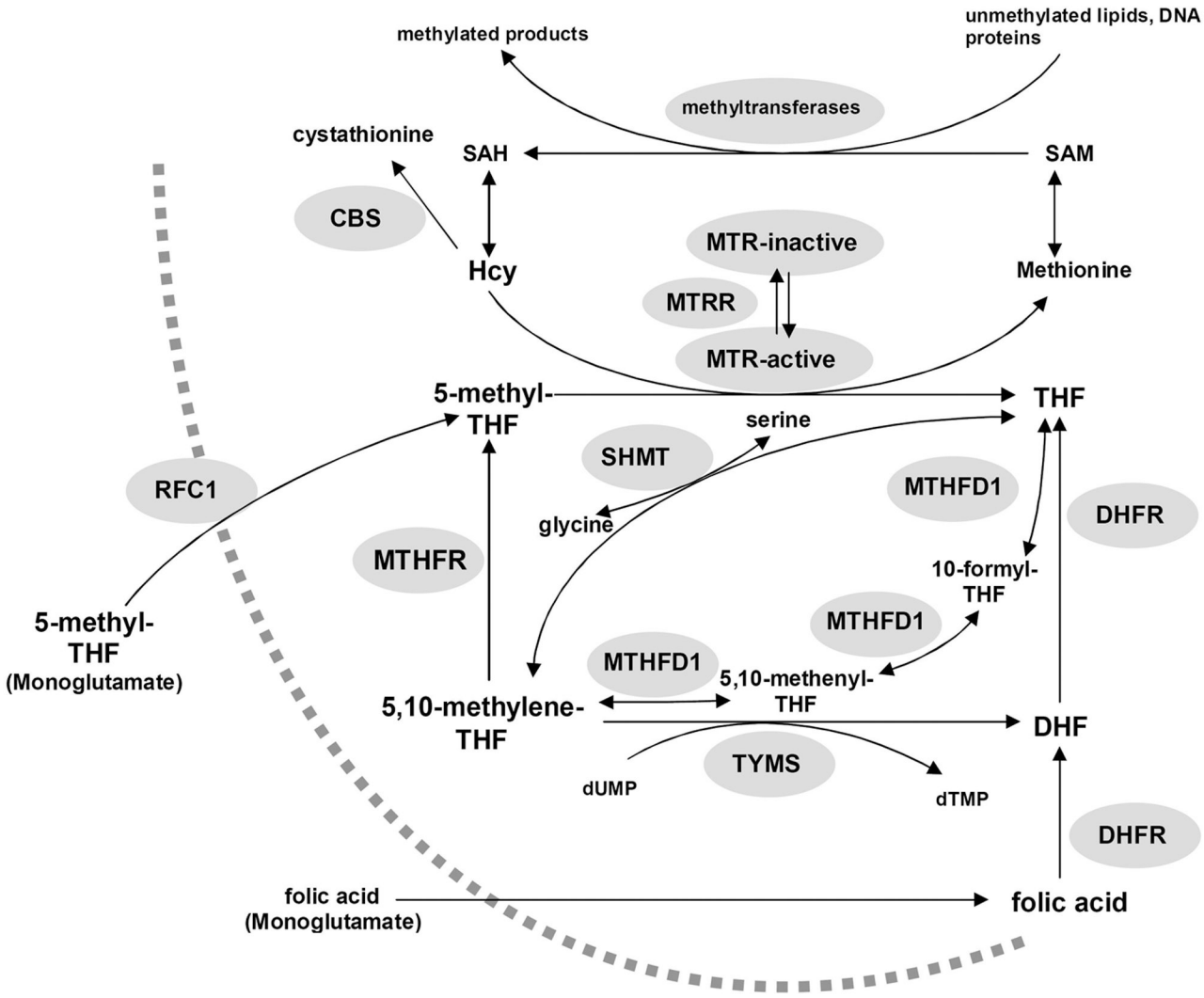

Figure 1.

Schematic representation of homocysteine/folate metabolism. CBS, cystathionine β-synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP deoxyuridine monophosphate; Hcy homocysteine; MTHFD1, methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolaseformyltetrahydrofolate synthetase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; RFC1, Reduced folate carrier; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, Serine hydroxymethyltransferase; THF, tetrahydrofolate; TYMS, thymidylate synthase.

Table 1

Characteristics of Participants in the Young Hearts 3 Study.

Variable	All (412)*	Males (N=225)	Females (N=186)
Biochemical Variables			
Homocysteine (μmol/l)			
N	401	220	180
Median (25^{th}–75^{th} percentile)	8.9 (7.5–10.9)	9.2 (7.7–11.0)	8.6 (7.4–11.0)
RBC folate (nmol/l)			
N	364	193	170
Median (25^{th}–75^{th} percentile)	644.1 (479.0–844.8)	704.8 (543.6–888.7)	562.9 (428.7–764.7)
Serum folate (nmol/l)			
N	352	192	159
Median (25^{th}–75^{th} percentile)	12.8 (9.6–18.9)	12.4 (9.4–18.9)	13.2 (9.6–19.1)
Lifestyle Variables			
Current use of cigarettes (N, %)			
Yes	156 (38.1)	84 (37.7)	72 (38.7)
No	253 (61.9)	139 (62.3)	114 (61.3)
Current use of alcohol (N, %)			
Yes	336 (81.8)	192 (85.3)	144 (77.4)
No	75 (18.3)	33 (14.7)	42 (22.6)
Current use of multivitamin supplements (N, %)			
Yes	93 (22.6)	44 (19.6)	49 (26.3)
No	318 (77.4)	181 (80.4)	137 (73.7)
Genotypes			
SLC19A1 c.80G>A (N, %)			
GG	122 (29.6)	67 (29.8)	55 (29.6)
GA	219 (53.2)	123 (54.7)	95 (51.1)
AA	71 (17.2)	35 (15.6)	36 (19.4)
MTHFR g.677C>T (N, %)			
CC	178 (43.5)	99 (44.4)	78 (42.2)
CT	176 (43.0)	100 (44.8)	76 (41.1)
TT	55 (13.4)	24 (10.8)	31 (16.8)

*Information on sex was not available for one subject.

Table 2

Proportion of the variation (R^2) in RBC folate, serum folate and homocysteine concentrations explained by SLC19A1 c.80G>A genotypes in YH3 study participants.

		All			Males			Females		
		R^2 (P-value)[1]	Variable Coefficient (SE)[2]	N	R^2 (P-value)[1]	Variable Coefficient (SE)[2]	N	R^2 (P-value)[1]	Variable Coefficient (SE)[2]	N
RBC Folate										
SLC19A1 c.80G>A		0.02 (0.04)		363	0.01 (0.43)		192	0.05 (0.02)		169
	GA		0.05 (0.02)			0.003 (0.03)			0.09 (0.03)	
	AA		0.07 (0.03)			0.05 (0.04)			0.10 (0.04)	
Serum Folate										
SLC19A1 c.80G>A		0.004 (0.54)		350	0.001 (0.95)		191	0.01 (0.32)		158
	GA		0.02 (0.03)			−0.01 (0.03)			0.06 (0.04)	
	AA		0.04 (0.04)			−0.10 (0.05)			0.06 (0.05)	
Homocysteine										
SLC19A1 c.80G>A		0.003 (0.53)		400	0.01 (0.50)		219	0.001 (0.89)		179
	GA		−0.02 (0.02)			−0.03 (0.02)			−0.01 (0.03)	
	AA		−0.02 (0.02)			−0.03 (0.03)			−0.003 (0.03)	

[1] P-value for test of hypothesis of no linear association between the predictor variable and RBC folate, serum folate or homocysteine.

[2] Estimated regression coefficients and standard errors. Variable coefficients estimate the change in RBC folate, serum folate or homocysteine concentration per unit change in the predictor variable.

Table 3

Median RBC folate, serum folate and homocysteine values in subgroups of subjects defined by sex and RFC1 80G>A genotype.

Biochemical Phenotype	*RFC*1 80G>A genotype[1]					
	Males			Females		
	GG	GA	AA	GG	GA	AA
RBC folate (nmol/l)	658.6 (55)	715.5 (109)	739.5 (29)	481.1 (51)	586.2 (85)	624.1 (34)
Serum Folate (nmol/l)	12.4 (59)	12.4 (105)	12.9 (28)	12.1 (49)	13.7 (81)	13.5 (29)
Homocysteine (μmol/l)	9.2 (65)	8.9 (120)	9.3 (35)	9.0 (54)	8.5 (90)	9.5 (36)

[1]The number given in parentheses is the number of individuals.

Table 4

Summary of logistic regression modeling of log-RBC folate in Young Hearts 3 study participants.

Model[1]	Predictor Variables	Males			Females		
		Variable Coefficient (SE)	Adjusted R^2	Change in Adjusted R^2	Variable Coefficient (SE)	Adjusted R^2	Change in Adjusted R^2
RBC folate							
1	*SLC19A1* c.80G>A		−0.0049	**0.0734**		0.0365	0.0143
	GA	0.0002 (0.03)			0.09 (0.03)		
	AA	0.04 (0.04)			0.10 (0.04)		
2	*MTHFR* g.677C>T		0.0706	−0.0021		0.0175	**0.0333**
	CT	−0.06 (0.02)			−0.04 (0.03)		
	TT	−0.16 (0.04)			−0.09 (0.04)		
3	*MTHFR* g.677C>T +*SLC19A1* c.80G>A		0.0685			0.0508	
	CT	−0.06 (0.02)			−0.04 (0.03)		
	TT	−0.16 (0.04)			−0.09 (0.04)		
	GA	0.008 (0.03)			0.09 (0.03)		
	AA	0.05 (0.04)			0.09 (0.04)		

[1] Change in R^2 was assessed relative to model 3.