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# Gender and Single Nucleotide Polymorphisms in *MTHFR, BHMT, SPTLC1, CRBP2, CETP,* and *SCARB1* Are Significant Predictors of Plasma Homocysteine Normalized by RBC Folate in Healthy Adults[1–3]

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## Abstract

Using linear regression models, we studied the main and 2-way interaction effects of the predictor variables gender, age, BMI, and 64 folate/vitamin B-12/homocysteine (Hcy)/lipid/cholesterol-related single nucleotide polymorphisms (SNP) on log-transformed plasma Hcy normalized by RBC folate measurements (nHcy) in 373 healthy Caucasian adults (50% women). Variable selection was conducted by stepwise Akaike information criterion or least angle regression and both methods led to the same final model. Significant predictors (where *P* values were adjusted for false discovery rate) included type of blood sample [whole blood (WB) vs. plasma-depleted WB; $P < 0.001$] used for folate analysis, gender ($P < 0.001$), and SNP in genes *SPTLC1* (rs11790991; $P = 0.040$), *CRBP2* (rs2118981; $P < 0.001$), *BHMT* (rs3733890; $P = 0.019$), and *CETP* (rs5882; $P = 0.017$). Significant 2-way interaction effects included gender × *MTHFR* (rs1801131; $P = 0.012$), gender × *CRBP2* (rs2118981; $P = 0.011$), and gender × *SCARB1* (rs83882; $P = 0.003$). The relation of nHcy concentrations with the significant SNP (*SPTLC1, BHMT, CETP, CRBP2, MTHFR,* and *SCARB1*) is of interest, especially because we surveyed the main and interaction effects in healthy adults, but it is an important area for future study. As discussed, understanding Hcy and genetic regulation is important, because Hcy may be related to inflammation, obesity, cardiovascular disease, and diabetes mellitus. We conclude that gender and SNP significantly affect nHcy. J. Nutr. 142: 1764–1771, 2012.

## Introduction

Folate functions in 1-carbon metabolism and is an essential nutrient. Adequate folate intake is associated with a lowering of plasma homocysteine (Hcy)[13], a risk factor for vascular disease (1), dementia (2), and hypertension (3). The demonstration that folate-marginal or folate-deficient nutritional status was also associated with a high incidence of developmental disorders, including neural tube defects (NTD) (4,5), prompted the FDA to mandate that most grain and cereal products in the United States be fortified with folic acid (6). Implementation of this fortification mandate (6) led to a reduction in the number of NTD-affected births (7) and to an increase in serum and RBC folate concentrations as well as to a reduction in plasma Hcy concentrations (8).

The discovery that the C677T single nucleotide polymorphism (SNP) in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene (9) was also a risk factor for NTD (10) led others to focus on additional SNPs in the genes of folate- and vitamin B-12–relevant enzymes and carriers: *MTHFR* rs1801133, *MTHFR* rs1801131, methionine synthase (*MTR*) rs1805087, methionine synthase reductase (*MTRR*) rs1801394, transcobalamin II (*TCN2*) rs1801198, reduced folate carrier (*SLC19A1*) rs1051266, and betaine hydroxymethyltransferase (*BHMT*) rs3733890. Since the identification of significant SNPs, the investigation of genetic variation of folate metabolism, transport,

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[3] Supplemental Tables 1–4 and Figures 1–3 are 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.
[13] Abbreviations used: AIC, Akaike information criterion; CVD, cardiovascular disease; DM, diabetes mellitus; dSL, deoxysphingolipid; Hcy, homocysteine; LARS, least angle regression; LASSO, least absolute shrinkage and selection operator; LC-MS/MS, liquid chromatography tandem MS; nHcy, normalized homocysteine; NTD, neural tube defect; PDC, plasma deplete cell; QC, quality control; RA, retinoic acid; SNP, single nucleotide polymorphism; WB, whole blood.
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and regulation of Hcy has been and continues to be of considerable interest. Recently, a genome-wide association study was completed in healthy study populations to investigate which of 484,115 autosomal SNPs may be associated with differential concentrations of folate, vitamins B-12 and B-6, and Hcy. The authors identified SNPs in genes *ALPL* (vitamin B-6), *FUT2* (vitamin B-12), and *MTHFR* (11). However, the main and 2-way interaction effects of these and many other, and as-yet-unidentified, SNPs on in vivo levels of RBC folate or plasma Hcy are not well known despite recent quantitation of in vivo folate metabolism (12).

Therefore, we tested the hypothesis that the main and interaction effects of individual traits of gender, age, BMI, and 64 genotypes of SNP (**Table 1**) on plasma Hcy normalized by RBC folate (13) (nHcy) were significant in a healthy Caucasian adult population consuming a folic acid-fortified diet. Plasma Hcy is an important indicator of potential health outcomes; the purpose of normalizing the Hcy concentration by RBC folate was to account for the high amounts of folate consumed in the American diet while still understanding the potential effects of age, gender, BMI, and 64 relevant SNPs on Hcy levels. Prioritizing the main and 2-way interaction effects of the individual traits on plasma nHcy status would suggest a strategy for minimizing Hcy effects on health outcomes.

## Participants and Methods

*Participant enrollment.* The Johns Hopkins University Bloomberg School of Public Health Committee on Human Research approved the study procedures and protocol, which was in accord with Good Clinical Practice guidelines and the Declaration of Helsinki, version 1989. The population consisted of 510 adults (257 females, 253 males) aged 30–69 y residing in the greater Washington, DC area. Participants were recruited to participate in a study of dietary recall between July 2002 and June 2004 (14) and were then included in the present study as an adjunct to the main study. Potential participants were recruited through email, advertisements in local newspapers, and announcements on USDA Agricultural Research Service websites.

**TABLE 1** Information regarding the 64 SNPs included in the analysis that passed the missing-ness criteria[1]

| Name | Gene, function | Chromosome | dbSNP Identifier | $n^2$ |
|---|---|---|---|---|
| apoA1 | *APOA1*, protein component of HDL, promotes cholesterol efflux from tissues to liver | 11 | rs2727784 | 1 |
| apoA-V | *APOA5*, protein component of HDL, regulates plasma TG | 11 | rs3135506, rs662799, rs2727784 | 3 |
| ATP-binding cassette, subfamilyA, member 1 | *ABCA1*, transport AA, sugars, vitamins, cholesterol, pt-choline to lipid acceptor apoA1 | 9 | rs2230806, rs2230808, rs4149267, rs4149327 | 4 |
| $\beta$-Carotene 15,15'-monooxygenase 1 | *BCMO1*, cleave $\beta$-Carotene | 16 | rs12934922, rs6564851, rs7501331 | 3 |
| $\beta$-Carotene oxygenase 2 | *BCMO2*, cleave $\beta$-Carotene | 11 | rs11214139, rs2250417, rs35361223 | 3 |
| Betaine-homocysteine *S*-methyltransferase | *BHMT*, methylate Hcy | 5 | rs3733890 | 1 |
| Cholesteryl ester transfer protein, plasma | *CETP*, transfer cholesterol | 16 | rs12708980, rs5880, rs5882, rs7205804, rs7499892 | 5 |
| Cystathionine $\beta$-synthase | *CBS*, synthesize cystathionine | 21 | rs5742905 | 1 |
| Cytochrome P450, family 4, subfamily F, polypeptide 2 | *CYP4F2*, involved in cholesterol synthesis | 19 | rs3093156, rs2108622, rs3093168, rs3093194 | 4 |
| Folate hydrolate (prostatespecific membrane antigen) 1 | *FOLH1 (GCPII)*,hydrolyze folate | 11 | rs61886492 | 1 |
| Methionine synthase reductase | *MTRR*, remethylate Hcy | 5 | rs1801394 | 1 |
| Methylenetetrahydro-folate reductase | *MTHFR*, distribute one-carbon units | 1 | rs1801131, rs1801133 | 2 |
| 5-Methyltetra-hydrofolate-homocysteine methyltransferase | *MTR*, remethylate Hcy | 1 | rs1805087 | 1 |
| Microsomal triglyceride transfer protein | *MTTP*, lipoprotein assembly | 4 | rs10516445, rs1057613, rs3828542, rs881980 | 4 |
| Niemann-Pick disease, type C1, gene-like 1 | *NPC1L1*, absorption of intestinal cholesterol and $\alpha$-tocopherol transport | 7 | rs11763759, rs217420, rs217430, rs217432 | 4 |
| Retinol binding protein 1, cellular | *CRBP2 (RBP2)* | 3 | rs2118981, rs35674260, rs3772875 | 3 |
| Scavenger receptor class B, member 1 | *SCARB1*, intestinal absorption of carotenoids | 12 | rs10773105, rs12582221, rs7306660, rs7967521, rs838892 | 5 |
| Serine palmitoyltransferase, long chain base subunit 1 | *SPTLC1*, sphingolipid biosynthesis | 9 | rs11790991, rs2297568, rs7858659 | 3 |
| Solute carrier family 19 (folate transporter), member 1 | *SLC19A1*, reduced folate carrier | 21 | rs13050920, rs3788205 | 2 |
| Solute carrier family 46 (folate transporter), member 1 | *SLC46A1*, proton coupled folate transporter | 17 | rs11080058, rs1128162, rs17719944, rs35714695, rs739439 | 5 |
| Thrombospondin receptor | *CD36*, platelet surface glycoprotein, binds to oxidized LDL | 7 | rs1358337, rs3211834, rs3211931, rs3211956 | 4 |
| $\alpha$-Tocopherol transfer protein | *$\alpha$-TTP*, transport $\alpha$-tocopherol | 8 | rs4501570, rs4587328, rs4606052 | 3 |
| Transcobalamin II | *TCII*, transport vitamin B-12 | 22 | rs1801198 | 1 |
| Total SNP that passed the missing-ness criteria | | | | 64 |

[1] AA, amino acid; SNP, single nucleotide polymorphism.
[2] Number of SNPs per gene.

Participants attended an informational meeting concerning study procedures. Participants completed a health history questionnaire (including general medical, personal, and family histories) and a medical screening evaluation, including measurement of height, weight, blood pressure, and laboratory analysis of fasting blood and urine. Age, BMI (calculated from height and weight), and gender were also recorded. The medical history questionnaire and laboratory results were reviewed by investigators and a cooperating physician to confirm that participants were in good health and had no evidence of underlying disease, untreated thyroid disorders, gastrointestinal disease, malabsorption syndromes, history of eating disorders, active cancer, or diabetes. All participants were weight stable, were not actively pursuing a weight-loss regimen, were not taking medications known to affect food intake or appetite, and were not taking diuretics or other medications that may affect water balance. Pregnant and lactating women were excluded from the study. Participants were compensated according to requirements of the main study (15).

*Blood analysis and genotyping.* Blood samples were analyzed for RBC folate with the use of a newly developed liquid chromatography tandem MS (LC-MS/MS) method (13,15). Information regarding quality assurance/quality control procedures for the chemical analysis of RBC folate was detailed in this previous work. The whole-blood (WB) folate concentrations determined by this method were divided by packed cell volume and expressed as RBC folate (nmol/L). In most cases (80%, or 410 of 510), only plasma-deplete WB (PDC) samples were available from the study population. The RBC folate in these samples were determined using LC-MS/MS (13) but were not normalized by packed cell volume.

The plasma Hcy measurement was completed using the Homocysteine kit with the IMMULITE 1000 immunoassay system (Siemens Healthcare Diagnostics). Plasma Hcy was divided by RBC folate concentrations and expressed as plasma nHcy.

High-quality DNA was extracted from white blood cells using the Gentra PureGene Blood DNA Purification kit (Qiagen). Seventy-four SNP in 23 genes were genotyped using allele discrimination by a MALDI-TOF MS platform (Sequenom MassARRAY) at GeneSeek (Neogen/GeneSeek). Candidate gene selection was performed based on a literature search focusing on different pathways associated with the folate, lipid, and vitamins A, E, and B-12 metabolism. SNPs in these genes were obtained from the dbSNP (16) and Ensembl (17) databases (Table 1). A detailed description on the quality control (QC) filters used to select SNPs for the association study is included in the next section.

*Statistical methods: data preparation.* The statistical analyses were conducted using the software R (version 2.12.1). The distribution of nHcy was positively skewed, so a natural-log transformation was applied as a preprocessing step. Natural-log–transformed nHcy was then used as the dependent (response) variable for regression modeling. Only participants in the Caucasian group with no more than 10 missing values in the SNP genotypes and nHcy were included in the final statistical analysis. The sample size of some of the ethnic-racial groups of the recruited population was too small for inclusion in the final statistical analysis. Thus, the final population for statistical analysis included 374 of the 510 participants of the study population. An outlier check by univariate histograms for the response (nHcy) led to the removal of one participant, leaving a total of 373 participants for the analysis.

The independent variables (predictors) included an indicator for the type of measurement for RBC folate (PDC = 0, WB = 1), age, gender (male = 0, female = 1), BMI (continuous), and genotype of SNP (where wild type = 0, hetero type = 1, and variant type = 2 for $n$ = 64 SNP). Originally, 74 SNPs were genotyped, but 10 SNPs did not pass the QC filters and they had call rates <90%, allele frequencies <0.05, or significantly deviated from Hardy Weinberg equilibrium and were discarded before further analysis. Imputing was done with Multivariate Imputation by Chained Equations (MICE 2.3, R package). Therefore, RBC folate type, age, gender, BMI, and 64 SNPs (Table 1) were included as independent (predictor) variables.

*Statistical modeling.* We fitted linear regression models with response = log(nHcy) and predictors = RBC folate type (PDC only or WB), age, gender, BMI, and SNP. All predictors were initially included. We conducted variable selection by the stepwise backward selection based on Akaike information criterion (AIC) and also least angle regression [LARS (18,19)].

Ideally, one would perform best subsets regression to select significant variables, but that is not practical, because with 64 SNPs, a large number of models would need to be fitted. Because all subsets selection was not feasible, we instead used stepwise backward elimination (or selection) based on AIC and LARS (a refinement of forward selection) for initial screening and, after adding interaction terms, we again used stepwise backward selection based on AIC, followed by backward elimination based on $P$ values to keep only significant predictors.

Here, stepwise backward selection denotes a selection procedure, where one starts with the full model, which includes all predictors and at each step determines whether to eliminate a predictor that is currently in the model or whether to add back a predictor that has been eliminated in a previous step. LARS is an efficient way to implement the least absolute shrinkage and selection operator (LASSO), an attractive version of Ordinary Least Squares that constrains the sum of the absolute regression coefficients. LARS is a refinement of stepwise forward selection, where instead of including variables at each step, the estimated coefficients are increased in a direction equiangular to the predictor correlations with the residuals. Both LARS and LASSO have been shown to be particularly useful for variable selection in high-dimensional situations under sparsity and low correlation assumptions.

Both methods led to the same final model presented in Results. All terms (**Table 2**) for the final model were significant at $P < 0.05$. All $P$ values were calculated based on the 2-sided $t$ test. $P$ values were adjusted to control the false discovery rate (20,21) (the last column of Table 2). The effects of the predictors on log(nHcy) are modeled additively. If we transform log(nHcy) back to nHcy, the effect is multiplicative, i.e., the estimated coefficients $b$ (column 3 in Table 2) can be interpreted as changing log(nHcy) for $b$ units additively when the predictor is increased by one unit or, alternatively, changing nHcy by a factor exp($b$) multiplicatively. For the final model fits, the model assumptions of normality, independence, and constant variances of residuals were checked by the usual residual plots and Q-Q plots and were found not to be seriously violated (**Supplemental Figs. 2 and 3**).

## Results

The goal of the statistical analysis was to identify those SNP among the 64 SNPs (Table 1) genotyped for these populations to jointly form the best subset to predict the response, log(nHcy). The resulting linear regression model was aimed at providing

**TABLE 2** Summary of the final model

| Predictor | SNP | Estimate[1] | SE | $t$ Value | $P$ value | Adjusted $P$ value[2] |
|---|---|---|---|---|---|---|
| Intercept | — | −5.042 | 0.056 | −89.836 | 0.000 | 0.000 |
| Blood group, WB vs. PDC | — | −0.247 | 0.054 | −4.572 | 0.000 | 0.000 |
| Gender | — | −0.600 | 0.075 | −7.973 | 0.000 | 0.000 |
| *SPTLC1* | rs11790991 | −0.076 | 0.037 | −2.066 | 0.040 | 0.040 |
| *CRBP2* | rs2118981 | −0.164 | 0.043 | −3.832 | 0.000 | 0.000 |
| *BHMT* | rs3733890 | 0.084 | 0.035 | 2.395 | 0.017 | 0.019 |
| *CETP* | rs5882 | 0.081 | 0.033 | 2.484 | 0.013 | 0.017 |
| Gender × *MTHFR* | rs1801131 | 0.136 | 0.052 | 2.642 | 0.009 | 0.012 |
| Gender x *CRBP2* | rs2118981 | 0.170 | 0.062 | 2.743 | 0.006 | 0.011 |
| Gender × *SCARB1* | rs838892 | 0.145 | 0.046 | 3.165 | 0.002 | 0.003 |

[1] Estimates are given per allele (where wild type = 0, hetero type = 1, and variant type = 2 for $n$ = 64 SNPs). PDC, plasma deplete cell; SNP, single nucleotide polymorphism; WB, whole blood.

[2] $P$ values are adjusted by the false discovery rate (20, 21).

good predictive ability with a relatively small set of SNPs. A stepwise backward selection based on AIC for predictors without interaction was initially used, which provided a set of candidate SNPs of particular interest (**Supplemental Table 1**). This initial selection was repeated with LARS (**Supplemental Table 2, Supplemental Fig. 1**). The interaction terms with gender of these candidate SNPs were then explored and further model selection was performed with stepwise backward selection based on AIC (Table 2; **Supplemental Tables 3** and **4**) followed by backward elimination based on *P* values, retaining only predictors with *P* values <0.05. The final results were the same, regardless of whether AIC or LARS was used in the initial screening step.

For the final model (Table 2), the residuals compared with the fitted values were plotted (Supplemental Fig. 2) and we also obtained the Q-Q plot of residuals from the final model (Supplemental Fig. 3). The model assumptions of normality, independence, and homoscedasticity were found not to be seriously violated as indicated by Supplemental Figures 2 and 3.

The terms and coefficients included in the final model are presented in Table 2. All of the terms in this final model are significant at $P < 0.05$. All the predictors together explain 23.7% of the total variance. The *P* values were calculated based on the 2-sided *t* test. The final column of the table lists the *P* values adjusted by the false discovery rate (20,21). The blood group (PDC vs. WB) indicator was significant with an estimated value of −0.247. This estimated value indicated that the mean log (nHcy) value for the WB group was smaller than that for the PDC group. The gender indicator was also significant with an estimated value of −0.600, which meant that the mean log (nHcy) value for women was smaller than that for men.

Three SNPs, rs11790991 (*SPTLC1*), rs3733890 (*BHMT*), and rs5882 (*CETP*), were found to have significant main effects. Thus, one unit increase (increasing from 0, wild type, to 1, heterotype, or 1 to 2, homozygous variant) in one of these SNPs tended to change the value of log(nHcy) by −0.076, 0.084, and 0.081, respectively. Equivalently, one unit increase in one of these SNPs tended to change nHcy by a multiplicative factor of $0.927 = \exp(-0.076)$, $1.088 = \exp(0.084)$, and $1.084 = \exp(0.081)$, respectively. The effects were the same for women and men.

Only one SNP (rs2118981; *CRBP2*) had a main effect as well as an interaction with gender. The estimated coefficient for the main effect was −0.164 and the interaction with gender was 0.170. Thus, for women, one unit increase in SNP value would increase the value of log(nHcy) by 0.006 (= −0.164 for main effect + 0.170 for gender × SNP interaction effect). For men, however, one unit increase in SNP value would tend to decrease the value of log(nHcy) by 0.164.

Finally, 2 SNPs (rs1801131; *MTHFR* and rs838892; *SCARB1*) were found to have interactions only with gender. Thus, for women only, one unit increase of SNP value would tend to increase the value of log(nHcy) by 0.136 and 0.145, respectively.

## Discussion

The strengths of our study included using an accurate LC-MS/MS method for RBC folate analysis, fasting blood samples from healthy adults aged 30–69 y, and controlling for false discovery rate. In this study, we selected 74 SNPs in 23 candidate genes to perform a marker trait association study with plasma Hcy normalized by RBC folate measures in Caucasian male and female samples. Selected genes corresponded to 8 genes associated with folate metabolism, 5 genes associated with vitamins B-12, A, and E metabolism, and 10 genes associated with cholesterol pathways or lipid metabolism. Most of the gene variants associated with folate metabolism were previously investigated in relation to diseases such as cancer, cardiovascular disease (CVD), NTD, congenital malformation, osteoporosis, or drug response phenotypes.

The model developed here utilized log(nHcy) as the response variable and the aim was to develop a linear regression model that provided a good predictive ability with a relatively small number of SNPs. Interestingly, a few SNPs associated with diabetes mellitus (DM), CVD, and maintenance of the cholesterol pathway or lipid metabolism were identified: *SPTLC1* (rs11790991), *CETP* (rs5882), and *SCARB1* (rs838892). SNPs associated with transfer of antioxidant vitamins, including rs2118981 of *CRBP2* (for vitamin A and retinoids) and rs838892 *SCARB1* (for tocopherols and tocotrienols), were also included in the final model. Immportantly, SNP in *BHMT* (rs3733890) and *MTHFR* (rs1801131), which are both involved in 1-carbon metabolism, were also included in the final model.

The various genes and associated SNPs are described in subsequent sections and the relation of Hcy with inflammation, CVD, obesity, and DM is discussed. The relation of nHcy concentrations with the significant SNPs (*SPTLC1*, *BHMT*, *CETP*, *CRBP2*, *MTHFR*, and *SCARB1*) may be tenuous, especially because we surveyed the main and interaction effects in healthy adults, but is an important area of future study. Understanding Hcy and genetic regulation is very important, because Hcy contributes to the development of atherosclerosis and vascular injury and may contribute to the formation of the atherosclerotic processes of DM, which may originate from inflammatory activity (22–24). The underlying mechanisms by which Hcy promotes CVD are not well understood (25) but may include endothelial cell injury, increased platelet adhesiveness, and enhanced LDL oxidation with deposition in the arterial wall (22). Also, hyperhomocysteinemia may be a risk factor for CVD in patients with DM and the potential regulation of Hcy metabolism by insulin and other hormones may provide a link to understand the relationship between DM and Hcy (23). Recent work has indicated that high Hcy levels may also be attributable to high-fat diets, so more work is required to understand Hcy regulation (26). Importantly, obesity may affect the body distribution of folate (27), which in turn may have an effect on Hcy regulation. However, Vayá et al. (28) found that abdominal obesity and fasting glucose levels > 5.6 mmol/L were independent predictors of hyperhomocysteinemia.

***SPTLC.*** Serine palmitoyltransferase, long-chain base subunit-1 catalyzes the first step in the biosynthesis of sphingolipids, including ceramide and sphingomyelin (29). Sphingolipids are important components of plasma membranes and are useful as messengers to modulate apoptosis, proliferation, and differentiation of cells (30). Increased glucosyl ceramide synthesis in lymphoblast cells in affected individuals is associated with massive cell death during neural tube closure through ceramide-induced apoptosis (29). High ceramide levels are also formed as a response to proinflammatory cytokines, oxidative stress, and FFA levels (31).

Recent evidence has suggested that sphingolipids, especially ceramides, may be important contributors to the development of insulin resistance and DM (32). Also, de novo sphingolipid synthesis is an important metabolic cross point where amino acid, lipid, and carbohydrate metabolic pathways intersect. Use of amino acid substrates L-alanine or glycine (vs. L-serine or palmitoyl-CoA) result in the formation of atypical deoxysphin-

golipids (dSL) upon metabolic activation. High levels of dSL are found in individuals with hereditary sensory neuropathy type 1 (HSN1), which closely resembles diabetic peripheral neuropathy (32). Levels of dSL, which are almost exclusively present in VLDL and LDL, are significantly elevated in individuals with nondiabetic metabolic syndrome or type 2 DM compared with controls. In addition to TG and HDL levels, dSL are critically important to explaining metabolic syndrome and may be formed in a prediabetic state. These dSL may also be potential pathogenic agents in diabetic peripheral neuropathy (32). Six mutations in *SPTLC1* (gene symbol) cause HSN1 (33,34). The SNP *SPTLC1* rs11790991 was associated with lower log(nHcy) in the model presented in this study. One unit increase in this SNP would decrease the value of log(nHcy) by 0.076 units in both genders. The interplay between sphingolipid synthesis, lipid metabolism, Hcy levels, insulin resistance, and DM is an important area of future study (32).

***BHMT.*** BHMT catalyzes the transfer of methyl groups to Hcy to form methionine (35), is found in the liver and kidney (36), and is responsible for ~50% of the remethylation of hepatic Hcy (37). *BHMT* expression affects organ development in model organisms (38) and the common SNP, rs3733890, has been associated with increased risk for having an NTD-affected child (35). This SNP has been associated with a decreased risk for CVD (39), though recent work suggested that in the presence of this SNP, low consumption of folate and vitamin B-6 may increase coronary artery disease risk (40). Teng et al. (35) established, using a $BHMT^{-/-}$ murine model, that Hcy in the liver and plasma increased 6-fold and 8-fold, respectively, compared with $BHMT^{+/+}$ controls. By 5 wk of age, the $BHMT^{-/-}$ mice had lower total hepatic phospholipids and significantly increased hepatic TG concentrations due to a decrease in secretion of VLDL. BHMT is thus important in Hcy and 1-carbon metabolism.

In this work, the final model established using the AIC stepwise selection included the common SNP, rs3733890. One unit increase in this SNP would increase the value of log(nHcy) by 0.084 units in these participants (both men and women) with hetero type or variant genotypes. Such phenotypes may also be affected by overall folate intake as suggested (40) or zinc deficiency (41), though the populations included in this study were consuming folate-fortified foods or may have used supplements, so further investigation may be required.

***CETP.*** Interestingly, the SNP rs5882 of the *CETP* gene was associated with higher log(nHcy) in the model presented in this study. One unit increase in this SNP would increase log (nHcy) by 0.081 units in both genders with hetero type or variant genotypes. CETP is involved in cholesterol homeostasis and the rs5882 SNP has been associated with lower CETP serum concentration and activity, increased HDL cholesterol levels, and increased lipoprotein sizes and these changes may be protective against CVD (42). This SNP may also be associated with lower risk for dementia and memory decline (42). Given these previously established positive outcomes of the rs5882 SNP of *CETP* on cardiovascular health and reduced risk of memory decline and dementia, it is surprising that our study found an increase in log(nHcy) values with this SNP. However, individual SNPs may have very small effects, but the cumulative effect of many SNPs may help to explain some of the variance in phenotype (43), so understanding the protective role of the rs5882 SNP is of further research interest.

***CRBP.*** CRBP2 is expressed in the intestine and binds hydrophobic retinol. Retinoids are required for normal physiological function (growth, reproduction, differentiation, embryogenesis, immune function, and vision) and are obtained solely from the diet. Retinol is the oxidation product of retinaldehyde (resulting from $\beta$-carotene cleavage) and is packaged with dietary lipids to form chylomicrons upon esterification (44). Importantly, CRBP2 channels newly absorbed retinol [>90% (45)] to the enzyme lecithin:retinol acyltransferase for esterification to retinyl esters (44). In fetal tissues, retinol supplied maternally is converted to retinal and then retinoic acid (RA), a critical regulator of gene expression during embryonic development (46). One SNP, rs12724719A, has been associated with substantially increased RA concentrations in umbilical cord blood in infants with the homozygous variant genotype. Because *CRBP2* expression is compromised in the homozygous state for *CRBP2* rs12724719A, decreased levels of RA may be delivered to the nucleus and will be found in higher concentrations in blood (46).

In serum, retinol is bound with serum retinol binding protein (RBP), allowing this water-insoluble vitamin to circulate freely. High concentrations of RBP in plasma induce insulin resistance (47). Stimulated by retinoid acid gene 6, a plasma membrane-bound protein, allows for retinol transport into cells and when activated by the retinol-RBP complex may be important in regulating insulin responses and lipid homeostasis (47,48).

In this study, both main effects for the SNP rs2118981 of *CRBP2* and interaction effects for gender × *CRBP2* were significant. Thus, for males, log(nHcy) was reduced by −0.164 units, whereas for females, where the interaction with gender was 0.170, log(nHcy) increased by 0.006 units.

***MTHFR.*** Here it was determined that the SNP rs1801131 of *MTHFR* was a significant interaction term (with gender) in this model. Thus, for women only, one unit increase in SNP value (from wild type to hetero type or from hetero type to variant type) would result in an increase in log(nHcy) values of 0.136 units. It has been established that *MTHFR* hetero (CT) or variant genotypes (TT) of the SNP rs1801133 lead to reduced enzyme activity of MTHFR (9) and an increased risk of NTD (10). The *MTHFR* rs1801133 SNP has consistently been associated with high plasma Hcy when folate intake or nutritional status is low, but this association is diminished when folate nutritional status is adequate (49). The *MTHFR* rs1801131 SNP has a reduced enzyme activity that is more pronounced in the homozygous CC genotype than in the heterozygous AC genotype; however, unlike the *MTHFR* rs1801133 TT genotype, the *MTHFR* rs1801131 CC genotype alone has not been associated with changes in plasma Hcy or plasma folate (50). D'Angelo et al. (50) suggested that folate and vitamin B-12 status and MTHFR thermolability were the main determinants of hyperhomocysteinemia phenotype, that the contribution varied from 2 to 9%, and that folate and vitamin B-12 accounted for 35% of that range. An earlier report suggested that Hcy increases when both *MTHFR* rs1801133 CT and *MTHFR* rs1801131 AC genotypes were present (51). Combined heterozygosity was associated with a reduced MTHFR activity ($P < 0.0001$) and decreased serum folate concentrations ($P < 0.03$) (51). A recent study also suggested that an increase in plasma Hcy and decrease in serum folate concentrations were seen in the presence of the *MTHFR* rs1801131 AC or CC genotypes (52). However, in that study, only 3 of the 13 SNPs were in Hardy-Weinberg equilibrium.

The influences of *MTHFR* genotypes for SNPs rs1801133 and rs18091131 on other health outcomes, including lung

cancer (53), head and neck squamous cell carcinoma (54), and type 2 DM are well established (24).

***SCARB.*** The scavenger receptor class B type 1 (SR-BI) protein is essential for HDL cholesterol uptake and excretion and is a key regulator of the reverse cholesterol transfer pathway (55,56) and participates in the metabolism of LDL and VLDL (56). Deletion of *SCARB1* (gene) in mice leads to increased atherosclerosis when fed a Western-type diet and, in humans, accelerated atherosclerosis may result in the presence of *SCARB1* mutations combined with a second atherogenic factor (55). Recent evidence also suggests that the ability of cholesterol efflux through HDL mediation via SR-BI was significantly impaired in patients with Alzheimer's disease (57). Thus, the importance of *SCARB1* in human HDL metabolism is of interest (55,58).

Here it was determined that the SNP rs838892 of *SCARB1* was a significant interaction term (with gender) in this model. Thus, for women only, one unit increase in SNP value (from wild type to hetero type or from hetero type to variant type) would result in an increase in log(nHcy) values of 0.145 units. Many of the studies that have investigated *SCARB1* SNPs have shown different effects in men and women, suggesting that sex hormones may play a mediating role (56). Recently, in a study of 498 postmenopausal Caucasian women, the SNP rs838893 × estradiol interaction showed the strongest association with HDL cholesterol, TG, and the TG:HDL cholesterol ratio. The rs838893 SNP is within a haplotype block that spans from intron 11 to intron 12 and other SNPs in this block include rs701106, rs7977729, rs838892, rs838893, rs838895, rs838896, and rs9919713. The variant alleles of this block were associated with increased TG and decreased HDL in an endogenous estrogen-dependent manner. Thus, changes in estradiol levels may affect the association of *SCARB1* genotypes on serum lipid levels (56).

Other *SCARB1* SNPs (rs9919713, rs838880) have been associated with increased levels of HDL, lower LDL, insulin resistance, and/or higher BMI. The SNP rs11057830 was recently significantly associated with $\alpha$-tocopherol levels in a genome-wide association study (59). Recent pharmacogenetic research indicated that individuals with the SNP rs4765615 had improved outcomes in statin use (60). *SCARB1* is thought to be involved in the transfer of $\alpha$-tocopherol from HDL to tissues to protect cellular membranes and other molecules from oxidative damage (59). The relationships of SR-BI, HDL, estradiol, and SNPs that modify the activity of SR-BI are of further research interest.

Finally, we chose to normalize Hcy by RBC folate rather than plasma folate, because RBC folate concentrations are ~40 times higher than serum or plasma folate concentrations RBC folate reflects an integrative measure of folate status whereas serum or plasma folate reflects recent intake, and folate-related polymorphisms are more likely to relate to RBC over serum (61,62).

In conclusion, significant predictors of plasma Hcy included type of blood sample (WB vs. PDC) used for folate analysis, gender, and SNPs in *SPTLC1* (rs11790991), *CRBP2* (rs2118981), *BHMT* (rs3733890), and *CETP* (rs5882). Significant 2-way interaction effects included gender × *MTHFR* (rs1801131), gender × *CRBP2* (rs2118981), and gender × *SCARB1* (rs83882). We conclude that gender and SNP significantly affect nHcy.

Understanding folate metabolism and methylation processes may be critically important to elucidating these seemingly disconnected pathways. Epigenetic modifications may be critically important to understanding disease-related processes and perhaps even more important than SNPs themselves (63). Investigation into other risk factors relating to Hcy risk factors or to levels of cysteine, which exhibit similar chemical properties to Hcy, is warranted (25).


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