Interactions between genetic variants of folate metabolism genes and lifestyle affect plasma homocysteine concentrations in the Boston Puerto Rican population

Tao Huang1,2,3, Katherine L Tucker4, Yu-Chi Lee2, Jimmy W Crott2, Laurence D Parnell2, Jian Shen2, Caren E Smith2, Jose M Ordovas2, Duo Li1,3,* and Chao-Qiang Lai2,*

1Department of Food Science and Nutrition, Zhejiang University, Hangzhou, People’s Republic of China; 2Jean Mayer–USDA Human Nutrition Research Center on Aging at Tufts University, Boston, 711 Washington Street, Boston, MA 02111, USA; 3APCNS Centre of Nutrition and Food Safety, Hangzhou, People’s Republic of China; 4Department of Health Sciences, Northeastern University, Boston, MA, USA

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Abstract

Objective: To investigate genetic and lifestyle factors and their interactions on plasma homocysteine (Hcy) concentrations in the Boston Puerto Rican population.

Design: Cross-sectional study. Plasma concentrations of Hcy, folate, vitamin B12 and pyridoxal phosphate were measured, and genetic polymorphisms were determined. Data on lifestyle factors were collected in interviews.

Setting: A population survey of health and nutritional measures.

Subjects: A total of 994 Puerto Rican men and women residing in the Boston metropolitan area.

Results: Smoking status was positively associated with plasma Hcy. Genetic polymorphisms MTHFR 677C→T, FOLH1 1561C→T, FOLH1 rs647370 and PCFT 928A→G interacted significantly with smoking for Hcy. MTHFR 1298A→C (P<0.040) and PCFT 928A→G (P=0.002) displayed significant interactions with alcohol intake in determining plasma Hcy. Subjects with PCFT 928GG genotype had significantly higher plasma Hcy concentrations compared with carriers of the A allele (AA, AG; P=0.030) among non-drinking subjects. When consuming alcohol, GG subjects had lower plasma Hcy levels compared with AA1 AG subjects. Physical activity interacted significantly with MTR 2756A→G in determining plasma Hcy (P for interaction =0.002). Smoking interacted with physical activity for plasma Hcy (P for interaction =0.023).

Conclusions: Smoking and drinking were associated plasma Hcy concentrations. Genetic variants involved in folate metabolism further modify the effects of lifestyle on plasma Hcy.

Keywords

Gene–environment interaction
Folate metabolism
Homocysteine
Genetic polymorphism

Elevated plasma Hcy concentration is generally considered an independent risk factor for various pathologies such as CVD, endothelial damage, venous thrombosis, atherosclerosis, abnormal collagen cross-linking, oxidative stress, osteoporotic fracture, preterm birth, neural-tube defects, pregnancy complications and several disorders of the central nervous system(5–9), but the causes of high plasma Hcy are not fully understood. The aetiology of hyperhomocysteinaemia (HHcy) is considered to be multi-factorial, including dietary and lifestyle and genetic factors(10). In the USA, two-thirds of cases with HHcy in an elderly population were associated with inadequate plasma/serum concentrations of one or more of the vitamin B group(11). In addition, low intake of n-3 PUFA(12–14), smoking(15,16), drinking(17,18) and physical activity(19) were...
also related to elevated plasma Hcy. The genetic causes of HHcy include rare inborn errors of Hcy metabolism, such as variants affecting cystathionine β-synthase and MTHFR\(^{20-22}\). Genetic polymorphisms in folate metabolism genes have been reported to be associated with elevated Hcy levels\(^{22,28}\). Of the genes involved in folate uptake and retention, that encoding folate hydrolase 1 (FOLH1), which hydrolyses dietary folate, has received the most attention with regard to its potential to modulate plasma folate status\(^{22}\). Studies reported that the 1561C→T single-nucleotide polymorphism (SNP) in FOLH1 was associated with elevated plasma folate concentrations\(^{24-26}\). Genetic variation in the proton-coupled folate transporter (PCFT), which was recently identified to absorb folate from the gut\(^{27}\), may also be associated with plasma folate and Hcy concentrations. We previously also reported that methionine adenosyltransferase variants (MTHFA) affect plasma Hcy levels and CVD\(^{28}\).

Although numerous studies have investigated the relationship between lifestyle factors such as smoking, drinking and physical activity, and genetic variants involved in folate metabolism and plasma Hcy levels\(^{16}\), the results are inconsistent across different populations. Little is known about the combined effects of lifestyle factors and genetic polymorphisms on plasma Hcy in the general population. In the present study, we hypothesized that genetic variants in genes involved in the folate metabolic pathway modulate the effects of smoking, drinking and physical activity on plasma Hcy concentration. The goal of the present study was to examine the interactions between lifestyle factors and selected genetic variants in genes of the folate metabolic pathway in relationship to plasma Hcy in an adult population of Puerto Ricans.

**Methods**

**Study design and subjects**

The current study was conducted in the ongoing Boston Puerto Rican Health Study (BPRHS) as described previously\(^{20}\). Briefly, areas of high Hispanic density in the Boston metropolitan area were identified from the year 2000 census, and one Puerto Rican adult from each household with at least one Puerto Rican person between 45 and 75 years of age was randomly selected for participation. Nine hundred and ninety-four participants with complete phenotypes and genotype data were included in the present study. Interviews were conducted in the home. In addition to health-related and anthropometric data, detailed dietary intake data were collected using a questionnaire previously adapted from the National Cancer Institute/Block food frequency form and validated for this population\(^{30}\). Fasting blood samples were collected the morning following the health interviews in the volunteer’s home. Approval for the BPRHS was obtained from the Institutional Review Board of the Tufts Medical Center and Tufts University Health Sciences.

**Genetic analysis**

DNA was isolated from blood samples using QIAamp DNA Blood Mini kits according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). Seven SNP of four methionine metabolism genes – MTHFA 1298A→C (rs1801131), MTHFA 677C→T (rs1801133), FOLH1 1561C→T (rs202712), FOLH1 (rs647370), MATFA 3U150 (rs7087728), MATFA_A15752 (rs4933327) and PCFT 928A→G (rs2239907) – chosen for genotyping are listed in Supplemental Table 1. These variants were genotyped using the TaqMan SNP genotyping system (Applied Biosystems, Foster City, CA, USA). Based on our internal quality control and that estimated independently by external laboratories, the genotyping error rate was <1%.

**Measurement of anthropometric and plasma biochemical parameters**

Anthropometric data including height and weight were measured in duplicate consistent with the technique used by the National Health and Nutrition Examination Survey\(^{31}\). BMI was calculated as weight in kilograms divided by the square of height in metres. Physical activity was estimated as physical activity score using the Paffenbarger questionnaire of the Harvard Alumni Activity Survey\(^{32}\). The physical activity score is constructed by weighting time spent in various activities by factors that parallel increasing oxygen consumption rates associated with physical activity intensity\(^{33}\). Fasting blood samples were collected by venepuncture from all participants. Total plasma Hcy was measured using HPLC with fluorescence detection as previously described\(^{34}\). Plasma pyridoxal phosphate (PLP) was determined using the radio-enzymatic method of Camp et al.\(^{35}\). Plasma folate and vitamin B\(_12\) were measured using Immulite chemiluminescent kits according to the manufacturer’s instructions (Diagnostic Products Corporation/Siemens, Los Angeles, CA, USA).

**Dietary assessment**

Dietary intake was assessed using an FFQ that was designed for and validated in this population\(^{30}\). Dietary data were linked to the Minnesota Nutrient Data System 1999, version 25 (University of Minnesota Nutrition Coordinating Center, Minneapolis, MN, USA) for nutrient analysis. Intakes of fatty acids were expressed as percentages of total energy intake.

**Statistical analyses**

The data analyses were performed using the SAS for Windows statistical software package version 9.1 (SAS Institute, Cary, NC, USA). All continuous variables were examined for normal distribution. Men and women were analysed together to ensure adequate statistical power.
Gene–lifestyle interactions and plasma homocysteine

The χ² test was used to examine whether the genotype frequencies of the selected SNP were in Hardy–Weinberg equilibrium. Correlations between smoking status, plasma parameters and anthropometric measures were performed using ANOVA. Interactions between lifestyle factors and genotypes were tested in a general linear model while adjusting for potential confounders (age, sex, BMI, population admixture, drinking, energy intake, plasma folate, plasma vitamin B₁₂ and plasma PLP, MTHFR 1298A→C, MTHFR 677C→T). The main effect of lifestyles as categorical or binary variables and genotypes as categorical variables were included in the model. All data are expressed as mean and standard deviation. Differences between groups were considered to be statistically significant at P ≤ 0.05.

Population admixture

For BPRHS participants, population admixture was estimated using principal component analysis(36,37). We estimated population admixture based on a panel of 100 SNP as informative ancestral markers that were genotyped in this population(37). All analyses were adjusted for the estimated population admixture using the first major principal component with linear regression models(37).

Results

Demographic, anthropometric and biochemical data in Boston Puerto Rican population

All seven SNP, where minor allele frequencies ranged from 0.05 to 0.42, were in Hardy–Weinberg equilibrium (χ² test; Supplemental Table 1). Plasma Hcy, plasma folate and BMI differed significantly between men and women (P < 0.01; Table 1). Plasma Hcy concentration for all subjects ranged from 3.9 to 30.4 μmol/l. Men had higher Hcy levels than women (P < 0.001; Table 1). In addition, plasma Hcy was positively correlated with age (P < 0.001).

Associations between lifestyle and plasma homocysteine

Plasma Hcy (P = 0.001), folate (P = 0.011) and vitamin B₁₂ (P = 0.012) were significantly associated with smoking status. Current smokers had higher plasma Hcy (9.64 (± 1.18) μmol/l), but lower plasma folate and PLP compared with non-smokers (Table 2). We did not observe significant associations between drinking status and plasma Hcy, folate, PLP and vitamin B₁₂ when smoking status was compared across three groups (current drinkers, former drinkers and non-drinkers) or when current drinkers were compared with non-drinkers and former drinkers combined (Table 2). Physical activity was positively associated with plasma PLP (P = 0.001) and vitamin B₁₂ (P = 0.041), but not with plasma Hcy (P = 0.068; Table 2).

Interactions between smoking status and genetic variants on plasma homocysteine

Four polymorphisms (MTHFR 677C→T, FOLH1 1561C→T, FOLH1 rs647370, PCFT 928A→G) interacted significantly with smoking in determining plasma Hcy while adjusted for age, sex, drinking, BMI, plasma folate, PLP and vitamin B₁₂, dietary energy and population admixture (P = 0.002 to 0.038; Table 3). In subjects with the genotype FOLH1 1561TT, plasma Hcy concentrations were significantly different among the three smoking statuses (P for trend = 0.011); subjects who were smokers had the highest plasma Hcy, while subjects who never smoked had the lowest plasma Hcy. However, in subjects with FOLH1 1561CT genotype, there was no significant difference in plasma Hcy levels among the three smoking statuses (P for trend = 0.631). For PCFT 928A→G, in GG subjects smoking significantly affected the plasma Hcy

Table 1: Demographic, anthropometric and biochemical characteristics of participants by sex among men and women in the Boston Puerto Rican Health Study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men (n 292)</th>
<th>Women (n 702)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean or n</td>
<td>Mean or n</td>
</tr>
<tr>
<td></td>
<td>sd or %</td>
<td>sd or %</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57-6</td>
<td>57-8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29-7</td>
<td>33-0*</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>80</td>
<td>126*</td>
</tr>
<tr>
<td>Current drinker, n (%)</td>
<td>132</td>
<td>219*</td>
</tr>
<tr>
<td>Physical activity (score)</td>
<td>32-6</td>
<td>31-1</td>
</tr>
<tr>
<td>Alcohol (g/d)</td>
<td>9-2</td>
<td>1-5*</td>
</tr>
<tr>
<td>Energy intake (kilojoules)</td>
<td>11277</td>
<td>9099</td>
</tr>
<tr>
<td>Energy intake (kcal)</td>
<td>2695.8</td>
<td>2174.6*</td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td>31-9</td>
<td>30-8*</td>
</tr>
<tr>
<td>Plasma folate (ng/ml)</td>
<td>17-7</td>
<td>20-1*</td>
</tr>
<tr>
<td>Plasma vitamin B₁₂ (pg/ml)</td>
<td>526-5</td>
<td>549-6</td>
</tr>
<tr>
<td>Plasma PLP (nmol/l)</td>
<td>61-4</td>
<td>59-2</td>
</tr>
<tr>
<td>Plasma Hcy (μmol/l)</td>
<td>10-7</td>
<td>8-8*</td>
</tr>
</tbody>
</table>

PLP, pyridoxal phosphate; Hcy, homocysteine.

Data are presented as mean and sd or %.

*Significantly different from men (P < 0.01).
level ($P$ for trend = 0.05), while we did not find any significant effect in subjects with $PCFT\ 928AG$ or $AA$ ($P$ for trend = 0.795; Table 3).

### Interactions between drinking status and $PCFT\ 928A\rightarrow G$ on plasma homocysteine

$PCFT\ 928A\rightarrow G$ displayed significant interactions with drinking status for plasma Hcy ($P$ = 0.002) after adjusting for age, sex, smoking, population admixture, dietary energy, dietary total fat, plasma folate, plasma vitamin B$_{12}$ and plasma PLP. Further analysis showed that $GG$ homozygotes had significantly higher plasma Hcy concentrations compared with homozygous ($AA$) and heterozygous ($AG$) subjects combined ($P$ = 0.050) among non-drinkers. Among current drinkers, $GG$ homozygotes had lower plasma Hcy concentrations when compared with homozygous ($AA$) and heterozygous ($AG$) subjects (Fig. 1).

### Influence on plasma homocysteine of the interaction between physical activity and the $MTR\ 2756A\rightarrow G$/smoking association

$MTR\ 2756A\rightarrow G$ significantly interacted with physical activity in influencing plasma Hcy level ($P$ = 0.002). When the physical activity score was low (<40), there was no significant difference between $MTR\ 2756AA$ and minor allele ($AG+GG$) carriers. Interestingly, minor allele carriers had significantly higher plasma Hcy than homozygous ($AA$) subjects ($P$ = 0.034) when the physical activity score was ≥40 (Fig. 2). Smoking interacted significantly with physical activity to influence plasma Hcy level ($P$ = 0.023; Fig. 3). When the physical activity score was low (<40), there was no significant difference between Hcy concentrations in smokers and non-smokers. However, when physical activity score was high (≥40), we observed that minor allele carriers ($AG+GG$)

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**Table 2:** Association between lifestyle and plasma vitamins and metabolites among men and women in the Boston Puerto Rican Health Study

<table>
<thead>
<tr>
<th>Lifestyle factor</th>
<th>Plasma Hcy (µmol/l)</th>
<th>Plasma folate (ng/ml)</th>
<th>Plasma vitamin B$_{12}$ (pg/ml)</th>
<th>Plasma PLP (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker (n = 753)</td>
<td>9.0 3.5</td>
<td>20.0 7.0</td>
<td>552.1 281.3</td>
<td>63.16 24.34</td>
</tr>
<tr>
<td>Current smoker (n = 241)</td>
<td>9.6 4.2</td>
<td>17.4 9.0</td>
<td>512.3 241.1</td>
<td>49.66 26.76</td>
</tr>
<tr>
<td>Drinking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-drinkers (n = 527)</td>
<td>9.1 3.6</td>
<td>19.5 9.4</td>
<td>535.4 249.0</td>
<td>56.9 26.2</td>
</tr>
<tr>
<td>Current drinker (n = 349)</td>
<td>9.4 4.3</td>
<td>19.0 8.8</td>
<td>554.8 302.7</td>
<td>64.1 29.4</td>
</tr>
<tr>
<td>Physical activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA score ≤30 (n = 392)</td>
<td>9.5 3.7</td>
<td>18.8 9.7</td>
<td>520.8 273.7</td>
<td>52.4 19.2</td>
</tr>
<tr>
<td>PA score &gt;30 (n = 498)</td>
<td>9.0 3.9</td>
<td>19.7 8.8</td>
<td>559.9 287.5</td>
<td>65.6 20.6</td>
</tr>
</tbody>
</table>

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**Table 3:** Effect of the interaction between folate metabolic gene polymorphisms and smoking status on plasma homocysteine (Hcy) among men and women in the Boston Puerto Rican Health Study

<table>
<thead>
<tr>
<th>Single-nucleotide polymorphism</th>
<th>Non-smoker (n = 451)</th>
<th>Past smoker (n = 302)</th>
<th>Current smoker (n = 241)</th>
<th>$P$ for trend</th>
<th>$P$ for interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$MTHFR\ 677C\rightarrow T$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8.7 3.7</td>
<td>9.2 3.7</td>
<td>9.6 4.5</td>
<td>0.425</td>
<td>0.002</td>
</tr>
<tr>
<td>$MTHFR\ 1298A\rightarrow C$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>8.7 3.7</td>
<td>9.5 3.7</td>
<td>9.8 3.9</td>
<td>0.192</td>
<td>0.332</td>
</tr>
<tr>
<td>$FOLH1\ 1561C\rightarrow T$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>9.4 4.6</td>
<td>9.2 3.7</td>
<td>9.5 3.9</td>
<td>0.631</td>
<td>0.038</td>
</tr>
<tr>
<td>$FOLH1\ (rs647370)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>8.7 3.3</td>
<td>9.4 3.6</td>
<td>9.5 3.7</td>
<td>0.422</td>
<td>0.024</td>
</tr>
<tr>
<td>$PCFT\ 928A\rightarrow G$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>8.7 3.6</td>
<td>9.4 3.3</td>
<td>9.9 4.5</td>
<td>0.050</td>
<td>0.029</td>
</tr>
</tbody>
</table>

$MTHFR$, 5-methyltetrahydrofolate reductase; $FOLH1$, folate hydrolase 1; $PCFT$, proton-coupled folate transporter. $\ddagger$Adjusted for age, sex, BMI, population admixture, smoking, dietary energy, plasma folate, plasma vitamin B$_{12}$ and plasma pyridoxal phosphate. $\ddagger\ddagger$Adjusted for age, sex, BMI, population admixture, smoking, dietary energy, plasma folate, plasma vitamin B$_{12}$ and plasma pyridoxal phosphate, $MTHFR\ 2756C\rightarrow T$. $\ddagger\ddagger\ddagger$. Adjusted for age, sex, BMI, population admixture, smoking, dietary energy, plasma folate, plasma vitamin B$_{12}$ and plasma pyridoxal phosphate, $MTHFR\ 1298A\rightarrow C$ and $MTHFR\ 677C\rightarrow T$. After adjusting for n-3 PUFAs in the model, the results remained the same.
had significantly higher plasma Hcy than major allele homozygotes (AA; \( P = 0.012 \)).

**Discussion**

In the present study conducted in the BPRHS, we demonstrated that lifestyle factors such as smoking were associated with elevated plasma Hcy concentration but that drinking and physical activity were not directly associated with plasma Hcy. Moreover, we observed that smoking, drinking and physical activity each interacted with genetic polymorphisms in genes involved in the methionine metabolic pathway in determining plasma Hcy levels.

Elevated plasma Hcy plays an important role in the pathology of CVD\(^\text{33}\). HHcy is a complex, multi-factorial condition and its environmental and genetic contributors continue to be identified and refined. However, these factors often have been evaluated mainly independently of one another. In the present study, we examined previously unreported interactions between lifestyle and genetic variants in modulating plasma Hcy levels.

Plasma Hcy concentrations were significantly higher in men than in women, which is consistent with previous studies\(^\text{16,38}\). This sex difference can be explained in part by folate intake, as the plasma folate concentrations in women were significantly higher than in men in this population. Other factors such as sex hormones may also contribute to gender-related differences in Hcy. Furthermore, plasma Hcy is higher in postmenopausal women compared with premenopausal women and is reduced in response to oestrogen replacement therapy\(^\text{34,35}\). All of these factors may contribute to the higher plasma Hcy concentrations observed in men compared to women.
In accordance with other studies in large populations (14,36) and a randomized control trial (39), we also observed that cigarette smoking was positively associated with plasma Hcy concentrations, while smoking was negatively associated with plasma folate and PLP. The mechanism for this relationship is not fully understood, but one possibility is smoking might reduce the availability of folate for the remethylation of Hcy to methionine (40). Additional proposed mechanisms are smoking, which induces local effects in cells exposed to cigarette smoke, may influence the Hcy concentration by changing plasma thiol redox status, or could inhibit enzymes involved in the metabolism of Hcy (40).

In the present study, we observed the same interaction between smoking status and MTHFR 677C→T as reported previously (14). In addition, we identified previously unreported interactions between smoking and genetic polymorphisms (FOLH1 1561C→T, FOLH1 rs647370, PCFT 928A→G) for plasma Hcy. The suboptimal plasma folate status among smokers observed in our study might explain this interaction, because plasma folate has been shown to interact with MTHFR 677C→T (15). Another study which reported that smoking interacted with dietary folate further supports this relationship (41).

Moderate alcohol consumption in social drinkers increased plasma Hcy levels (40). MTHFR 677T, MTRR 66A, GCPII 1561T and alcohol intake were also associated with higher plasma Hcy among South Indians (46). However, we did not confirm these results in the present study. One possible explanation for this discrepancy is genetic variants involved in folate metabolism may modify the effect of drinking on plasma Hcy and folate levels. Therefore, to evaluate our hypothesis, we examined the interactions of drinking and critical genes involved in folate metabolism on plasma Hcy. Interestingly, we observed that PCFT 928A→G interacts significantly with drinking status for plasma Hcy, which suggests that the effect of drinking on plasma Hcy depended on PCFT genotype, and may account for the inconsistencies with previous studies. Studies also suggested that higher plasma Hcy concentrations in consumers of large quantities of alcohol may be related to trapping of 5-methyltetrahydrofolate by alcohol and impaired remethylation associated with genetic polymorphisms (17) or to ethanol-induced B vitamin depletion (40) and interference of alcohol with intestinal absorption of folate (42). Therefore, these results suggest that the effect of drinking on plasma Hcy depends on folate metabolism-related gene polymorphisms.

Despite the inconsistent results of effects of physical activity on plasma Hcy levels, several studies have reported beneficial effects on Hcy in response to exercise. Plasma Hcy level is inversely related to level of physical activity, especially in older subjects (38). In contrast, other studies reported that physical exercise does not reduce plasma Hcy levels or may even be associated with higher concentrations (43). In the present study we also observed that physical activity was not associated with plasma Hcy. Therefore, questions about the nature of the relationship between Hcy and physical activity remain unanswered (45). However, these inconsistencies reported in previous studies can be explained by our present results which identified a previously unreported interaction between MTR 2756A→G and physical activity on Hcy. This result suggests that not all subjects in a population have the same response to physical activity, as the effect of physical activity on plasma Hcy depends on MTR genotype. Additionally, it has been shown that the thermolabile variant of MTHFR 677C→T (rs1801133, a regulating enzyme in Hcy metabolism) modified the relationship between several lifestyle factors and Hcy (44). Thus, the effect of the changes in lifestyle-related factors on Hcy may depend on MTHFR C677T genotype (45). Better understanding of the complex relationships between lifestyle and Hcy will require additional studies focusing on the interaction between physical activity and genetic polymorphisms involved in folate metabolic pathways. The biological mechanism through which physical activity lowers plasma Hcy levels remains to be determined.

In summary, the present study reinforces earlier evidence demonstrating that lifestyle factors are important modifiers of plasma Hcy concentration. In addition, our work extends this knowledge to demonstrate that genetic polymorphisms in genes whose proteins function in the methionine metabolic pathway further modulate the effects of lifestyle factors which influence plasma Hcy. It is essential to determine whether changes in these lifestyle factors reinforce effects in the context of interaction with genetic variation on Hcy metabolism and to clarify the potential mechanism(s) by which this can take place.

Acknowledgements

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References

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