Plasma Homocysteine Concentrations in Greek Children Are Influenced by an Interaction between the Methylenetetrahydrofolate Reductase C677T Genotype and Folate Status

Constantina Papoutsakis, Nikos Yiannakouris, Yannis Manios, Evaggelos Papaconstantinou, Faidon Magkos, Kleopatra H. Schulpis, Antonis Zampelas, and Antonia L. Matalas

Laboratory of Nutrition and Clinical Dietetics, Department of Nutrition and Dietetics, and *Department of Home Economics and Ecology, Harokopio University, Athens, Greece; and †Institute of Child Health, Aghia Sofia Children’s Hospital, Athens, Greece

ABSTRACT Risk factors established at young ages may set the stage for later cardiovascular disease (CVD). Elevated total homocysteine (tHcy) in blood is an emerging risk factor for CVD, yet few studies have been conducted in children, especially in the Mediterranean. We described plasma tHcy concentrations in a group of healthy Greek children and examined its relation with physiologic, metabolic, and genetic variables. Fasting blood samples were collected from 186 students, 11.6 ± 0.4 years old, and tHcy, folate, vitamin B-12, and routine biochemistry variables in plasma were measured. The methylenetetrahydrofolate reductase (MTHFR) C677T genotype was determined and anthropometric and dietary data were obtained. The distribution of tHcy was positively skewed with a median of 7.9 μmol/L (mean: 8.2 ± 2.3 μmol/L; range: 4.4–22.2 μmol/L). tHcy was inversely related to plasma folate (r = −0.34, P < 0.0001), vitamin B-12 (r = −0.20, P = 0.008), and glucose (r = −0.15, P = 0.045). An interaction between the MTHFR genotype and plasma folate on tHcy was detected (P = 0.047). Specifically, the homocysteinemia mutant TT genotype was associated with higher tHcy only in children with lower plasma folate (<19.9 nmol/L) (P = 0.012). In our sample of healthy Greek children, plasma tHcy concentrations were higher than values reported in children of Northern European descent and were associated with folate, vitamin B-12, and glucose in plasma. The results also show that, similar to adults, plasma folate concentration is important in determining the contribution of the MTHFR C677T mutation to tHcy concentrations in children.

KEY WORDS: homocysteine • folate • MTHFR C677T genotype • children • Greece

Heart disease is the leading cause of death in Greece, with a mortality rate that increased between 1980 to 1997 from 181 to 304 per 100,000 (1). However, not too long ago, in the 1960s, Greeks had impressively low cardiovascular disease (CVD) rates as described in the Seven Countries Study (2). The increase of CVD in Greek adults and the fact that risk factors established at young ages can set the stage for adult disease necessitate the study of CVD risk factors in young populations (3).

Cigarette smoking, hypertension, an abnormal lipid profile, obesity, diabetes, physical inactivity, an atherogenic diet, family history, age, and male gender are well-known risk factors that explain a substantial proportion but not all of coronary heart disease. Thus, the identification of new risk factors continues to be an area of active research. An increased total homocysteine (tHcy) concentration in the blood has been recognized as such an independent CVD risk factor (4). tHcy is a sulfur-containing amino acid whose concentrations are controlled by 2 metabolic pathways: tHcy remethylation to methionine or tHcy transsulfuration to cysteine, which require folate and vitamins B-12 and B-6 as cofactors (5). Whether homocysteine-lowering efforts will translate into lowering CVD rates remains to be shown by clinical intervention trials. The prospect for prevention supports the need to study tHcy in young populations (6), especially in countries where CVD rates are of concern and tHcy has never been described. This is not to imply that elevation of CVD can be solely attributed to tHcy or the factors that influence it. Most likely, adverse changes in classic risk factors (such as obesity and worsening of blood lipids) are responsible for the majority of the CVD increase observed in Greece (7). However, the possibility that elevated tHcy in childhood contributes to future risk of CVD cannot be discounted.

Elevated tHcy is influenced by nutrition, most notably inadequate status of the B vitamins, folate and vitamins B-12 and B-6, as well as male gender, increasing age, and genetic background (8). The most common genetic defect of tHcy metabolism is the 677C → T mutation in the gene for 5,10-methylenetetrahydrofolate reductase (MTHFR) (EC 1.7.99.5),
where an alanine-to-valine substitution takes place. The 677C → T mutation is found in a thermolabile variant of the MTHFR enzyme and results in about half of the usual enzyme activity (9). Adults who are homozygous for valine (or TT genotype) have higher tHcy concentrations under conditions of inadequate folate status (10). Also, folate is the most robust nutritional predictor of tHcy, and thus hyperhomocysteinemia can occur as a consequence of a gene-nutrient interaction (4,8,10). Recent studies suggest that age could modify the contribution of the MTHFR mutation to tHcy concentrations (11), yet few studies have investigated the effects of the MTHFR-folate interaction on tHcy in children (12).

Plasma tHcy concentrations may vary according to geographic location (13), but data for healthy children in Mediterranean countries are minimal (14). To our knowledge, tHcy has not been studied in presumed healthy children living in Greece. In the present study, we measured plasma tHcy in a sample of Greek sixth-grade schoolchildren and we investigated putative associations with folate, vitamin B-12, and other metabolic variables in plasma, as well as dietary intake and the MTHFR C677T polymorphism.

SUBJECTS AND METHODS

Population of the study. The present study was part of a survey investigating cardiovascular risk factors in sixth-grade students. Participants were residents of Volos, a semiurban city, which is the capital of Magnesia County in central mainland Greece. Permission to carry out the study was provided by the Greek Ministry of Education and the Bioethics Committee of Harokopio University, Athens, Greece. Twelve elementary public schools of Volos were randomly selected to participate in the study. All sixth-grade students of these schools were considered eligible for the study and were asked to join. After being informed of the purpose and procedures of the study, each participant and his/her parent or guardian signed an informed consent form. Trained personnel conducted face-to-face interviews and used a structured questionnaire to record information such as birth date, birthplace, nationality, and medical history (e.g., history of chronic disease). Body weight and height were measured and BMI was calculated as weight/height\(^2\) (kg/m\(^2\)). Reasons for exclusion from the study were chronic or present illness, absence from school, or relocation. Of the 271 students invited, 198 (73%) participated (106 girls and 92 boys). For 12 subjects, plasma or DNA samples were limited. Thus, we present data on 186 Greek children (99 girls and 87 boys) for whom we had complete information. Data and specimen collection took place during March 2001.

Specimen collection and biochemical analyses. Overnight fasting (>10 h) venous blood was collected from study participants. The samples were collected in EDTA-containing tubes and protected from light thereafter. Plasma was immediately separated (1800 \times g, 15 min) and theuffy coats of nucleated cells were saved for DNA extraction. After being divided into aliquots, plasma samples were shipped in ice under code to the Laboratory of Nutrition and Clinical Diabetics, Harokopio University, Athens. Aliquots were stored at −80°C until assayed. Among other routine blood indices, plasma total cholesterol, HDL cholesterol, triacylglycerol, and glucose were determined in duplicate using commercially available enzymatic colorimetric assays (Sigma Diagnostics) on an automated ACE analyzer (Schiapparelli Biosystems). Within-batch coefficients of variation for the determination of biochemical variables were all below 5%. LDL cholesterol was calculated by the Friedewald equation (15).

Plasma tHcy concentration (total of protein- and nonprotein-bound homocysteine) was measured via reverse-phase HPLC with fluorometric detection. Precolumn derivatization of plasma thiol with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate was implemented prior to HPLC and fluorometric detection. The method of Übink et al. (16) as originally described by Araki and Sako (17) was modified to include a novel phosphate reagent: tris(2-carboxyethyl)-phosphate (18). Fluorescent intensities were measured with excitation and emission wavelengths set at 245 and 515 nm, respectively. tHcy concentration was determined against a known plasma standard for tHcy (ClinCal-Calibrator, Plasma-Calibrator, Lyophil., for homocysteine from Recipe, Chemicals and Instruments). Also, controls (Clincheck-Control, Plasma Control, Lyophil., for homocysteine, Level I, II from Recipe, Chemicals and Instruments) were used for internal quality assurance of the assay. The intra- and interassay CV were 3 and 4%, respectively. Plasma folate and vitamin B-12 levels were measured using a commercially available RIA kit (Dualcount; solid phase no boil assay; Diagnostic Product) with a sensitivity of ~0.7 nmol/L for folate and 25 pmol/L for vitamin B-12.

Dietary information. In this survey, information was also collected regarding children’s current dietary intake via 2 nonconsecutive 24-h recalls. No child reported taking a vitamin and mineral supplement. Food intake data were analyzed using Nutritionist V software, version 2 (First Databank). The Nutritionist V food database was modified by adding traditional food recipes (19) and local processed food items using nutrient data provided by industry.

DNA isolation and genotyping. Genomic DNA was extracted from leucocyte nuclei by the salting-out method (20). The MTHFR C677T genotype was determined using the method of Frosst et al. (9). Briefly, a 198-bp PCR amplification product was generated using the following set of primers: 5'-TGA AGG AGA AGG TGT CTG CG-3' (forward); 5'-AGG ACG GTG CGG TGA GAG TG (reverse). The PCR conditions were 95°C for 5 min, 60°C for 1 min, and 72°C for 1 min for 1 cycle and subsequently 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 5 min. The PCR products were digested with the restriction endonuclease HinfI (New England Biolabs) and then electrophoresed on 3% agarose gels, prestained with ethidium bromide to visualize the different genotypes. Digestion of the 677C allele resulted in no cleavage products whereas digestion of the 677T allele produced 2 fragments with sizes 175 and 23 bp.

Statistical analysis. SPSS 11.0 for Windows (SPSS) was used for all statistical analyses. The descriptive presentation of data includes means and standard deviations or number of subjects and respective percentage. Unpaired two-tailed t tests were calculated to detect significant differences between 2 independent groups, and the chi-square test was applied to assess differences in frequencies of measured genotypes. Chi-square tests were also conducted to examine whether the genotype frequencies were in Hardy-Weinberg equilibrium. The normal distribution of the investigated variables was assessed through the Kolmogorov-Smirnov criterion. In all analyses, log-transformed values were used for tHcy, folate, vitamin B-12, triacylglycerol, and dietary intakes of various nutrients, due to their skewed distribution. However, in the tables untransformed means are provided. Pearson’s correlation coefficients were determined to identify significant correlations between continuous variables of interest. ANOVA was used to test for differences between genotypes and for interactions between genotype and vitamin concentrations. For post hoc comparisons of means, overall type I error was controlled using Tukey’s test. Also, the effect of several potential confounders (i.e., age, gender, BMI) was considered, yet adjustment for these variables had no influence on the observed results, and therefore only the unadjusted data are presented. Furthermore, in a recessive model for the T allele, the differences in measured variables in individuals with homozygous mutant genotypes (TT) versus carriers of the wild-type allele (C allele) were explored. Statistical significance was interpreted as values of \( P < 0.05 \).

RESULTS

Characteristics of the participants. The mean age of participating students was 11.6 ± 0.8 years (age range, 10.8–13.5 years). Age, BMI, cholesterol, HDL cholesterol, LDL cholesterol, glucose, tHcy, folate, and vitamin B-12 in plasma did not differ between females and males (Table 1). Females exhibited significantly higher plasma triacylglycerol concentrations (Table 1) and reported lower total energy intake, but nutrient intakes with adjustment for total energy intake did not differ between genders (data not shown). The distribution of the MTHFR genotype did not differ between females and males and was...
compatible with Hardy-Weinberg equilibrium ($P = 0.7$). Compared with other available data from adult Greek populations, there was a similar T allele frequency (0.37) in our pediatric sample.

**Homocysteine, folate, and vitamin B-12 concentrations.** The frequency distribution of plasma tHcy concentration in this sample was asymmetric with a calculated skewness factor of 2.5 (Fig. 1). tHcy values ranged from 4.4 to 22.2 μmol/L with a median of 7.9 μmol/L. Also, the values 5.4, 6.7, 7.9, 9.2, 11.5, and 21.7 μmol/L corresponded to the 5th, 25th, 50th, 75th, 95th, and 99th percentiles, respectively. Nine children (or 4.5% of tHcy observations) exceeded the 95th percentile of the presented distribution (with values of 11.5 μmol/L or above). The plasma folate and vitamin B-12 medians were 19.9 nmol/L (range 6.9–61.2 nmol/L) and 408 pmol/L (range 152–831 pmol/L), respectively. No subject was found to be below the laboratory minimum normal reference value for either folate (laboratory minimum normal reference = 3.4 nmol/L) or vitamin B-12 (laboratory minimum normal reference = 133 pmol/L). A positive correlation was identified between plasma folate and vitamin B-12 ($r = 0.17$, $P = 0.018$). tHcy concentrations correlated negatively with folate ($r = -0.34$, $P < 0.0001$), vitamin B-12 ($r = -0.20$, $P = 0.008$), and glucose ($r = -0.15$, $P = 0.045$) in plasma. No significant correlations were found between tHcy and the following variables: age, gender, plasma lipids, anthropometric measurements, and nutrient intakes (data not shown).

**Homocysteine concentrations, MTHFR genotype, and folate status.** Plasma tHcy concentration demonstrated a significant difference across the MTHFR variants, with individuals carrying the homozygous mutant genotype (TT) exhibiting 15% higher tHcy concentration compared to subjects with the CC or CT genotypes (Table 2). Similar results were obtained after controlling for age, gender, BMI, and plasma concentrations of folate and glucose (overall $P = 0.012$). Moreover, TT homozygotes exhibited lower plasma folate (14% lower) compared with carriers of the C allele (Table 2). In contrast, participants with different genotypes did not differ regarding vitamin B-12 (Table 2) or plasma lipids and glucose concentrations (data not shown).

To investigate the possible effect of folate status on the relation between the MTHFR polymorphism and concentrations of tHcy that has previously been shown in adults, study participants were divided by folate status (lower or upper) using the folate median as the cutoff criterion. An interaction between the MTHFR genotype (TT vs. CT and CC) and plasma folate concentrations on tHcy was found ($P$ for interaction = 0.047). Among children with lower plasma folate concentrations (<19.9 nmol/L), those with the TT genotype had tHcy concentrations that were 21% greater than children with the CC or CT genotypes (Table 3). For subjects in the upper folate stratum, however, mean tHcy was not affected by the MTHFR genotype.

### DISCUSSION

To our knowledge, this is the first population-based study that provides evidence on the concentrations of plasma tHcy in healthy Greek children. We show that plasma folate, vitamin B-12, and glucose are related to tHcy. Furthermore, we demonstrate that the MTHFR C677T genotype plays an important role in predicting tHcy concentrations via an interac-
tion with folate. These results support the notion that at young ages folate may be an important nutrient in reducing CVD susceptibility by protecting the human MTHFR from thermal inactivation.

The distribution of plasma tHcy concentrations was positively skewed in this group of children (Fig. 1) as it is in adult populations, whereas tHcy values were appreciably lower than those reported in adults (4). The homocysteine concentrations we present are relatively similar to those reported for healthy populations, whereas tHcy values were appreciably lower than those reported in other developed countries (age-appropriate reported values in Italy (21) and Spain (14,22), and higher than those reported in neighboring Mediterranean countries, namely Greece (23)). The homocysteine concentrations reported in other developed countries (age-appropriate reported values range from 5.0 to 6.8 μmol/L) (13,23–27). tHcy may depend on geographic location and/or ethnicity, reflecting differentiations of nutritional environment and/or genetic profile. Unfavorable socioeconomic characteristics may increase children’s tHcy even when vitamin status is adequate (23). Also, variations in reported tHcy results may be attributed to preanalytic conditions, whether data are presented as medians or arithmetic or geometric means or whether results are presented by gender or not. Some studies have fewer than 30 children in age groups or provide results without categorizing data by age (13). There is no standard definition for hyperhomocysteinaemia, but the 95th percentile value has frequently served as a relative cutoff point (26). Consistent with this definition, hyperhomocysteinaemia in our sample corresponded to concentrations exceeding 11.5 μmol/L. Osogian et al. (6) reported 95th percentile values of 8.5 μmol/L in US children (ages 13–14 y), and De Laet et al. (28) reported 95th percentile values of 10.2 μmol/L in Belgian children (ages 10–14 y). Even though our sample is not representative of the Greek population, and we did not study a comprehensive pediatric age range, an estimate of what may be deemed as hyperhomocysteinaemia in Greek children is clinically relevant because hyperhomocysteinaemia has been found to be a potent risk factor for thromboembolism in children (29).

Plasma tHcy was inversely associated with folate and vitamin B-12 concentrations, in agreement with existing research from different countries in adults (30–32) and children (6,12,13,23,24,26,28,33). The repeatability of the inverse relationship between tHcy and B-vitamin status suggests that even though there may be differences in tHcy concentrations between different populations, optimal B-vitamin status is universally important in achieving lower tHcy.

We did not find differences in plasma tHcy concentrations between males and females. Others have reported a lack of gender difference in similar ages, whereas such a difference begins to become important in postpubertal children (5). The absence of gender difference in earlier childhood seems to be related to the process of maturation: as age increases, muscle mass increases, and sex hormones change. We did not account for the influence of puberty stage. Additional research is needed to describe the specific relation between development of gender differences during adolescent years and tHcy. As we show, plasma glucose is inversely related to tHcy (34). Fonseca et al. found that in folate-replete individuals, such as in our sample, insulin sensitivity correlates positively with plasma tHcy concentrations (35). Insulin has recently been shown to directly influence homocysteine metabolism by inhibiting cystathionine β-synthase expression in liver; a key enzyme involved in the transulfuration pathway of homocysteine catalysis (36), and it is likely that such a mechanism underlies the above-observed relationships.

We did not find any significant relations between tHcy and

### TABLE 2

<table>
<thead>
<tr>
<th>MTHFR C677T genotype</th>
<th>CC (n = 79)</th>
<th>CT (n = 76)</th>
<th>TT (n = 31)</th>
<th>P-value by ANOVA</th>
<th>P-value (TT vs. CC + CT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>11.6 ± 0.4</td>
<td>11.6 ± 0.3</td>
<td>11.7 ± 0.6</td>
<td>0.269</td>
<td>0.137</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>20.2 ± 3.2</td>
<td>20.8 ± 4.0</td>
<td>20.0 ± 3.3</td>
<td>0.407</td>
<td>0.536</td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>7.9 ± 1.8</td>
<td>8.0 ± 1.7</td>
<td>9.2 ± 3.1</td>
<td>0.016</td>
<td>0.004</td>
</tr>
<tr>
<td>Folate, nmol/L</td>
<td>22.1 ± 8.6</td>
<td>21.9 ± 8.9</td>
<td>18.9 ± 8.6</td>
<td>0.088</td>
<td>0.027</td>
</tr>
<tr>
<td>Vitamin B-12, pmol/L</td>
<td>407 ± 107</td>
<td>423 ± 124</td>
<td>404 ± 109</td>
<td>0.734</td>
<td>0.594</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. * Different from CC (P = 0.014) and CT (P = 0.028) by Tukey’s post hoc test.
2 Based on ANOVA test for differences between means.
3 Tested using log-transformed data.

### TABLE 3

<table>
<thead>
<tr>
<th>MTHFR C677T genotype</th>
<th>All (n = 186)</th>
<th>CC (n = 79)</th>
<th>CT (n = 76)</th>
<th>TT (n = 31)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate &lt; 19.9 nmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>8.8 ± 2.6</td>
<td>8.2 ± 1.7</td>
<td>8.3 ± 1.6</td>
<td>10.0 ± 3.4</td>
<td>0.012</td>
</tr>
<tr>
<td>Folate ≥ 19.9 nmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>7.6 ± 1.8</td>
<td>7.7 ± 1.8</td>
<td>7.7 ± 1.8</td>
<td>7.4 ± 1.1</td>
<td>0.946</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. * Different from tHcy in upper folate stratum based on t test (P < 0.0001). † Different from CC (P = 0.021) and CT (P = 0.021) by Tukey’s post hoc test.
2 Based on ANOVA test for differences between means.
3 Tested using log-transformed data.
plasma lipid concentrations, BMI, or dietary intake. Osogian et al. (6) reported that tHcy was not associated with lipids and was only weakly related to BMI, but De Laet et al. (28) found BMI to relate positively with tHcy across a wide age range of children. The relation of dietary intake and tHcy has not been thoroughly studied in children. In our pediatric sample, we did not detect a relation of reported macro- and/or micronutrient intake with tHcy, folate, and vitamin B-12 in plasma. However, the use of two 24-h dietary recalls may not be sufficient to reveal such associations with micronutrients. Further studies on the diet-tHcy relation in children are warranted because adherence to a Mediterranean eating pattern has been related to lower tHcy concentrations (37) and tHcy may be modifiable through diet, even in children with adequate vitamin status (23).

For the MTHFR polymorphism, we report a frequency distribution of 37.1% for the T allele, similar to the frequency reported for Greek adults (35%) (37,38). Our MTHFR frequency data are in agreement with a higher prevalence of the homozygous mutant genotype TT in Mediterranean countries compared to other countries in Northern Europe (39). Also, our data support the concept of a north-to-south increasing gradient for the frequency of T677 in Europe (39). The relatively high tHcy concentrations observed in our group of Mediterranean children may be related to the increased frequency of the T allele in Southern Europe.

In adults, the TT genotype increases tHcy under conditions of lowered folate status (10), whereas CC adults usually exhibit higher plasma folate concentrations and have ≈25% lower concentrations of tHcy when compared to TT individuals (40). Such information in children is very limited. Balasa et al. (41) found that the C677T MTHFR polymorphism was an independent determinant of tHcy in 197 healthy U.S. children (aged 6 mo to 16 y), but these investigators did not measure plasma concentrations of B vitamins to examine possible interactions between the genetic profile and nutritional status on tHcy. In our study, TT children had about 16% higher concentrations of tHcy and ≈15% lower plasma folate concentrations compared to CC children. Moreover, TT status was important in exhibiting increased tHcy only in children who were stratified in the lower folate status group, suggesting that even at young ages the interaction between folate and the genotype influences tHcy concentrations. An interaction between the MTHFR C677T genotype and serum folate on tHcy was previously reported in the study of Tonstad et al. (42) among 92 Norwegian children with familial hypercholesterolemia during cholestyramine treatment. Delvin et al. (12) also reported that in a healthy French Canadian pediatric sample the TT genotype was associated with higher tHcy concentrations only in nutritionally stressed children who were older than 10 y. It is noteworthy that none of the children in our sample qualified as being folate deficient, suggesting that optimal folate status versus avoidance of subclinical or clinical deficiency ought to be the appropriate nutritional goal, especially for individuals carrying the mutant allele. Our study supports the hypothesis that comprehensive interventions to achieve optimal folate status ought to start early to maximize CVD prevention potential.

The cross-sectional study design limits our potential to reveal causal relations. Also, our study included a moderate number of participants and the sample is not representative of the Greek population. Nevertheless, this investigation is the first one to examine simultaneously in a Greek pediatric sample plasma tHcy, folate, and vitamin B-12 concentrations and the MTHFR polymorphism. We showed that, similar to what has been observed in adults, plasma folate is important in determining the contribution of the MTHFR C677T mutation to tHcy concentrations in children. The collection of representative data in young subjects is needed to address important issues such as establishing tHcy reference values in Greek children and investigating further the factors that affect tHcy.

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LITERATURE CITED


