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**Background:** Moderately increased plasma concentrations of total homocysteine (tHcy) have been shown to be an important risk factor for vascular diseases. Two common polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene, the thermolabile C677T and a more recently reported A1298C polymorphism, may contribute to hyperhomocysteinemia.

**Methods:** Using PCR and restriction fragment length polymorphism analysis, we studied the prevalence of the C677T and A1298C MTHFR genotypes and the combined effect of these polymorphisms on plasma tHcy concentrations, as measured by HPLC with fluorometric detection, both fasting and post-methionine load (PML), in 1238 individuals.

**Results:** The prevalences of the C677T and A1298C MTHFR genotypes did not differ significantly in 772 individuals with documented coronary artery disease (CAD), 137 individuals with deep-vein thrombosis (DVT), and 329 individuals without documented vascular disease. Individuals homozygous for the 677T allele had significantly increased fasting tHcy, particularly in the presence of low folate, compared with individuals homozygous for the wild-type allele. Neither the 1298AC nor the 1298CC genotype was associated with significantly increased fasting or PML tHcy concentrations irrespective of serum folate. Of the nine combined MTHFR genotypes, six were present in >10% of the population. Of these, the difference in mean fasting tHcy reached statistical significance (P < 0.005) only in individuals with the 677TT/1298AA genotype compared with individuals with the wild-type 677CC/1298AA genotype. Differences in mean fasting tHcy did not reach statistical significance in individuals heterozygous for both MTHFR variants. We detected two 677CT/1298CC and three 677TT/1298AC individuals; only one, an 677TT/1298AC individual, had increased tHcy (both fasting and PML). No individuals had the 677TT/1298CC genotype.

**Conclusions:** The prevalences of the C677T and A1298C polymorphisms did not differ among individuals with CAD, DVT, or those without documented vascular disease. In contrast to the C677T polymorphism, the A1298C polymorphism is not associated with increased fasting tHcy. Although the two polymorphisms usually exist in trans configuration, crossover may occur rarely to form recombinant chromosomes.

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The enzyme methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) plays a critical role in homocysteine metabolism by catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl-group donor in the B12-dependent remethylation of homocysteine to methionine. Severe deficiency of the MTHFR enzyme leads to homocystinuria, a rare inborn error of metabolism characterized by highly increased blood and urine homocysteine concentrations. Moderately reduced concentrations of MTHFR, often associated with the common thermolabile form of this
enzyme, may lead to hyperhomocysteinemia, as characterized by mild to moderately increased concentrations of plasma total homocysteine (tHcy). Hyperhomocysteinemia has been reported in individuals with occlusive vascular diseases (1, 2), and recent studies have demonstrated that moderately increased tHcy, whether measured after fasting or 2–6 h after a methionine load, is associated with an increased risk for coronary artery disease (CAD) (3–7). In addition, high plasma tHcy concentrations have been reported to be a risk factor for deep-vein thrombosis (DVT) in the general population (8, 9).

Two common polymorphisms that may contribute to hyperhomocysteinemia have been reported in the MTHFR gene. The C677T (Ala-to-Val) transition, which produces thermolability and somewhat reduced enzyme activity in vitro, was first described by Kang et al. (10). Individuals homozygous for the C677T mutation have moderately increased concentrations of fasting plasma tHcy, especially in the presence of low (<15.4 nmol/L) plasma folate (11), and this mutation is more prevalent in patients with CAD (17%) than in controls (5%) (10, 12). Although some studies have confirmed this observation (13), most recent studies do not support an association between the C677T polymorphism and CAD (14–17). Likewise, homozygosity for the C677T polymorphism has been implicated as a risk factor for venous thrombosis (18, 19); however, not all studies support this finding (20, 21).

More recently, a second prevalent polymorphism, which is associated with decreased enzyme activity in vitro, has been discovered in the MTHFR gene (22, 23). This genetic variant consists of an A→C transversion at nucleotide 1298, which produces a Glu-to-Ala substitution. Studies on a relatively small number of individuals have shown that ~10% of individuals are homozygous for the 1298C allele and roughly 20% of individuals are heterozygous carriers of both the C677T and A1298C polymorphisms (22, 23). Although it has been shown that the A1298C polymorphism, in either homozygosity or heterozygosity, is not associated with higher plasma tHcy concentrations in patients with neural-tube defects (NTDs) and their parents (22, 23), it has been reported that combined homozygosity for the C677T and A1298C variants is associated with reduced MTHFR specific activity and higher tHcy concentrations when compared with heterozygosity for either variant (22).

Previous studies on the A1298C polymorphism involved a relatively small number of patients with NTDs and their parents (22–25). In the current investigation, we studied the prevalence of the A1298C polymorphism in a population of 1238 individuals with and without documented vascular disease and the effect of this polymorphism, both alone and in combination with the C677T polymorphism, on fasting and post-methionine load (PML) tHcy concentrations.
MUTATION ANALYSIS

Genomic DNA was extracted from peripheral leukocytes isolated from acid-citrate-dextrose-anticoagulated blood by a commercially available DNA isolation method (Puregene; Gentra Systems).

To detect the presence or absence of the A1298C polymorphism, we selectively amplified a 256-bp fragment of the MTHFR gene by PCR. PCR reactions were performed with 50 ng of genomic DNA, 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Roche Molecular Systems), 10 mM Tris (pH 8.3), 1.0 mM MgCl2, 50 mM KCl, 0.2 mM of all four deoxynucleotide triphosphates, and 0.2 μM of each primer (sense, 5′-CTTCTACCTGAA-GAGCAAGTC-3′; antisense, 5′-CATGTCCACAGCATGCAG-3′), in a volume of 50 μL. After denaturation at 95 °C for 3 min, the temperature was cycled 30 times (95 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min), followed by extension at 72 °C for 5 min to amplify the target DNA.

The amplified fragment was digested with the restriction enzyme MbolI according to manufacturer’s instructions (Promega), electrophoresed on a 2% ultra PURE™ Agarose-1000 (Life Technologies) gel containing ethidium bromide, and visualized on a ultraviolet transilluminator (Fotodyne). DNA from a patient homozygous for the 1298A allele appears as a fluorescent band 176 bp in length relative to the size marker, with three smaller fragments of 30, 28, and 22 bp. Presence of the A1298C polymorphism abolishes an Mbol cut site; thus, DNA from a patient homozygous for the 1298C allele appears as a fluorescent band of 204 bp with smaller fragments of 30 and 22 bp.

The C677T mutation of the MTHFR gene was detected by PCR amplification followed by digestion with the restriction enzyme HinfI, as described by Frosst et al. (12).

STATISTICAL ANALYSIS

The fasting tHcy concentrations and the PML increase in tHcy had skewed distributions; thus, these variables were natural log-transformed, and geometric means were used. Mean tHcy values and 95% confidence intervals (CIs) were calculated after adjustment for age and gender. A t-test for the C677T and A1298C mutant genotypes compared with each wild-type genotype was used to determine whether there were significant differences in tHcy concentration between genotypes. P <0.05 was considered statistically different. Statistics were computed with SPSS for Windows (Release 7.5; SPSS).

Results

We determined the C677T and A1298C genotypes in 772 patients with documented premature CAD and 137 patients with DVT (Table 1); 329 apparently healthy individuals were used as controls. There were no significant differences in the prevalences of the different genotypes between CAD and DVT patients, between CAD patients and controls, or between DVT patients and controls (P >0.05 for all). Because of the similarities in genotype prevalence and mean age of the three populations, we combined the three groups to study the relationship between MTHFR genotypes and plasma tHcy concentrations.

The geometric mean fasting tHcy concentrations and the mean PML increases in tHcy in the combined population of 1238 individuals classified according to the C677T and A1298C genotypes, as well as the frequency of each genotype, are shown in Table 2. Of the 1238 individuals, 512 (41.3%) were homozygous for the 677C allele, 585 (47.2%) were heterozygous, and 141 (11.4%) were homozygous for the 677T allele. The mean fasting tHcy concentration was significantly higher in individuals who were homozygous for the 677T allele compared with those heterozygous for the polymorphism (P = 0.005).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Prevalence, n (%)</th>
<th>Fasting tHcy, μmol/L (95% CI)</th>
<th>PML Increase in tHcy, μmol/L (95% CI)</th>
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</thead>
<tbody>
<tr>
<td>677CC</td>
<td>324 (42.0)</td>
<td>9.1 (8.9–9.4)</td>
<td>20.7 (20.1–21.4)</td>
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<tr>
<td>677CT</td>
<td>364 (47.2)</td>
<td>9.3 (9.1–9.5)</td>
<td>20.7 (20.1–21.2)</td>
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<td>677TT</td>
<td>84 (10.9)</td>
<td>9.3 (9.1–9.5)</td>
<td>20.6 (20.0–21.1)</td>
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<tr>
<td>1298AA</td>
<td>360 (46.6)</td>
<td>9.3 (9.1–9.5)</td>
<td>20.8 (19.9–21.7)</td>
</tr>
<tr>
<td>1298AC</td>
<td>322 (41.7)</td>
<td>9.2 (9.0–9.4)</td>
<td>20.9 (20.3–21.5)</td>
</tr>
<tr>
<td>1298CC</td>
<td>90 (11.7)</td>
<td>9.2 (9.0–9.4)</td>
<td>20.3 (19.1–21.6)</td>
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</table>

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<thead>
<tr>
<th>Genotype</th>
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<th>PML Increase in tHcy, μmol/L (95% CI)</th>
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<tbody>
<tr>
<td>677CC/1298AA</td>
<td>148 (12.0)</td>
<td>9.0 (8.5–9.4)</td>
<td>20.3 (19.2–21.5)</td>
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<td>677CC/1298AC</td>
<td>235 (19.0)</td>
<td>9.1 (8.8–9.5)</td>
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<tr>
<td>677CC/1298CC</td>
<td>129 (10.4)</td>
<td>9.3 (8.8–9.8)</td>
<td>20.5 (19.3–21.8)</td>
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<tr>
<td>677CT/1298AA</td>
<td>298 (24.0)</td>
<td>9.1 (8.8–9.4)</td>
<td>20.6 (19.9–21.5)</td>
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<tr>
<td>677CT/1298AC</td>
<td>285 (23.0)</td>
<td>9.5 (9.2–9.8)</td>
<td>20.7 (19.9–21.7)</td>
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<td>677CT/1298CC</td>
<td>2 (0.2)</td>
<td>6.0 (4.0–9.1)</td>
<td>12.5 (7.8–20.2)</td>
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<tr>
<td>677TT/1298AA</td>
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<td>10.1 (9.5–10.5)</td>
<td>20.8 (19.7–22.0)</td>
</tr>
<tr>
<td>677TT/1298AC</td>
<td>3 (0.2)</td>
<td>10.7 (7.6–15.0)</td>
<td>22.0 (14.9–32.6)</td>
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<tr>
<td>677TT/1298CC</td>
<td>0 (0)</td>
<td>Not included in statistical analysis.</td>
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The interaction between folate status and MTHFR genotypes was studied in a subgroup of 387 individuals. Among those with RBC folate concentrations below the sample median (833 nmol/L), the mean fasting tHcy concentrations were significantly higher in individuals with the 677TT genotype (n = 25) than in individuals with the 677CC (P = 0.008; n = 67) or 677CT (P = 0.005; n = 101) genotype (data not shown). No association was observed between C677T genotypes and fasting tHcy concentrations in individuals with RBC folate concentrations at or above the median. The PML increase in tHcy concentrations was not significantly larger in individuals who were heterozygous or homozygous for the 677T allele (Table 2). Individuals with RBC folate concentrations <833 nmol/L and who were heterozygous or homozygous for the 677T allele did show a trend toward a larger PML increase in tHcy concentration, but the differences did not reach statistical significance (data not shown).

With respect to the A1298C polymorphism, Table 2 shows that 584 individuals (47.2%) were homozygous for the 1298A allele, 523 (42.2%) were heterozygous, and 131 (10.6%) were homozygous for the 1298C allele. There were no significant differences in mean fasting tHcy concentrations or PML increases in tHcy in individuals who were either heterozygous carriers or homozygous for the 1298C allele compared with individuals homozygous for the 1298A allele. Results were unchanged when RBC folate concentrations were taken into account.

Regarding the two common MTHFR polymorphisms, Table 2 shows that six of the nine combined genotypes were present in >10% of the population. No individuals were homozygous for both the 677T and 1298C alleles (677TT/1298CC genotype), and we detected only two individuals with the 677CT/1298CC genotype and three with the 677TT/1298AC genotype. Approximately 23% of the individuals (285 of 1238) were heterozygous for both MTHFR polymorphisms (677CT/1298AC genotype). Individuals with the 677TT/1298AA genotype had significantly increased fasting tHcy concentrations compared with individuals with the 677CC/1298AA, 677CC/1298AC, 677CC/1298CC, or 677CT/1298AA genotype. Individuals with combined heterozygosity for the two MTHFR variants (677CT/1298AC genotype) had higher fasting tHcy concentrations than individuals with the 677CC/1298AA genotype; however, the differences did not reach statistical significance (P = 0.061). The results were essentially the same in the subgroup of individuals whose RBC folate concentrations were <833 nmol/L; there were no significant differences in fasting tHcy concentrations among the six genotypes when RBC folate concentrations were ≥833 nmol/L.

No significant differences in the PML increase in tHcy were observed for any combination of genotypes. Individuals with the 677CT/1298CC or the 677TT/1298AC genotype were omitted from the statistical analysis because of the small size (two and three, respectively) of each group. Only one of these five individuals (677TT/1298AC genotype) had increased tHcy (both fasting and PML).

Discussion

Moderately increased plasma tHcy, whether fasting or PML, has been demonstrated to be an independent risk factor for CAD (3–7) and DVT (18, 19). Controversies exist, however, as to whether homozygosity for the C677T polymorphism of the MTHFR gene predisposes individuals to CAD or DVT. In the current study, our results confirm some of the more recent reports that homozygosity for the 677T allele is not associated with CAD or DVT. In contrast to our results, however, our study also confirms previous reports (10–12) that homozygosity for the C677T transition of the MTHFR gene is associated with increased fasting plasma tHcy and that this is particularly true for individuals with lower serum folate concentrations. In contrast to a smaller study in which individuals with the 677TT genotype showed markedly increased PML tHcy values compared with those with the 677CC genotype (13), our results, like those of Jacques et al. (11), show that the presence of the C677T polymorphism is not associated with significantly larger PML increases in tHcy irrespective of folate concentration.

Previous studies regarding the A1298C polymorphism have shown that presence of the mutated 1298C allele is not a significant risk factor for NTDs (22, 23, 25). In the current study, we show in a large population that there are no significant differences in the prevalences of the 1298AA, 1298AC, and 1298CC genotypes in individuals with CAD or DVT, and in apparently healthy controls. In our population, allelic frequencies for the A1298C polymorphism (68% for 1298A; 32% for 1298C) were similar to those reported previously (23, 25). Our study also confirms the results obtained in smaller studies (22, 23) that heterozygosity or homozygosity for the A1298C polymorphism alone or with increased fasting tHcy regardless of RBC folate concentrations. Additionally, we are the first to report that there is no significant association between the A1298C polymorphism and PML increase in tHcy concentration.

With respect to the two common polymorphisms (677T and A1298C) in the MTHFR gene, we found that six of the nine combined genotypes were present in >10% of our population. Of these, ~23% were compound heterozygotes. An earlier study reported that combined heterozygosity for the C677T and A1298C MTHFR variants predisposes individuals to increased tHcy (22). Although our results indicate that there is a tendency toward increased fasting tHcy concentrations in individuals with the 677CT/1298AC genotype compared with individuals with the 677CC/1298AA genotype, the mean differences in fasting tHcy values did not reach statistical significance, irrespective of folate concentration.

Various studies have examined the haplotype distribution of the two MTHFR polymorphisms. van der Put et al.
(22) reported that an individual with a 677TT genotype always has a 1298AA genotype and vice versa, thus concluding that the 677T and 1298C alleles are always in trans configuration. Other studies involving a group of Ashkenazi Jews (24) and a small German population (25) also detected no individuals who were homozygous for both polymorphisms or homozygous for one polymorphism and heterozygous for the other. Weisberg et al. (23), however, observed 1 individual with a 677TT/1298AC genotype among a Canadian population of 133 children with spina bifida, thus demonstrating that the two alleles, although rare, can exist in cis. We are the first to report two individuals with the 677CT/1298CC genotype. We also detected three individuals with the 677TT/1298AC genotype. Presumably, the two polymorphisms arose separately on different alleles, and because of the small distance that separates them on the chromosome, little crossover has occurred. However, our finding of five individuals who were homozygous for one variant and heterozygous for the other indicates that although the two polymorphisms usually are in trans configuration, crossover can occur rarely to form recombinant chromosomes. Despite the fact that our study included 1238 individuals, we, like other investigators (22–25), found no individual who was homozygous for both polymorphisms (677TT/1298CC genotype).

A recent study (27) comparing the prevalences of the C677T and A1298C polymorphisms in 119 neonatal and 161 fetal tissue samples reported that although the 677CT/1298CC and 677TT/1298CC genotypes were present in the fetal tissue samples (5 of 161 and 1 of 161, respectively), they were absent in the neonatal group. The authors surmised that these two genotypes with three and four mutant alleles in the MTHFR gene may impair the viability of the fetus. In contrast, the authors found a third group of individuals in whom three mutant alleles (677TT/1298AC genotype) were equally present in both the fetal and neonatal groups. The finding of two individuals with the 677CT/1298CC genotype and normal fasting and PML tHcy concentrations in the present study, along with the previous finding of Isotalo et al. (27) that a third group genotype with three mutant alleles (677TT/1298AC genotype) is equally present in fetal and neonatal tissue, cast doubt on the hypothesis that fetuses with three mutant alleles have decreased viability. Further studies, however, are needed to distinguish whether low crossover events or decreased fetal viability is responsible for the absence of the 677TT/1298CC genotype in the current study.

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References


