Plasma Homocyst(e)ine Concentration, But Not MTHFR Genotype, Is Associated With Variation in Carotid Plaque Area

J. David Spence, MD; M. Rene Malinow, MD; Peter A. Barnett, PhD; Ali J. Marian, MD; David Freeman, MD; Robert A. Hegele, MD

Background and Purpose—Elevated plasma homocyst(e)ine [H(e)] concentration is associated with premature atherosclerosis. A common cause of elevated plasma H(e) concentration is a thermolabile mutation (677T) in the gene encoding methylenetetrahydrofolate reductase (MTHFR). We sought to determine whether plasma H(e) concentration or MTHFR genotype would be more strongly associated with carotid plaque area (CPA), a potential intermediate phenotype of atherosclerosis.

Methods—In 307 subjects who were ascertained through a premature atherosclerosis clinic, we measured CPA with 2-dimensional ultrasound and also determined traditional atherosclerosis risk factors, in addition to plasma H(e) concentration and MTHFR genotypes.

Results—We found that the frequency of the MTHFR 677T allele was 0.363 in this sample. Mean plasma H(e) concentration was significantly higher in 677T/T homozygotes than in 677T/C heterozygotes and 677C/C homozygotes (17.1±13.7 versus 13.5±6.1 versus 12.6±5.9 μmol/L, respectively, P<0.001). Analysis of variance showed that CPA was significantly associated with age, sex, smoking, diabetes, hypertension, and hyperlipidemia (each P<0.05). When plasma H(e) concentration was included in the model, it was significantly associated with CPA (P<0.05). However, when the MTHFR genotype was included in the model, it was not associated with CPA (P=0.50). Furthermore, there was a significant correlation of CPA with plasma H(e) (r=0.23, P<0.0001). However, the mean CPA did not differ between subjects according to genotype.

Conclusions—Thus, plasma H(e), but not MTHFR genotype, is significantly associated with carotid atherosclerosis, suggesting that the biochemical test may be sufficient to identify patients who may be at increased risk of atherosclerosis through this mechanism. (Stroke. 1999;30:969-973.)

Key Words: carotid arteries ■ genetics ■ polygenic disease ■ risk factors

Numerous studies have demonstrated a significant relationship between elevated plasma homocyst(e)ine [H(e)] concentration and vascular disease.1–7 Of several enzymatic defects or deficiencies that can lead to elevated plasma H(e) concentration,1,2 the one that has attracted recent attention is the thermolabile variant of methylenetetrahydrofolate reductase (MTHFR). A genetic basis for the thermolabile variant of MTHFR, in addition to its relationship with elevated H(e) concentration and coronary heart disease (CHD), was inferred from biochemical studies in 1991.8 Since this variant is common in whites,9–11 it could represent an important genetic determinant of CHD risk in the general population. The molecular basis of the MTHFR thermolabile variant was later found to be a mutation (C677T) in the MTHFR gene.9 Furthermore, dietary folate deficiency has been found to be associated with elevated plasma H(e) concentration in subjects who are homozygous for the MTHFR thermolabile variant.10 Since then, there have been numerous genetic association studies of the MTHFR C677T variant, particularly in the homozygous state, which have shown both the presence10–16 of significant associations of such end points as CHD, myocardial infarction (MI), thrombo-occlusive disease, and cerebrovascular disease. However, the relationship between elevated plasma H(e) concentration and the end points of vascular disease appears to be consistent, even in those studies that failed to show an association with the MTHFR C677T variant. We hypothesized that the association of MTHFR genotype would be more obvious when using a more proximal, intermediate phenotype of atherosclerosis, such as ca-
clinoid plaque area (CPA), as determined by 2-dimensional ultrasound. We evaluated the association of both plasma H(e) concentration and MTHFR genotype with CPA with multivariate analysis of variance, using classical atherosclerosis risk factors as covariates in the model.

Subjects and Methods

Study Subjects
Three hundred seven subjects ranging in age from 28 to 78 years were recruited from a vascular disease prevention clinic. These subjects had been referred to the clinic because they had premature atherosclerosis, namely, the onset of clinical end points under the age of 55 years in men and under the age of 60 years in women. All subjects were white. 63% of subjects had premature clinical presentation of the end points of CHD (such as angina, myocardial infarction, and/or revascularization procedures), 27% had premature clinical presentation of the end points of cerebrovascular disease (such as transient ischemic attacks and/or stroke), and the remainder had premature clinical presentation of the end points of both coronary heart and cerebrovascular disease. Of these subjects, 61% also had a strong family history of premature atherosclerosis, namely, the onset of clinical end points under the age of 55 years in a male first-degree relative and under the age of 60 years in a female first-degree relative. In addition, a reference control population was studied with respect to plasma H(e). The reference control subjects were asymptomatic for vascular disease and were ascertained through voluntary participation in a study of psychological stress and blood pressure variation.28 All subjects gave their informed consent to participate. The protocol was approved by the Research Ethics Committee at the University of Western Ontario.

Clinical, Biochemical and Genetic Determinations
Questionnaires relating to past medical history were administered to and physical examinations were performed on the study subjects who were ascertained through the vascular disease prevention clinic. Fasting plasma H(e) concentration was measured by high performance liquid chromatography with electrochemical detection, which had been standardized against reference methods in both Oregon and Cleveland.29,30 From determinations performed in more than 1000 previous plasma samples, the coefficient of variation was <5% at the range of plasma H(e) concentrations observed in the present study. The MTHFR C677T genotypes were determined by DNA amplification and restriction digestion with HindI as described.9

Ultrasound Evaluations
Ultrasound measurements were made from 2-D B-mode images of the carotid arteries using a cursor and a microprocessor within the ultrasound equipment (Mark 9, ATL).28 CPA was defined as the sum of the cross-sectional areas of all plaques seen in longitudinal views of the common, external, and internal carotid arteries. Within-observer variability of CPA was assessed by having each of 2 technicians measure the CPA in 25 subjects on 2 separate occasions, at least a week apart. These ultrasound scans were then distributed in a blinded fashion within a large number of routine clinical scans, which resulted in their being scored again by the same individual. The intraobserver reliability of CPA measurement was assessed by having 2 technicians exchange their tapes on 25 patients and each measure the plaque area. The interobserver correlation coefficient between observers was 0.94.

Statistical Analysis
SAS (version 6.12) was used for all statistical analyses. Significance of the deviation of observed genotype frequencies from those predicted by the Hardy-Weinberg law was determined using \( \chi^2 \) analysis, with a nominal \( P<0.10 \). ANOVAs were performed with use of the general linear models procedure to determine the sources of variation for both plasma H(e) concentration and 2-D CPA. The

<table>
<thead>
<tr>
<th>TABLE 1. Clinical and Biochemical Characteristics of Study Sample</th>
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<tr>
<td><strong>Characteristic</strong></td>
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<tr>
<td>Subjects, n</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>Postmenopausal, %</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<td>Total cholesterol, mmol/L</td>
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<td>Triglycerides, mmol/L</td>
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<td>HDL cholesterol, mmol/L</td>
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<td>Plasma H(e) concentration, μmol/L</td>
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<tr>
<td>Smokers, %</td>
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<tr>
<td>No. of pack-years smoked</td>
</tr>
<tr>
<td>Diabetics, %</td>
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<tr>
<td>Treated for hypertension, %</td>
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<tr>
<td>Treated for hyperlipidemia, %</td>
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</table>

Values are mean±SD.

untransformed CPA had a distribution that was significantly nonnormal, but a cube root transformation resulted in a variable whose distribution was not significantly different from normal (Wilks \( W=0.96, \) NS).

To determine sources of variation for plasma H(e), 1 ANOVA was performed. The independent variable was the MTHFR genotype. If a significant association was found, Bonferroni \( t \) tests were performed to determine whether the mean plasma H(e) concentration differed between subjects with the 677T/T genotype and each of the other 2 genotypes.

To determine the sources of variation for CPA, 2 ANOVAs were performed. The dependent variable for each of the 2 ANOVAs was the transformed CPA. Plasma H(e) concentration was included as an independent variable in the first ANOVA. The 3 MTHFR genotypes were included as an independent variable (\( df=1 \)) in the second ANOVA. All factors were adjusted for other factors used in the model. The sample was divided by quartiles of plasma H(e) concentration to create subgroups to test for differences between CPA and MTHFR genotype frequencies between lowest and highest plasma H(e) concentration quartiles. \( \chi^2 \) tests were used to determine whether there was a between-groups difference in proportions of subjects who were homoygous for the MTHFR 677T allele.

Results

Characteristics of the Study Sample
The baseline clinical and biochemical features of the 307 subjects from the vascular disease prevention clinic, divided according to gender, are shown in Table 1. As expected, a high proportion of the 307 subjects from the vascular disease prevention clinic smoked and were receiving treatment for hyperlipidemia and hypertension. As expected, the overall mean plasma H(e) was higher in the 307 subjects from the vascular disease prevention clinic than in the 351 reference controls (13.7±7.3 versus 10.6±5.6 \( \mu \)mol/L, \( P=0.002 \)). Furthermore, 33% of subjects from the vascular disease prevention clinic had a plasma H(e) in excess of 14 \( \mu \)mol/L, compared with only 21% of the reference controls (\( P=0.009 \)). In the study subjects, the ranges for the lowest, second, third, and highest quartiles of plasma H(e) concentration were 3.90 to 9.27, 9.30 to 11.54, 11.6 to 14.5, and 14.6 to 69.2 \( \mu \)mol/L, respectively.
MTHFR Allele and Genotype Frequencies
The frequency of the MTHFR 677T allele in the 307 subjects from the vascular disease prevention clinic was 0.363. There were 124 MTHFR 677C/C homozygotes (40.4%), 143 677C/T heterozygotes (46.6%), and 40 677T/T homozygotes (13.0%). The observed MTHFR genotype frequencies did not deviate from those predicted by the Hardy-Weinberg equilibrium ($P=0.68$, NS).

Plasma H(e) Concentration and MTHFR Genotype ANOVA showed that MTHFR genotype was a significant determinant of plasma H(e) concentration ($P=0.0007$). Bonferroni t tests showed that there were significant differences, at a nominal $P<0.05$, in the mean plasma H(e) concentration of MTHFR 677TT homozygotes (17.1 ± 13.7 μmol/L) compared with both 677TC heterozygotes (13.5 ± 6.1 μmol/L) and 677CC homozygotes (12.6 ± 5.9 μmol/L). Furthermore, the frequencies of MTHFR 677TT homozygotes according to ascending quartiles of plasma H(e) concentrations were 0.087, 0.139, 0.101, and 0.192. There was a significant difference in the frequency of MTHFR 677TT homozygotes between the lowest and the highest quartile of plasma H(e) concentration ($P<0.001$).

Correlation of Plasma H(e) Concentration and CPA
The Pearson correlation coefficient between plasma H(e) concentration and transformed CPA was 0.23 ($P<0.0001$). Furthermore, the mean±SDs of transformed CPA according to ascending quartiles of plasma H(e) concentrations were 0.56 ± 0.33, 0.63 ± 0.35, 0.59 ± 0.30, and 0.76 ± 0.47, respectively. There was a significant difference in the transformed CPA between the lowest and the highest quartile of plasma H(e) concentration ($P<0.001$).

CPA and MTHFR Genotype
When subjects were classified according to MTHFR genotype, we found that the mean±SDs of transformed CPA in 677TT homozygotes, 677TC heterozygotes, and 677CC homozygotes were 0.65 ± 0.39, 0.61 ± 0.36, and 0.68 ± 0.38, respectively. Pairwise comparisons indicated that the mean CPA was not significantly different in 677TT homozygotes compared with 677TC heterozygotes and in 677TT homozygotes compared with 677CC homozygotes (each $P>0.50$).

Sources of Variation for CPA
Two ANOVAs were performed, with the only difference between them being the use of plasma H(e) concentration as a covariate in one and the use of MTHFR genotype as a covariate in the other. As shown in Table 2, the 2 ANOVAs had very similar $F$ values and $P$ values for the significantly associated independent variables age, sex, smoking, hypertension treatment, hyperlipidemia treatment, and diabetes history (each $P<0.05$). The plasma H(e) concentration was significantly associated with CPA in the first ANOVA ($P=0.049$), but the MTHFR genotype was not associated with CPA in the second ANOVA ($P=0.50$).

Table 2. Results of Two ANOVAs for Sources of Variation in Carotid Plaque Area

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1</th>
<th>Model 2</th>
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<tbody>
<tr>
<td>Age</td>
<td>84.3</td>
<td>92.3</td>
</tr>
<tr>
<td>Sex</td>
<td>4.05</td>
<td>5.44</td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
<td>15.3</td>
<td>17.3</td>
</tr>
<tr>
<td>Hypertension treatment</td>
<td>7.33</td>
<td>8.10</td>
</tr>
<tr>
<td>Hyperlipidemia treatment</td>
<td>16.5</td>
<td>18.9</td>
</tr>
<tr>
<td>Diabetes history</td>
<td>6.32</td>
<td>6.31</td>
</tr>
<tr>
<td>Plasma H(e) concentration</td>
<td>3.89</td>
<td>0.049</td>
</tr>
<tr>
<td>MTHFR genotype</td>
<td>0.69</td>
<td>NS</td>
</tr>
</tbody>
</table>

Discussion
A preponderance of recent literature supports the concept that plasma H(e) concentration is more strongly and consistently associated with clinical end points of atherosclerosis, such as myocardial infarction and stroke, than is the MTHFR genotype.10–27 Our findings extend this pattern of association to a more proximal, intermediate phenotype of atherosclerosis, namely, CPA. We found that plasma H(e) concentration, but not MTHFR genotype, was among the independent variables that were significantly associated with variation in CPA. Furthermore, there was a significant correlation between plasma H(e) concentration and CPA, with subjects in the highest plasma H(e) concentration quartile having a significantly greater mean CPA compared with subjects in the lowest plasma H(e) concentration quartile. Although the frequency of MTHFR 677TT homozygotes was also significantly greater compared with subjects in the lowest plasma H(e) concentration quartile and the mean plasma H(e) concentration was highest among MTHFR 677TT/T homozygotes, there was no association between CPA and MTHFR genotype either in the ANOVA or in pairwise comparisons of genotypic means. In particular, the CPA did not differ significantly between MTHFR 677TT homozygotes and MTHFR CC homozygotes. Furthermore, the results are consistent with the reported associations of plasma H(e) concentration with another intermediate phenotype of atherosclerosis, namely, carotid intimal-medial wall thickness.31,32

There are several possible reasons that MTHFR 677TT/T homozygotes did not have mean CPA significantly different from subjects with the other MTHFR genotypes despite having significantly higher mean plasma H(e) concentrations than subjects with the other MTHFR genotypes. First, this apparent disparity could simply have reflected a lack of statistical power to detect a difference due to the relatively small number of homozygotes in the overall study sample. Alternatively, it could have reflected the lack of specificity in the association between MTHFR genotype and plasma H(e) concentration. In particular, while the MTHFR 677TT genotype was associated with elevated plasma H(e) concentration in the overall study sample, the vast majority (ie, >80%) of subjects with elevated plasma H(e) concentration did not have the MTHFR 677TT genotype. This suggests that factors other than MTHFR genotype are the main determinants of
elevated plasma H(e) concentration. It therefore follows that factors other than MTHFR genotype are the main determinants of the association between elevated plasma H(e) concentration and increased CPA.

In addition, almost 10% of subjects in the lowest plasma H(e) concentration quartile had the MTHFR 677T/T genotype, which would also tend to attenuate the association between MTHFR genotype and CPA. It is widely assumed that a diet replete in folate can reverse the tendency to higher plasma H(e) concentration in MTHFR 677T/T homozygotes.2,19,21,33 Although we did not obtain dietary histories of folate intake in our study subjects, it is quite likely that a diet replete in folate could have explained the very low plasma H(e) concentration in at least some of the MTHFR 677T/T homozygotes. Thus, such a gene-diet interaction could have further obscured the association between the MTHFR genotype and CPA.

In our study sample, women were found to have had a lower mean plasma H(e) concentration compared with men (Table 1, P<0.01). The most likely explanation for this observation was the large number of women in our study sample who were taking hormone replacement therapy. It has been fairly well established that plasma H(e) concentration can be reduced by up to 20% in postmenopausal women who use exogenous estrogen.34 This could be yet another factor that, in addition to MTHFR genotype and diet, contributes to interindividual variation in plasma H(e) concentration in the present study and in others.

Is CPA useful to determine the extent of carotid atherosclerosis? In general, technical improvements in carotid ultrasound over the past 10 years have resulted in its wide use as a research tool.35–46 Some studies have demonstrated a close correlation between carotid ultrasound measurements, usually of carotid intimal-medial wall thickness, and angiographic measurements.35–46 Previous concerns regarding apparent discrepancies between angiography and carotid ultrasound have been alleviated by the recent understanding that factors affecting lesion development within the arterial wall do not necessarily affect the angiographic appearance of the lumen.47,48 As the plaque thickens, the artery enlarges, so that the intima maintains exposure to a constant shear rate; thus, significant plaque can develop before any stenosis occurs.47,48 Thus, the complexity of the atherogenic processes within the arterial wall could weaken the relationship between any single independent variable, the underlying pathology, and the resulting expression of disease. B-mode ultrasound measurement of CPA may thus be complementary to other measures, such as carotid intimal-medial wall thickness, in the assessment of the progression of preclinical atherosclerosis and the monitoring of its rate of progression.

Our results are somewhat limited by the absence of both dietary information and plasma folate concentrations in our study subjects. Knowledge of either of these might have helped to clarify some of the apparent discrepancy in the relationship between MTHFR genotype, plasma H(e) concentration, and CPA. Nonetheless, our data are consistent with the concept that elevated plasma H(e) concentration, from whatever cause, is more directly involved in atherogenesis than is the MTHFR genotype. While homozgyosity for MTHFR 677T/T was statistically associated with elevated plasma H(e) concentration, it was neither the major factor determining elevated plasma H(e) concentration nor was it specifically associated with an elevated plasma H(e) concentration. This is compatible with the idea that the MTHFR genotype can create a susceptibility to an elevated plasma H(e) concentration but that the actual expression of elevated plasma H(e) concentration requires other factors and does not specifically require the presence of homozygosity for MTHFR 677T/T. The results suggest that case-finding to identify patients at higher risk for atherosclerosis, and therefore those who might benefit from earlier diagnosis and treatment, would be more effective with measurement of plasma H(e) concentration instead of MTHFR genotyping. It remains to be shown whether an intervention plan based on knowledge of elevated plasma H(e) or treatment of an elevated plasma H(e) concentration itself will reduce the risk of vascular disease in a particular subject.

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