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## Plasma homocyst(e)ine concentration, but not MTHFR genotype, is associated with variation in carotid plaque area

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

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**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 ( $17.1 \pm 13.7$  versus  $13.5 \pm 6.1$  versus  $12.6 \pm 5.9$   $\mu\text{mol/L}$ , 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.<sup>1-7</sup> Of several enzymatic defects or deficiencies that can lead to elevated plasma H(e) concentration,<sup>1,2</sup> 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.<sup>8</sup> Since this variant is common in whites,<sup>9-11</sup> 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.<sup>9</sup> 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.<sup>10</sup> Since then, there have been numerous genetic association studies of the *MTHFR* C677T variant, particularly in the homozygous state, which have shown both the presence<sup>10-16</sup> and the absence<sup>17-27</sup> 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-

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rotid 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.<sup>28</sup> 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.<sup>29,30</sup> 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 *HinfI* as described.<sup>9</sup>

### 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).<sup>28</sup> 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 intraclass correlation coefficient for CPA by the same observer was 0.94. Between-observer reliability of CPA determination from the images was assessed by having 2 technicians exchange their tapes on 25 patients and each measure the plaque area. The intraclass correlation coefficient between observers was 0.99.

### 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 1. Clinical and Biochemical Characteristics of Study Sample**

Characteristic	Men	Women
Subjects, n	159	148
Age, y	49.7±11.8	54.0±10.5
Postmenopausal, %	...	76.8
Body mass index, kg/m <sup>2</sup>	27.1±3.87	26.1±5.02
Total cholesterol, mmol/L	5.49±1.06	5.52±0.96
Triglycerides, mmol/L	2.06±1.24	1.65±1.29
HDL cholesterol, mmol/L	1.03±0.29	1.35±0.40
Plasma H(e) concentration, $\mu$ mol/L	15.0±9.0	12.3±5.4
Smokers, %	46.3	39.9
No. of pack-years smoked	10.2±14.9	7.7±12.7
Diabetics, %	5.3	3.0
Treated for hypertension, %	44.3	43.0
Treated for hyperlipidemia, %	36.2	24.3

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 in 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 homozygous 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 ( $13.7 \pm 7.3$  versus  $10.6 \pm 5.6$   $\mu$ 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* 677T/T homozygotes ( $17.1\pm 13.7$   $\mu\text{mol/L}$ ) compared with both 677T/C heterozygotes ( $13.5\pm 6.1$   $\mu\text{mol/L}$ ) and 677C/C homozygotes ( $12.6\pm 5.9$   $\mu\text{mol/L}$ ). Furthermore, the frequencies of *MTHFR* 677T/T 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* 677T/T 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  $\pm$ SDs of transformed CPA according to ascending quartiles of plasma H(e) concentrations were  $0.56\pm 0.33$ ,  $0.63\pm 0.35$ ,  $0.59\pm 0.30$ , and  $0.76\pm 0.47$ , 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  $\pm$ SDs of transformed CPA in 677T/T homozygotes, 677T/C heterozygotes, and 677C/C homozygotes were  $0.65\pm 0.39$ ,  $0.61\pm 0.36$ , and  $0.68\pm 0.38$ , respectively. Pairwise comparisons indicated that the mean CPA was not significantly different in 677T/T homozygotes compared with 677T/C heterozygotes and in 677T/T homozygotes compared with 677C/C 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**

Variable	Model 1		Model 2	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Age	84.3	<0.0001	92.3	<0.0001
Sex	4.05	0.045	5.44	0.020
Smoking (pack-years)	15.3	<0.0001	17.3	<0.0001
Hypertension treatment	7.33	0.0072	8.10	0.0048
Hyperlipidemia treatment	16.5	<0.0001	18.9	<0.0001
Diabetes history	6.32	0.013	6.31	0.013
Plasma H(e) concentration	3.89	0.049		
<i>MTHFR</i> genotype			0.69	NS (0.50)

### 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.<sup>10–27</sup> 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* 677T/T 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* 677T/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* 677T/T homozygotes and 677C/C 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.<sup>31,32</sup>

There are several possible reasons that *MTHFR* 677T/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* 677T/T 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* 677T/T 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.<sup>2,19,21,33</sup> 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.<sup>34</sup> 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.<sup>35–46</sup> Some studies have demonstrated a close correlation between carotid ultrasound measurements, usually of carotid intimal-medial wall thickness, and angiographic measurements.<sup>35–46</sup> 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.<sup>47,48</sup> 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.<sup>47,48</sup> Thus, the complexity of the atherogenic processes within the arterial wall could weaken the relationship between any single indirect 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 homozygosity 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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