The Methylene tetrahydrofolate Reductase C677T Polymorphism Does Not Associate with Susceptibility to Abdominal Aortic Aneurysm

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Objectives. To test whether the T variant of the C677T polymorphism in the gene for 5,10-methylenetetrahydrofolate reductase (MTHFR) would associate with three distinct forms of vascular disease, abdominal aortic aneurysm (AAA), coronary artery disease (CAD) and peripheral vascular disease (PVD).

Background. Increases in homocysteine induce elastolytic activity in the arterial wall, a condition which may favour vascular pathogenesis including aneurysm formation. Homozygosity of the common T variant of the C677T polymorphism in the gene for MTHFR has been shown to associate with increased levels of homocysteine. Thus, this functional polymorphism may lead to an increased propensity to develop cardiovascular disease and, in particular, AAA.

Methods. An association study was conducted across 1207 subjects; 428 patients with AAA, 271 CAD patients, 226 PVD patients and 282 controls being genotyped for the C667T variants of MTHFR.

Results. There were no significant differences in the frequency of the MTHFR C677T variant between any of the groups examined. AAA patients who were homozygotes for the 677T allele did, however, appear to have significantly larger aneurysms than C allele carriers.

Conclusion. This study provides no evidence that the T variant of MTHFR is associated with susceptibility to AAA, CAD or PVD. It may, however, be a contributory factor in AAA severity as indicated by aneurysm size.

Keywords: Genetics; Cardiovascular disease; 5,10-Methylenetetrahydrofolate reductase (MTHFR); Abdominal aortic aneurysm; Peripheral vascular disease; Coronary artery disease.

Introduction

Abdominal aortic aneurysm (AAA) afflicts a surprisingly large portion of the older population, the incidence increasing with smoking. Though many exacerbating factors have been identified, the basic cause of aneurysms is largely unknown but almost undoubtedly multifactorial with contributions from both environment and heredity. An increase in the plasma level of the amino acid homocysteine (hyperhomocysteinemia) is a common genetic condition strongly influenced by diet. The association between hyperhomocysteinemia and the development of atherosclerosis and coronary artery disease has become well known.

Destruction and degradation of the elastic layers of the abdominal aorta is the sine qua non of AAA. Recently, homocysteine was shown to induce the endogenous vascular elastase (EVE) produced by the smooth muscle cells of the arterial vasculature. The induction of EVE by homocysteine resulted in fragmentation of the internal elastic lamina and fraying and splitting of muscle and elastic fibers of the media. Thus, hyperhomocysteinemia may be a factor in the development of aneurysms as well as atherosclerotic occlusive disease.

A common C→T point mutation in nucleotide 677 of the gene for methylenetetrahydrofolate reductase (MTHFR) converts an alanine to a valine in the protein product of the gene. This results in a thermolabile form of MTHFR and a consequent hyperhomocysteinemia, which can be normalized by a daily low-dose supplement of folic acid. In recent years, much evidence has accumulated implicating the C677T variant of MTHFR as the most common cause of hyperhomocysteinemia and linking it to a wide variety of adverse cardiovascular effects. Recently, the 667T allele of MTHFR has been found to associate with abdominal aortic aneurysm in a Polish population, though it should be noted that...
both studies were, by current standards, underpowered for genotype association analysis. Considering that the 677T variant of MTHFR has been shown to associate with increased levels of homocysteine and in light of the research showing a possible association between hyperhomocysteinemia and AAA, genomic DNA from an Otago population previously characterized for AAA\(^1\)\(^4\)\(^,\)\(^5\) was genotyped for the 677T variant of MTHFR to determine whether this common variant would associate with AAA. For further comparison and elucidation, the Otago coronary artery disease (CAD) and peripheral vascular disease (PVD) populations were similarly genotyped.

**Methods**

**Subjects**

The Otago population of AAA patients\(^1\)(\(n=428\)) was compared with patients with peripheral vascular disease (PVD; \(n=226\)), coronary artery disease (CAD; \(n=271\)) and a healthy control group (\(n=282\)). All patients provided written informed consent and the study was undertaken with the approval and oversight of the Otago Ethics Committee. All AAA patients had aneurysms greater than 5 cm in diameter, necessitating surgical repair. The largest single diameter of the infrarenal aorta was measured by ultrasound and taken to be the size of the aneurysm. A subset of 75 AAA patients (17.5%) had a family history (one or more first-degree family members with an AAA). PVD was defined as significant stenosis in multiple segments, including clinical symptoms such as claudication, rest pain, or tissue loss. Diagnosis was further confirmed with a resting ankle-brachial index less that 0.7, pulse volume recordings and arteriography. CAD subjects all had angiographically proven coronary artery stenosis \(\geq 50\%\) of the vessel internal diameter in at least one vessel. Percent diameter stenosis was visually estimated as the maximal percent reduction in the vessel diameter expressed as a percentage of the angiographically normal adjacent vessel. The number of vessels involved (single, double or triple) and the American College of Cardiology/American Heart Association (ACC/AHA) classification\(^16\) was used to evaluate the morphology of coronary lesions at the index event.

Patients with PVD or CAD also underwent abdominal ultrasound examination, to identify concurrent AAA and were excluded if maximum anteroposterior aortic diameter was greater than 2.5 cm. Control subjects were recruited from local community groups, with inclusion criteria of age greater than 55 years, no history of ischemic heart disease and being currently in good general health.

All subjects were given a questionnaire to ascertain demographic risk factors. Information collected included age, gender, history of hypertension, hyperlipidemia, diabetes (type collected but grouped for analysis), stroke, ischemic heart disease and PVD. Smoking habits (current and past) were assessed, and the number of pack-years was calculated (1 pack-year equalled 20 cigarettes per day for 1 year). Plasma samples were also collected, and lipoprotein A (Lp(a)) levels were analyzed with sandwich enzyme-linked immunosorbent assay.

**PCR genotyping**

Genomic DNA was extracted from blood samples using a modified salting out procedure, and diluted to 50 ng/\(\mu\)l. PCR primers were designed using PrimerSelect (DNASTAR, Inc.) applied to template sequence GenBank accession number AF105980 which contains exon 4 of the MTHFR gene and partial sequences of introns 3 and 4. The C677T single nucleotide polymorphism (SNP) reported in the first paper to observe its effect\(^17\) is at base 129 in AF105980. The AGG-GAGCTTTGAGGCTGACCTGAA (\(C\)) and GGGGACGATGGGGCAAGTGAT (\(K\)) primers amplify a 151 bp-fragment. The restriction enzyme \(Hin\)fl (New England Biolab, Inc., MA, USA) recognises and cuts a GAT\(\text{TC}\) sequence. T allele (underlined) carriers have this sequence and are, therefore, cut into 51 and 100-bp fragments. Alternatively, the equivalent region in C allele carriers has a GAT\(\text{CC}\) sequence which is not recognised by \(Hin\)fl and the PCR product is, therefore, left intact. Subsequent to PCR amplification, \(Hin\)fl digestion and electrophoresis in 1\(\times\) TAE on 3% SeaKem LE agarose gels (BMA Products, Rockland, ME, USA), the fragments from patients and controls were detected by ethidium bromide staining (Fig. 1).

**Statistical analysis**

Statistical analysis was performed using StatView version 5.01 (SAS Institute Inc.). Genotype and allele frequencies were compared using chi-squared tests. For continuous variables ANOVA with Fisher’s PLSD test were performed. Multiple logistic regression was used to test interactive effects of variables. A \(p\) value less than 0.05 was considered significant and all data is presented as means ± one standard deviation.
The frequencies of the common C and rarer T alleles were comparable among all four populations examined (Table 1) and were also similar to those reported in other Caucasians populations. There were no significant differences in the frequency of the T allele or the T homozygote genotype between the control group and the AAA, CAD and PVD patient populations. The distribution of genotypes was comparable among all populations \( (\chi^2 \text{ p value for trend } 0.78) \). All genotypes were in Hardy–Weinberg equilibrium with predicted versus actual genotype \( \chi^2 \text{ p values of: controls 0.98, AAA 0.41, CAD 0.61, PVD 0.55.} \)

The familial AAA subgroup had a T allele frequency of 0.29 with 9.3% being T homozygotes, neither being significantly different from either the control or other vascular disease groups. In the coronary artery disease group, there was no association between either T allele frequency or T homozygotes and the severity of CAD, as assessed by either the number of vessels containing significant stenoses \( (p=0.87) \) or the index ACC/AHA lesion severity scores \( (p=0.68) \). Similarly, this polymorphism did not associate with severity of peripheral vascular disease as indicated by ankle brachial index \( (p=0.32) \). There were no obvious associations observed between this polymorphism and any of the demographic characteristics assessed including age, gender, body mass index, waist circumference or waist–hip ratio. There were, however, significant differences in the demographic risk factor profiles of the four groups examined (Table 2). Using multiple logistic regression to test for these potentially interactive effects a model which accounted for age, gender, non-fasting triglycerides, HDL, Lp(a), smoking pack years, history of hypertension and hypercholesterolemia was applied using the control population and the common C homozygote genotype as references. The resulting adjusted odds ratios and 95% confidence intervals for an association between the T homozygote genotype and disease groups were 1.12 \( (0.56–2.22, p=0.76) \) for AAA, 1.81 \( (0.83–3.93, p=0.13) \) for CAD and 0.90 \( (0.34–2.35, p=0.83) \) for PVD.

Interestingly, abdominal aortic aneurysm diameter at time of diagnosis was significantly greater in T homozygotes compared to C allele carriers \( (6.6 \pm 1.9 \text{ cm versus } 6.0 \pm 1.6 \text{ cm, } p=0.037) \). The resulting odds ratio for T homozygotes and aneurysm size was 1.35 \( (1.05–1.72, p<0.02) \) after adjusting for age, gender, non-fasting triglycerides, HDL, smoking pack years, diabetes, history of hypertension and hypercholesterolemia. There was no such association between the MTHFR C677T genotype and abdominal aortic diameter in either the CAD or PVD patient groups examined.

**Discussion**

The allele frequencies of the C677T variants of MTHFR differ widely among populations around the world, and there is even a distinct gradient within Europe, the proportion of the T allele increasing from North to South.
versus CAD or PVD. ††

0.0001 versus AAA. ‡

Association between disease groups. Most notable was the absence of association between diabetes and AAA. *

Cardiovascular demographic risk factors were not only associated with all three forms of atherosclerotic disease but also showed differential association between disease groups. Most notable was the absence of association between diabetes and AAA.

<table>
<thead>
<tr>
<th>G Lp(a) (nmol/l)</th>
<th>Triglycerides (mmol/l)</th>
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<td>43.9</td>
<td>2.1</td>
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Hypercholesterolemia (%) 19.4 22.6

Hypertension (%) 25.9 55.5* 51.0*§ 66.1*‡

Smoking (pack years) 11.3

Diabetes (%) 8.2 5.9** 21.8* 23.7*

Age (years) 69.5

Gender (%male) 42.6 77.8* 72.3* 57.8*†

<table>
<thead>
<tr>
<th>Controls (n = 282)</th>
<th>AAA (n = 428)</th>
<th>CAD (n = 271)</th>
<th>PVD (n = 226)</th>
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<tbody>
<tr>
<td>Gender (%male)</td>
<td>42.6</td>
<td>77.8*</td>
<td>72.3*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>69.5 ± 7.6</td>
<td>71.7 ± 7.6*</td>
<td>64.0 ± 9.5*</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>25.9</td>
<td>55.5*</td>
<td>51.0*§</td>
</tr>
<tr>
<td>Lp(a) (nmol/l)</td>
<td>43.9 ± 61.6</td>
<td>64.5 ± 90.5*</td>
<td>76.2 ± 95.6*</td>
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Table 2. Demographic characteristics of the control, AAA, CAD and PVD populations

Cardiovascular demographic risk factors were not only associated with all three forms of atherosclerotic disease but also showed differential association between disease groups. Most notable was the absence of association between diabetes and AAA. *p < 0.001 versus controls. †p < 0.0001 versus AAA. ‡p < 0.005 versus AAA or CAD. §p < 0.005 versus AAA. ¶p < 0.005 versus CAD or PVD. ¶¶p < 0.0001 versus CAD or PVD.

South. 18 However, the allele frequencies of the C677T variants in our study subject populations were in accord with those typical in European populations. 11 Most importantly, there were no significant differences in C677T allele frequencies or zygosities among all four populations we studied; AAA, CAD, PVD and controls.

The T variant of the C677T functional SNP in the gene for MTHFR is the most common cause of genetic hyperhomocysteinemia. 9,19 Several small studies have found an association between the incidence of AAA and both the frequency of the MTHFR 677T allele 12,13 and circulating levels of homocysteine. 10,13 These observations appear consistent with reports linking increased homocysteine with risk of cardiovascular disease. 4,6,10 Hyperhomocysteinemia has been specifically associated with PVD, 10,20 CAD, 6,21,22 as well as with AAA. 10,13 Thus, we anticipated possible associations between the T allele of C677T and all three of the phenotypically distinct vascular disease groups examined in this study. It is sometimes the case that associations fail to reach significance because the study population is too small. This may well account for our negative observations in both the CAD and PVD groups. However, at 428, our AAA population is the largest tested, to date, for association with the MTHFR 677T allele. In contrast, Brunelli et al. 13 studied only 58 AAA patients and Strauss et al. 12 studied 63. It is clear that the relationship between hyperhomocysteinemia and vascular disease is complex and multifactorial with influences from both environment and genetics, thus making it difficult to pinpoint the basis of the apparent divergence between this study and the previously reported positive associations. 12,13 For example, variation in allele frequency due to ethnicity 18 cannot be excluded as a confounding factor. Nevertheless, the most probable explanation for the failure of this study to corroborate an association between MTHFR C677T genotype frequency and AAA is the underpowered nature of the previous studies.

It is worth noting that while we did not see an association between this MTHFR polymorphism and apparent susceptibility to AAA there did appear to be an association between this genetic variant and aneurysm size. This effect persisted after correction for possible confounders including age and gender. While this study was not specifically designed to examine this relationship, it appears to be consistent with that reported by Brunelli and co-workers 13 in which they noted that hyperhomocysteinemic AAA patients had significantly larger aneurysms compared to those with normal homocysteine levels (5.09 ± 0.84 versus 5.79 ± 1.5 cm; p < 0.05). The aneurysms observed in this study were significantly larger than those reported by Brunelli et al., with the previous study clearly including a significant proportion of subjects with aortic diameters less than 5 cm. The vast majority of aneurysms in this current study, while being of a size suitable for surgery, were detected incidentally at routine examination or as a result of investigations for other problems and were, therefore, without obvious selection bias. If the association between the MTHFR 677T allele and aneurysm size is borne out, in appropriately designed aneurysm growth surveillance studies, such data would suggest that this allele is influential in the expansion process rather than in the events initiating AAA.

With regard to potential environmental confounders, dietary supplementation with folic acid ameliorates increases in plasma homocysteine in MTHFR 677T homozygotes. Individuals homozygous for the T variant of MTHFR require around 400 μg per day of folic acid (in addition to their usual dietary folate intake) to achieve homocysteine levels comparable with the other genotypes. 3 It is highly unlikely that people living in NZ would have been receiving 400 μg folic acid in their diets unless they were supplement users. Some foods (mainly breakfast cereals) are voluntarily fortified to provide 100 μg folic acid per serve. Studies of Otago residents, the region from
which over 90% the subjects genotyped in this study were recruited, indicate that, based on changes in red blood cell folate concentrations, the average consumption of folic acid is approximately 100 µg per day (personal communication with Dr Bernard Venn, Department of Human Nutrition, University of Otago). Thus, it is unlikely that any impact of the T allele has been reduced by dietary folate consumption in the Otago populations we studied.

A limitation of this study is that control subjects, unlike CAD or PVD patients, were not screened for AAA. Screening studies indicate that less than 5% of our control group is likely to have a AAA (≥3 cm), with less than 0.5% (of men) being expected to have a surgical (≥5 cm) aneurysm equivalent to the AAA patients examined in this study. This would, therefore, correspond to only one or two subjects and it is highly improbable that such ‘contamination’ by asymptomatic AAA’s could represent a major confounder. Moreover, the CAD and PVD groups were screened to exclude AAA and there was no difference in MTHFR genotypes between these groups.

The relationships between hyperhomocysteinemia and vascular disease appear well established with many papers supporting a causal association between increases in plasma homocysteine and cardiovascular pathology since the initial observation in 1969. Though there have also been negative results reported, meta-analysis indicates that there is a likely association of folic acid is approximately 100 g per day (personal communication with Dr Bernard Venn, Department of Human Nutrition, University of Otago). Thus, it is unlikely that any impact of the T allele has been reduced by dietary folate consumption in the Otago populations we studied.

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