THERMOLABILE METHYLENETETRAHYDROFOLATE REDUCTASE C677T MUTATION AND ITS ASSOCIATION WITH VASCULAR DISEASE

by

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CERTIFICATE OF APPROVAL

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Abstract

The goal of this study is to determine whether the prevalence of a common mutation in the gene encoding 5,10-methylenetetrahydrofolate reductase (MTHFR) is higher among vascular disease patients than among control subjects. MTHFR is an enzyme involved in the remethylation pathway of homocysteine (Hcy) to form methionine. As such, it is important in the regulation of methyl donors such as methionine and S-adenosylmethionine (SAM). The particular variant under study is a C to T substitution at locus 677 (C677T) that results in a 50% reduction in MTHFR enzyme activity, in the homozygous state, as well as thermolability of that enzyme (tl-MTHFR). The resulting decrease in catalytic efficiency may result in higher homocysteine levels due to decreased remethylation of homocysteine to form methionine (figure 1).

The amino acid homocysteine is a known risk factor for the development of arteriosclerotic and thrombotic vascular disease. By elevating homocysteine levels in the blood, genetic variation in the MTHFR gene may be a risk factor for vascular disease (Mudd, Skovby et al. 1985; Kang, Wong et al. 1992; Bienvenu, Ankri et al. 1993; Falcon, Cattaneo et al. 1994; den Heijer, Blom et al. 1995; Fermo, Vigano' D'Angelo et al. 1995; den Heijer, Koster et al. 1996). Early evidence linking homocysteine and vascular disease came from studies of homocystinuria and hyperhomocysteinemia where autopsies demonstrated widespread vascular lesions and thromboses (McCully 1969; Mudd, Skovby et al. 1985). It has become apparent from more recent studies that Hcy, and dietary intake of folic acid, B12 and B6, along with mutations in the genes regulating the Hcy biochemical pathway, have complex interactions that may play a part in making an individual at increased risk for vascular disease (Rozen 1996; Welch and Loscalzo 1998).

The results of the present study do not suggest a role for C667T tl-MTHFR in the development of ischemic and thromboembolic vascular disease. The combined vascular disease subjects from several studies with varying clinical outcomes (late-onset vascular disease, acute stroke, and venous thrombosis) demonstrated no significant differences in distributions for the C667T polymorphism (X^2 =2.0, p = 0.37). When the genotype frequencies between those combined case groups and a control group were compared, there was no apparent association between the genotype and disease (X^2 = 5.4, p = 0.25).

Additional stratified analyses, performed to examine the possibility of confounding or interaction with known risk factors for vascular disease (age, gender, and smoking history), uncovered no significant alterations in the genotype frequencies (p > 0.05) or odds ratios (95%CIs include 1.0) across risk factor strata, except among a small group (62 controls, 29 cases) of never-smokers. The C677T genotype frequency was significantly different between the never-smoking cases and controls (X^2 = 8.2, p = 0.02). Homozygotes for the C677T mutation were at a decreased odds of disease (OR = 0.1, 95%CI = 0.01 - 1.99) and heterozygotes at an increased odds (OR = 1.93, 95%CI = 0.79 - 1.63), but neither comparison was significant.

The possibility that the genotype-risk factor-disease association was more complex was further explored by multiple logistic regression to account for the effects of genotype and risk factors simultaneously. The results of this multivariate analysis confirmed the previous analyses, and implied no significant association for either the heterozygous or homozygous C677T MTHFR polymorphism with vascular disease (heterozygote OR = 1.2, p = 0.35, homozygote OR = 0.6, p = 0.20).

Limitations

One of the limitations of this study is the result of the inclusion of both prevalent and incident cases, which could have confounding effects of genotype on disease risk and survival. Another limitation is the lack of a control group that was selected specifically for this case group, which could affect the comparability of the cases and controls. In addition, the lack of information on important co-factors, such as folic acid limits the identification of the individuals at increased risk of disease due to the known gene-environment interactions.

Background

Classic Homocystinuria and Severe Hyperhomocysteinemia

In classic homocystinuria, patients are characterized by high levels of free Hcy in the blood. This elevation occurs in the context of early onset of neurological disorders, arteriosclerosis, arterial and venous thrombosis and premature mortality (McCully 1969; Kang, Wong et al. 1991). Frequently, homocystinuria is the result of severe deficiencies in either the methylation or transsulfuration pathways (see figure 1) (Rozen 1996). In the transsulfuration of homocysteine to cysteine (via cystathionine), deficiency in the enzyme cystathione-ß-synthase (CBS) was found to result in homocystinuria (McCully 1969; Mudd, Skovby et al. 1985). Autopsy of patients with CBS dysfunction reveals disseminated vascular lesions including occlusive sclerotic vascular disease and thromboembolism (McCully 1969; Mudd, Skovby et al. 1985). In the remethylation pathway, severe deficits in the MTHFR enzyme that result in 2% of normal activity are also associated with elevated Hcy levels, thromboembolism and occlusion of cerebral, coronary, and renal arteries (Mudd, Uhlendorf et al. 1972; Baumgartner, Wick et al. 1980).

Moderate and Mild Hyperhomocysteinemia

Hyperhomocysteinemia, in contrast to homocystinuria, is characterized by moderate elevations in protein-bound, rather than free, Hcy in plasma (Kang, Wong et al. 1979). Hyperhomocysteinemia is more difficult to detect because Hcy levels are only moderately elevated and conventional amino acid analysis cannot detect these changes. Moderate hyperhomocysteinemia may result from several genetic and non-genetic factors. Obligate CBS heterozygotes as well as homozygotes for tl-MTHFR are reported to have moderate hyperhomocysteinemia (Kang, Wong et al. 1991; Kluijtmans, van den Heuvel et al. 1996). In the transsulfuration pathway (Figure 1), reductions in pyroxidal 5'-phosphate (a coenzyme of CBS) via a dietary insufficiency of pyroxidine (vitamin B6) can elicit hyperhomocysteinemia by decreasing transsulfuration. In the remethylation pathway, alterations in available folate (Hcy methyl donor) and B12 (methionine synthase coenzyme) in the diet, as well as inborn errors of B12 metabolism, affect the levels of Hcy due to reduced remethylation of Hcy to methionine via MTHFR [(Kang, Wong et al. 1992; Rosenblatt 1995).

Thermolabile MTHFR and Vascular Disease

The association of the thermolability of the MTHFR (tl-MTHFR) enzyme with vascular disease has been described in numerous epidemiologic studies (Rozen 1996). In 1991, Kang et. al., reported that 17% of cardiac patients versus 5% of controls demonstrated thermolabile MTHFR by biochemical assay (Kang, Wong et al. 1991). More recently, a common mutation in MTHFR (tl-MTHFR, C677T) was described (Frosst, Blom et al. 1995). This mutation was correlated with thermolability and reduced enzyme activity (approximately 50% reduction in lymphocyte extracts). The isolation of the cDNA for this

gene made the development of polymerase chain reaction (PCR) genotyping routinely possible (Goyette, Sumner et al. 1994). It is interesting to note that obligate heterozygosity for severe MTHFR deficiency results in approximately 50% of normal enzyme activity (Kang, Wong et al. 1991), which is the same proportion associated with homozygosity for the C677T tl-MTHFR polymorphism (Rozen 1996). Since the prevalence of the C677T homozygous mutation (8.3-16.5%, Fletcher, Kessling 1998) is much greater than the frequency of heterozygotes (estimated from biochemical analyses) for the severe deficiency (0.02%), it represents a far greater potential public health impact. One study suggests that the C677T polymorphism may account for 28% of moderate hyperhomocysteinemia among patients with premature vascular disease (Blom 1998). In a meta-analysis examining the relationship between Hcy and vascular diseases Boushey et. al., computed that a 5µmol/l increase in plasma Hcy corresponded to an odds ratio of coronary artery (CAD) disease for men of 1.6, and 1.8 for women. Based upon the known association of Hcy with CAD they reported a population attributable risk of 10% for men (Boushey, Beresford et al. 1995).

C677T tl-MTHFR and Vascular Disease

In a previous study from our laboratory, the relationship between tl-MTHFR, C677T, folate, and Hcy in 247 vascular disease subjects and 594 healthy subjects without vascular disease was examined (Deloughery, Evans et al. 1996). Linear regression analysis revealed that subjects homozygous for the tl-MTHFR, C677T mutation had a significantly more negative slope relating folate and Hcy ($r = -0.52$, $p = < 0.001$, see figure 2). This suggests that subjects homozygous for the mutation have an elevated response to folic acid depletion as well as a potentially greater response to folate replacement (Deloughery, Evans et al. 1996). Furthermore, the analysis implies that heterozygotes for the polymorphism

are not at the same risk of elevated Hcy (as compared to wild types) even when they are folate deprived (unpublished observation). In addition, elevation in Hcy among homozygotes was folate dependent and only significant at the lower folate values. In another study, Jacques et. al., stratified their analysis by folate levels and found that Hcy differences among genotypes occurred at lower plasma folate concentrations (<15.4 nmol/l) but not at higher levels, providing further evidence of a dynamic interaction between folate, genotype and Hcy levels (Jacques, Bostom et al. 1996).

To date, the association of the C677T MTHFR homozygous genotype with vascular disease has been mixed (Deloughery, Evans et al. 1996; Kluijtmans, van den Heuvel et al. 1996; Wilcken, Wang et al. 1996). Some of these studies have attempted to control for dietary factors that modulate Hcy including folate, B12, B6, and smoking (Deloughery, Evans et al. 1996; Harmon, Woodside et al. 1996; Jacques, Bostom et al. 1996; Ma, Stampfer et al. 1996; Schmitz, Lindpaintner et al. 1996; Schwartz, Siscovick et al. 1997). It is difficult to compare the studies of the C677T mutation and vascular disease due to the variety of subjects examined, the inclusion or exclusion of Hcy modifying dietary factors (folate, B12, B6), and risk factors that increase Hcy (e.g., smoking, alcohol). Furthermore, single measurements of folate, B12, B6, and Hcy may not be an adequate substitute for lifetime Hcy exposure.

Rationale

Although there has been substantial evidence that moderately elevated Hcy levels, as seen in hyperhomocysteinemia, are correlated with vascular disease (Welch and Loscalzo 1998), the association with the tl-MTHFR C677T mutation and disease sequelae remains uncertain (Fletcher and Kessling 1998). Since most studies have shown that only homozygotes have significantly elevated Hcy (Rozen 1996; Blom 1998) it is possible that homozygotes would

be over-represented in the groups of patients with stroke, ischemic heart disease, and occlusive vascular disease. In our study previous study (Deloughery, Evans et al. 1996), and in others (Jacques, Bostom et al. 1996), homocysteine was only significantly elevated at lower serum folate values, suggesting that only folate-depleted C677T homozygotes may be at increased risk of vascular disease. Because of this gene-environment interaction, demonstrating an effect of the C677T requires greater statistical power than if the gene alone were sufficient to cause vascular disease.

One way to increase the power of a study is to increase the sample size by broadening the case definition. Except for the vascular disease study, (Deloughery, Evans et al. 1996) our previous studies have evaluated the presence of the C677T mutation and the association with particular clinical outcomes such as stroke and venous thrombosis. That analysis is appropriate when a particular disease outcome is of interest. However, with respect to the C677T polymorphism and elevated Hcy, the outcome of interest may be vascular disease generally, including ischemic and thromboembolic mechanisms. The justification for combining these study groups comes from the results of autopsies of patients with homocystinuria (McCully 1969), and studies of moderate hyperhomocysteinemia (Fermo, Vigano' D'Angelo et al. 1995), where elevated Hcy has been shown to cause atherosclerotic damage, and to increase the tendency for thrombosis (Welch and Loscalzo 1998).

Materials and Methods

Subjects

Cases

The subjects for this study come from several previously published case-control studies of the tl-MTHFR C677T mutation and vascular disease. The three studies evaluated acute stroke (Press, Beamer et al. 1999), atherosclerotic peripheral and cerebrovascular disease (Deloughery, Evans et al. 1996), and thrombotic venous disease (Ocal, Sadeghi et al. 1997). For the purposes of this analysis, cases from each of the three studies will be evaluated in terms of any type of vascular disease, rather than for a particular disease outcome (e.g., stroke) as in the original analyses. There were 530 cases available from the 3 case groups (Table 1), and 519 of those were used for the genotype analyses (Table 2).

Acute Stroke Study

The Acute Stroke study prospectively recruited elderly subjects from the wards and outpatient clinics at the Portland Veterans Affairs Medical Center and the Oregon Health Sciences University (Press, Beamer et al. 1999). Subjects for the study were classified into three groups; acute stroke, those with risk factors for stroke, and healthy elderly individuals. Only subjects with an ischemic stroke within 7 days of enrollment were included in the acute stroke group. The Stroke Data Bank (Jacobsen, Gatautis et al. 1994) criteria were used to classify the stroke mechanisms (athero-embolic 28%, cardio-embolic 27%, lacunar 43%). Subjects with cerebral venous thrombosis, or subarachnoid or intracerebral hemorrhage, were excluded. The stroke risk group was composed of individuals with two or more risk factors for stroke such as, prior history of stroke or TIA, hypertension, diabetes mellitus, tobacco use, atrial fibrillation, ischemic or valvular heart

disease, or peripheral vascular disease. For the stroke study, the acute stroke group will all be included as cases in this analysis. Subjects in the stroke risk group will be included as cases only if they have a specific history of vascular disease (previous stroke, peripheral vascular disease, transient ischemic attacks (TIA). The total number of stroke study subjects with some form of vascular disease is 185 (Table 1). Individual information on race was not available, but about 98% of the participants were Caucasian (personal communication). All cases were included in these analyses (Table 2).

Vascular Disease Study

In a prospective study of the effects of homocysteine and atherosclerosis, 247 patients were recruited from the vascular surgery clinic at Oregon Health Sciences University (OHSU) (Table 1). Subjects were between 40 and 80 years of age (mean, 68 ± 11 years) with documented evidence of symptomatic atherosclerotic, cerebrovascular, or peripheral vascular disease. Patients with end-stage vascular disease were not eligible. Other atherosclerotic risk factors in this group included hypertension (60%), diabetes (22%), smoking (33% current smokers, 56% ex-smokers), heart disease (48%), hypercholesterolemia (mean cholesterol level, 221±45 mg/dl), and male (63%). For the analyses for this study only Caucasians from the vascular disease study (Deloughery, Evans et al. 1996) were used (n=236) (Table 2).

Venous Thrombosis

The venous thrombosis group was assembled from 685 patients who were originally referred to a diagnostic molecular pathology lab for suspected Factor V Leiden deficiency. Of the 685 patients, 331 (48%) tl-MTHFR C677T were randomly selected for genotyping. There were brief clinical histories available for 212 (31%) of the 685 original subjects. Histories from 157 (74%) cases suggested venous thrombosis, while 32 (15%) indicated

arterial thrombosis as the reason for the diagnostic referral. The remaining subjects could not be classified based upon the available histories (23 subjects, 11%). The complete group of patients had a mean age of 44 ± 16 years, were 44% male, and 21% were either heterozygous or homozygous for the factor V R506Q (Leiden) mutation. The subjects referred for factor V (Leiden) evaluation will only be eligible for this study if their brief clinical histories included a diagnosis of thrombotic disease (venous or arterial, n=189). Genotype analysis for C677T MTHFR was available for 98 of the 189 subjects with brief clinical histories (Table 2). No race or smoking history information was available for the Venous Thrombosis subjects. Race data was not available for the Venous Thrombosis group, but was approximately 98% (personal communication).

Control Subjects

The control group for this study was originally recruited as part of a study investigating common polymorphisms in cancer risk (courtesy of Dr. David Henner, Hematology and Medical Oncology, OHSU). The subjects were recruited by advertisement in local newspapers, and through the distribution of flyers to public locations (e.g., libraries). Controls were frequency matched to cases with head and neck cancer based upon age, and smoking history. Eligible controls were over age 18, and were free of any history of malignancy (except non-melanoma skin cancer or carcinoma in situ of the uterine cervix). To enrich the control group with minority races, for the purpose of evaluating varying rates of polymorphisms across racial and ethnic groups, no age or smoking criteria were required for minority controls. There were 349 total controls available for genotyping (Table 1). The subjects were predominantly Caucasian (87%), and female (53%), and had significant smoking history (mean pack years ± SD = 35 ± 27)), though many had never smoked (23%). For the purposes of comparison to the case subjects in this study, only Caucasian subjects were selected for the primary analysis (N = 272). The Caucasian

subgroup was 51% male, and had a mean pack years smoking history of 29 (SD = 29) among smokers (Table 2). The selected controls had 62 (23%) never-smokers, and 208 ever-smokers (77%) within the group.

Study subjects gave informed consent for the respective studies, and each study was approved by the human studies Institutional Review Board at OHSU.

PCR Methods

For the subjects from each study that had not been genotyped for the C677T tl-MTHFR mutation previously, PCR analysis was performed as previously described by Frosst et. al.. (Frosst, Blom et al. 1995) with minor modifications. This method was previously used to genotype the cases from each of the three case groups.

DNA samples were obtained (courtesy of Dr. Richard Press, Department of Pathology, Oregon Health Sciences University) as 200ng/µl stocks. 50ng of DNA, 200ng of each primer (forward: 5' TGAAGGAGAAGGTGTCTGCGGGA 3', reverse: 5'AGGACGGTGCGGTGAGAGTG 3'), 10mM deoxynucleoside triphosphates, 5µl of 10X reaction buffer (Roche Biochemicals), and 1.25 units of Taq polymerase were combined. Reactions were incubated for 35 cycles in a thermocylcer (MJ Research) for 2 minutes at 95C to denature, followed by 30 seconds primer annealing at 60°C, and 60 seconds of primer extension at 72°C.

Following PCR amplification, samples were fragmented by incubating with 5 units of the restriction enzyme Taq1 (New England Biolabs) at 65°C for 3 hours. Fragments were resolved on a 3% agarose gel run at 4°C for 2 hours at 100 volts. Following ethidium bromide staining the gel was photographed under ultraviolet illumination.

Data Analysis

Before genotype distributions were examined, basic descriptive analyses were performed. These included measures of central tendency (mean, median) and dispersion of the data (interquartile range, and minimum and maximum values), and frequency distributions of each of the study variables (age, gender, smoking history) by case-control status (data not shown). This preliminary analysis provided an understanding of the trends within the dataset, and was useful in subsequent analyses for determining cut-off values for stratified variables.

The genotype distributions for each of the study groups was examined to determine if they were in Hardy-Weinberg Equilibrium. Provided that there is random mating within a population, the Hardy-Weinberg Equilibrium predicts that the frequency of genotypes will come to equilibrium that is stable over time.

Subsequently, the genotype distributions between the cases and controls were stratified on the risk factors (age, gender, and smoking history) to look for gross changes in the genotype frequencies that might suggest interaction or confounding.

Following the preliminary analysis, the genotype-disease association was examined by chi-square analysis. In particular, genotype distributions for the tl-MTHFR C677T mutation for vascular disease subjects and controls were compared. Following basic analysis of the genotype-disease relationship, stratified analysis was also performed to determine whether there is any confounding or interaction (effect-modification) in the relationship between tl-MTHFR C677T mutation, vascular disease, and known risk factors such as age, gender, and smoking history.

Following stratified chi-square analysis, the data was examined by multivariate analytical methods. The genotype-disease association, adjusted for potential confounding variables such as gender, age, and smoking was estimated by multiple logistic regression analysis (Table 15). All statistics were performed using the Statview 5.0 (SAS) statistical package.

The distributions of the MTHFR C677T genotypes among case groups were also compared and combined using the Mantel-Haenszel method. The distributions of the genotypes were first examined using a test of homogeneity to determine if the different case groups could be combined. Subsequently, a summary odds ratio (OR) was computed, followed by a test of significance for the OR.

Results

Selection of Study Subjects

For this study the total group of subjects genotyped for the C677T polymorphism included 349 control subjects (Control group), and 530 combined cases from three separate case groups (Table 1). Of the 530 total cases (Combined Case group) there were 247 from the prospective study of ischemic vascular disease (Stroke group), and 98 were from venous thrombosis patients (Venous Thrombosis group), and 185 with a history of ischemic or thromboembolic vascular disease from a stroke study (Vascular Dx group).

Race information was only collected for the Vascular Dx and Control groups (Table1). Both groups were primarily Caucasians (Controls = 87%, Vasc Dx = 96%). All other analyses for this study were performed on a selected set of cases and controls (Table 2). The Control group and the Vascular Dx cases were limited to Caucasians only, because the

number of subjects of other races was insufficient for analysis. Exclusion based on race was not possible for the Venous Thrombosis or Acute Stroke groups since that data was not available from the authors of those studies, but both groups were estimated to be 98% Caucasian (personal communications). Stratifying genotype analysis based upon race is important because of the varying genotype frequencies for MTHFR across racial groups. For example, it is known that the frequency of the homozygous C677T genotype among Black Americans is much lower than that for Caucasians (0% compared to 8-16% for Caucasians) ((McAndrew, Brandt et al. 1996; Stevenson, Schwartz et al. 1997)). Among Japanese the frequency is 10-11% %, (Nishio, Lee et al. 1996; Morita, Taguchi et al. 1997). In the control group for this study Black Americans had a significantly reduced frequency (0% compared to 14% for Caucasian controls) of the homozygous C677T genotype (Table 3, $p = 0.004$). By selecting for all Caucasians among the control group, any subsequent analyses comparing the controls to the cases will tend to be biased toward the null hypothesis of no association (larger Type II error). The few Black Americans and other races in the analyses would most likely decrease the overall frequency of C677T genotypes among the case group, thereby reducing the likelihood of demonstrating an increased prevalence of the C677T mutation among the case groups.

Demographics

The demographic distributions of age, gender, and smoking history for the selected cases and controls are shown in Table 2. There were 272 Caucasian controls and 519 combined cases included in the groups for the study analyses. Of the 519 total cases, 236 were Caucasians from the Vascular Dx group, 98 were from the Venous Thrombosis study, and 185 were subject with a history of ischemic disease from the Stroke study.

Age Distributions

The median ages for the various case groups were similar between the Vascular Dx and Stroke groups (69 vs. 67), while the Venous Thrombosis groups was younger (49). The minimum and maximum ages for the case groups shows that the Venous Thrombosis group includes some younger subjects than the other groups (min-max = 14-79), but that it also includes older subjects. Another measure of the dispersion of ages, the interquartile-range (IQR), suggests that, excepting the Venous Thrombosis group, the range of ages is similar across the study groups. The median age of the control group (51) was 17 years younger than for the combined cases (67). Although the median ages were different between the cases and controls, the minimum and maximum ages between the combined cases and controls were similar (controls = 24 - 86, cases = 14 - 88) (14).

Gender Distributions

While Vascular Dx and Stroke groups had a greater frequency of men than women (64% and 89% men) the majority of subjects from the Venous Thrombosis group were women (56%). The control group had a nearly equal proportion of men and women (51% and 49%), while the combined case group contained a greater percentage of men (69%), possibly reflecting the increased risk of vascular disease among men.

Smoking History

Smoking history was collected for the Vascular Dx and Stroke groups, but was not available for the Venous Thrombosis group. The Stroke group had fewer mean pack years of smoking than did the Vascular Dx group (40 vs. 53), and had a greater proportion of never-smokers (17% vs. 0.5%). Ever-smokers in the control group less exposure than did smokers in the combined case group (29 vs. 47), and a much larger fraction of controls were never-smokers (23% vs. 8%). The combined cases had more significant missing

smoking data (27%), mainly due to the absence of smoking information on the Venous Thrombosis group.

Genotype Distributions

Genotypes for the case groups (Table 4) show some variation in the frequencies of each allele across the groups. The Stroke group had the highest heterozygote (het) rate (55%) and the lowest wild-type (wt) (38%) and homozygous mutant (mut) rates (8%). Among the cases, the Venous Thrombosis group had the highest proportion of mut genotypes (13%). The Control group as compared to the combined cases was roughly similar, with the Combined Cases having slightly more het genotypes (50%) as compared to the Control group (45%), while the Control group had a greater percentage of the mut genotype (14% vs. 9%).

Hardy Weinberg Equilibrium

Under the assumption of random mating in the population, the frequencies of different genotypes will come to a distribution that is stable over time. Deviations from Hardy-Weinberg equilibrium can occur due to differences in survival for each genotype, but they may also be observed as a result of population structure, such as selection of a group of subjects with varying ethnic backgrounds. Each of the case and control groups was examined for Hardy-Weinberg equilibrium and only the Stroke cases significantly deviated ($p = 0.006$). If the genotype distribution in the control subjects was not in equilibrium, it would be difficult to interpret comparisons based upon them. The genotype distributions in the case groups may be out of equilibrium because of an association between the disease and a specific genotype.

Case Groups: Comparison of Genotype Frequencies

Before comparing the controls to the selected-case subject groups, initial analyses were performed to determine the similarity of the case groups with respect to the C677T MTHFR polymorphism. This analysis was first performed using a 3X3 chi square analysis (Table 5) comparing the frequencies of the genotypes across the three case groups (Vascular Dx, Acute Stroke, and Venous Thrombosis). The results indicate no significant difference in genotype distribution among the groups ($p = 0.25$). The case groups were also compared using the Mantel-Haenszel method, which permits the computation of a summary OR for the three case groups compared with the control group (Table 16). Before combining the data, the ORs for the individual case groups (as compared to the control group) were tested for homogeneity. The ORs for the individual case-control comparisons were not statistically different ($p=0.21$). The Mantel-Haenszel summary OR was 0.71 for C677T homozygotes compared with wild-type homozygotes (95%CI = 0.49 to 1.05). The Mantel-Haenszel test of association revealed no genoytpe-disease association ($p=0.26$).

Another way to express genetic differences among a population is by allele frequencies (Table 6). In this case, the mutations are not expressed as genotypes, but rather as the frequency of allele frequencies in a particular group. For each person there are two alleles possible, so there are twice as many alleles as subjects. The relative frequency of alleles, like the genotype frequencies, were not significantly different across case groups, again suggesting that the groups are similar with respect to the C677T polymorphism ($p = 0.37$).

Genotype Distributions: Combined Cases and Controls

The primary purpose of this study is to determine if there is an association between the C677T polymorphism and ischemic vascular disease. As a first test of this relationship the genotype frequencies (Table 7) between the combined cases and the control group were compared by chi-square analysis (Table 8). The results suggest that there is no association between the genotype and vascular disease ($X^2 = 3.5$, $p = 0.17$). The odds ratios (ORs) and confidence intervals (CIs) for the comparisons of individual genotypes wt (ala/ala), het (ala/val), and mut (val/val) reveal no significant association for either the heterozygous or homozygous mutant genotypes as compared to the wild type. Although the ORs in this analysis differ from one, their corresponding CIs include one, indicating that the association could be due to chance.

Genotype Distributions: Stratified Analyses for Combined Cases and Controls

To look for evidence of confounding, or effect modification, in the genotype-disease association the selected cases and controls were stratified on several known risk factors for ischemic vascular disease (age, gender, and smoking history). By looking for a change in the genotype frequencies and odds ratios across strata, effects of the risk factor on the disease-genotype association may be detected. If the ORs between strata change (e.g., Table 14), then there is evidence for interaction between the genotype and the risk factor, if the ORs across strata are similar, but different from the overall OR (e.g., Table 10) then there is evidence for confounding. Chi square analyses were performed on each of the stratified groups. When the genotypes were stratified on gender (Table 11) no significant

disease-genotype association (Table 12) was detected for women or for men (women: p = 0.45, men p = 0.27). The ORs and 95%CIs for the individual genotype comparisons stratified by gender also suggested no association between the genotypes and vascular disease. Similar lack of association was found when the analyses were stratified on age (p > 0.05 for all genotype comparisons, 95%CIs all include 1.0) (Table 9,10).

Only when genotype was stratified according to smoking history (Table 13) was there any evidence of interaction (Table 14). Never-smoking controls were more often wild-type (wt) and homozygous mutant (mut) and less often heterozygous (het) than cases (p = 0.02). Although an exact odds ratio could not be calculated since the combined cases included no never-smoking homozygous mutants (val/val), by adding 0.5 to each cell in the analysis it is possible to obtain an OR (OR = 0.10; 95%CI = 0.01 - 1.99). Among ever-smokers, there was no association between case-control status and genotype. Although this observed difference between ever-smokers and never smokers is intriguing, it is based on a very small number of never-smokers.

Genotype-Disease Association: Multivariate Analysis

While the overall analysis as well as the stratified results concluded that there was no association between the C677T polymorphism and vascular disease, other than for a small group of non-smokers, it could still be possible that the relationship was more complex. To examine this possibility multivariate analyses were undertaken to simultaneously control for each of the potentially confounding risk factors (age, gender, and smoking history) while evaluating the effect of the genotype on vascular disease (Table 15). The results of the logistic regression analysis indicated no significant association between the genotype and vascular disease for either of the polymorphic genotypes (het or mut) as compared to wild-type (het: OR = 1.2, 95%CI = 0.8 - 1.9; mut: OR=0.6, 95%CI = 0.3 - 1.3). There

was also no convincing evidence for confounding based upon the logistic ORs and CIs for the included risk factor variables (Table 8 vs. Table 15).

Genotype-Disease Association: Individual Case Groups Compared to Controls

Another way to examine the genotype-disease association among the various case-groups would be to compare each case group separately with the controls to look for associations specific for the particular study that the cases were drawn from (Table 16). Genotype frequency comparisons between the controls and Venous Thrombosis and Vascular Dx groups show no significant associations ($p > 0.05$ for both comparisons). The comparison with the cases from the Stroke study are nearly significant ($X^2 = 5.88$, $p = 0.054$), though the comparisons by individual genotype show no similar trend (95%CIs include 1.0).

Discussion

There seems to be little doubt now that elevated homocysteine is a risk factor for vascular disease. It has been suggested that moderate hyperhomocysteinemia confers the same risk of coronary artery disease as moderate increases in serum cholesterol (Boushey, Beresford et al. 1995). It is also apparent that rare mutations in either the methylation or transsulfuration pathways result in elevated Hcy, and subsequent, early onset, vascular disease. The evidence for the tl-MTHFR C677T polymorphism and vascular disease risk is somewhat less clear. The reason for this may be that the polymorphism only reduces the enzyme activity by 50% per allele, so that only homozygotes exhibit significantly elevated Hcy. Another factor, effectively reducing the penetrance of the mutation, is that dietary intake of folic acid moderates Hcy values, and if sufficient, can reduce Hcy levels in C677T homozygotes to normal levels (Boushey, Beresford et al. 1995; Deloughery, Evans

et al. 1996; Jacques, Bostom et al. 1996). Since only folate-depleted homozygotes are at risk of elevated Hcy, it is conceivable that many case/control studies have not had the power to detect a small change in risk among this subset of homozygotes. Given the relatively small proportion of the population at risk, a proportionately large case-control study is required to detect the elevated risk.

Although the results of this study reveal no convincing evidence of an association between the C677T tl-MTHFR polymorphism and ischemic and thromboembolic vascular disease, it cannot be concluded that no relationship actually exists. The present study combined case groups to increase the statistical power of the analyses, so that an increase in disease risk among the subset of homozygous mutants, with decreased folic acid in their diets, could be detected as an increase in the proportion of homozygotes in the combined case group. While, in theory this would be possible, the realities of combining multiple case and control groups originally designed to answer different scientific questions limited the precision and validity of the studies analyzed and conclusions that were realistically achievable. This criticism is not unique to this study alone, however, and in some ways is responsible for the ambiguity over tl-MTHFRs association with vascular disease across the many studies that have attempted to associate the mutation with vascular disease (Table 17). In fact, to design and implement a case-control study to adequately address the issues associated with polymorphism genotype-disease association studies is a difficult task. Firstly, because the frequencies of polymorphisms tend to be group specific, the cases and controls ideally need to be drawn, not simply from the same racial group, but rather from the same ethnic group. This point is illustrated in a review of the C677T literature (Fletcher and Kessling 1998), where the rates of the polymorphism across Caucasians is shown to vary from 7.3% (Kirke, Mills et al. 1996) for healthy Irish women to 16.3% for Italians (Fletcher and Kessling 1998). Secondly, in studying complex diseases with multiple etiologies, and

because polymorphisms tend to be weakly penetrant, the sample sizes of studies must be rather large to accommodate adjustment for multiple factors.

The combined number of cases from the three different case groups in this analysis was 519, and the total number of controls was 272. If it is true that the homozygous mutants for the C677T MTHFR polymorphism represent the "at-risk" group, then based upon of the prevalence of 14% homozygous mutants among the combined control group, the proposed analyses would have 80% power to detect an odds of 1.78 or greater (p=.05). If however, the folic acid profile is a necessary factor in determining the risk of homozygotes, then only the folate-deprived homozygotes would be at risk, and the power calculation should be based upon them. If 50% of the homozygotes were folate-depleted, then this analysis would have 73.6% power to detect an OR of 2.0 (p = 0.05). If the number of cases and controls were equal, 414 cases and controls would be required to detect an OR of 2.0 (power = 80%; p = 0.05). Based upon this estimate, no study to date, including this one, has examined the genotype-disease association with sufficient numbers of subjects to be certain that a clinically significant association did not in fact exist.

Although crude, this analysis demonstrates the importance, in genotype-disease associations, of including environmental components that modulate risk when determining sample sizes. Fletcher and Kessling in their review of the association of the C677T polymorphism and vascular disease risk make a similar point (Fletcher and Kessling 1998). Their analysis is based on the meta-analysis of Boushey et. al., which calculated for every 5μmol/l increase in Hcy an increased odds ratio of disease of 1.6 for men, and 1.8 for women. From those figures they calculate that, even given the highest reported increase in Hcy seen in any study (1.8μmol/l), there was not sufficient power to detect a difference in the proportion of homozygous mutants between cases and controls.

Other Limitations

While this study was designed to overcome the difficulty of detecting the C677T-related risk of vascular disease among a subset of vascular disease subjects, there are serious flaws with such an approach beyond issues of sample size. An important limitation is that the subjects for each of the combined case groups were ascertained in different ways. In the vascular disease and venous thrombosis studies, cases could be either prevalent or incident, while in the stroke study recurrence of stroke was the primary criterion for recruitment. These differences may be important if the C677T mutation affects survival after an event, such that prevalent cases are less likely to have the mutation because it is associated with early mortality, such as a fatal first heart attack or stroke. In this way, the prevalence of the mutation would be lower in prevalent case groups than in incident groups, and the mutation may even appear to be protective (OR <1.0) if it is truly a factor in early-onset, fatal disease, because the polymorphic alleles would be removed from the study case groups.

Along with problems associated with case group selection, the control group is also not ideal, because subjects were not selected as controls for any of the case groups. The controls were selected to be free of cancer for a study of common genetic polymorphisms and cancer risk. It is not known what fraction of the controls were free of vascular disease, and given that vascular disease is common in the population, it is likely that some of them had some sort of vascular disease, particularly among the older controls. If the C677T polymorphism is associated with vascular disease, a comparison of the cases and controls may not detect a difference in genotype frequency due to a significant number of controls with unknown vascular disease.

Another critical limitation is the lack of measurements of other factors known to alter plasma Hcy, and therefore may have a potential confounding or modifying effect on the genotype-disease relationship. Since it has been repeatedly shown that the effect of C677T on Hcy can be alleviated by higher folate intake (and B6 and B12), and that Hcy is increased by increasing age and smoking, measurement of the levels of all of those factors would be necessary to correctly define what proportion of the risk due to elevated Hcy was the result of the C677T mutation. Although some studies have measured Hcy, folic acid, B6 and B12 along with the MTHFR genotype, none have done it prospectively. Single measurements of these dietary factors and plasma Hcy may not be an appropriate substitute for a lifetime of Hcy exposure as a result of the interaction between an individual's daily dietary intake of folic acid, B6 and B12, age, smoking history, and the C677T MTHFR mutation.

Future Directions

The ideal study would be a prospective cohort study where serum homocysteine and dietary intake of B6, B12, and folic acid were serially measured. It would also be important to account for the age and smoking habits of the cohort. Under these conditions, the effect of the tl-MTHFR on vascular disease risk could be isolated from the other factors affecting homocysteine metabolism. It is possible to improve upon the current case-control study by recruiting incident cases to avoid potential bias by genotype-specific survival, and selecting a control group free of vascular disease, but matched on smoking history and age. Measurements of folic acid or a historical dietary assessment would also improve the current case-control study design. Ultimately the demonstration that tl-MTHFR C677T is a risk factor for disease resides in demonstrating that it consistently elevates the proven risk factor, Hcy, independent of other effectors of Hcy. For tl-MTHFR, consistently elevated Hcy will only occur under the conditions of low folate

intake. Thus, the primary analysis of such a study should evaluate whether there is an excess risk of disease due to the C677T polymorphism among subjects with low folate intake. Such goals could be achieved with ongoing longitudinal studies such as the Physician's Health Study, that provide accurate ascertainment of new vascular disease, and have serially collected plasma or blood samples available for prospective folic acid, B6 and B12, and Hcy measurements, and for genotype analysis of the C677T MTHFR polymorphism (Stampfer, Malinow et al. 1992).

Summary

This investigation's intended purpose was to examine the genotype-disease association for the C677T polymorphism in MTHFR across vascular disease subjects from multiple case groups with varying clinical endpoints as their focus, and compare them to a control group with similar risk factors, including age, gender, and smoking history. The case subjects from three different studies of vascular disease (late-onset vascular disease, acute stroke, and venous thrombosis) demonstrated no significant differences between them in their distributions for the C667T polymorphism (X^2 =2.0, p = 0.37). Combining those case groups and comparing them to the control group revealed no apparent association between the genotype and disease (X^2 = 5.4, p = 0.25).

The other purpose of the current study was to stratify the genotype-disease analysis to examine any potential effect-modification or confounding from known risk factors, as a way of understanding the interplay between genotype, environment, and behavior in disease risk. Only among never-smoking cases and controls was there any significant suggestion of a genotype-disease association (p = 0.02) and effect-modification, but the numbers for that analysis were very small (n = 0 cases among the never-smoking

homozygotes). Since so many tests were performed during this study, it is possible that the result was a chance association reflecting only the current sample, and therefore not broadly applicable.

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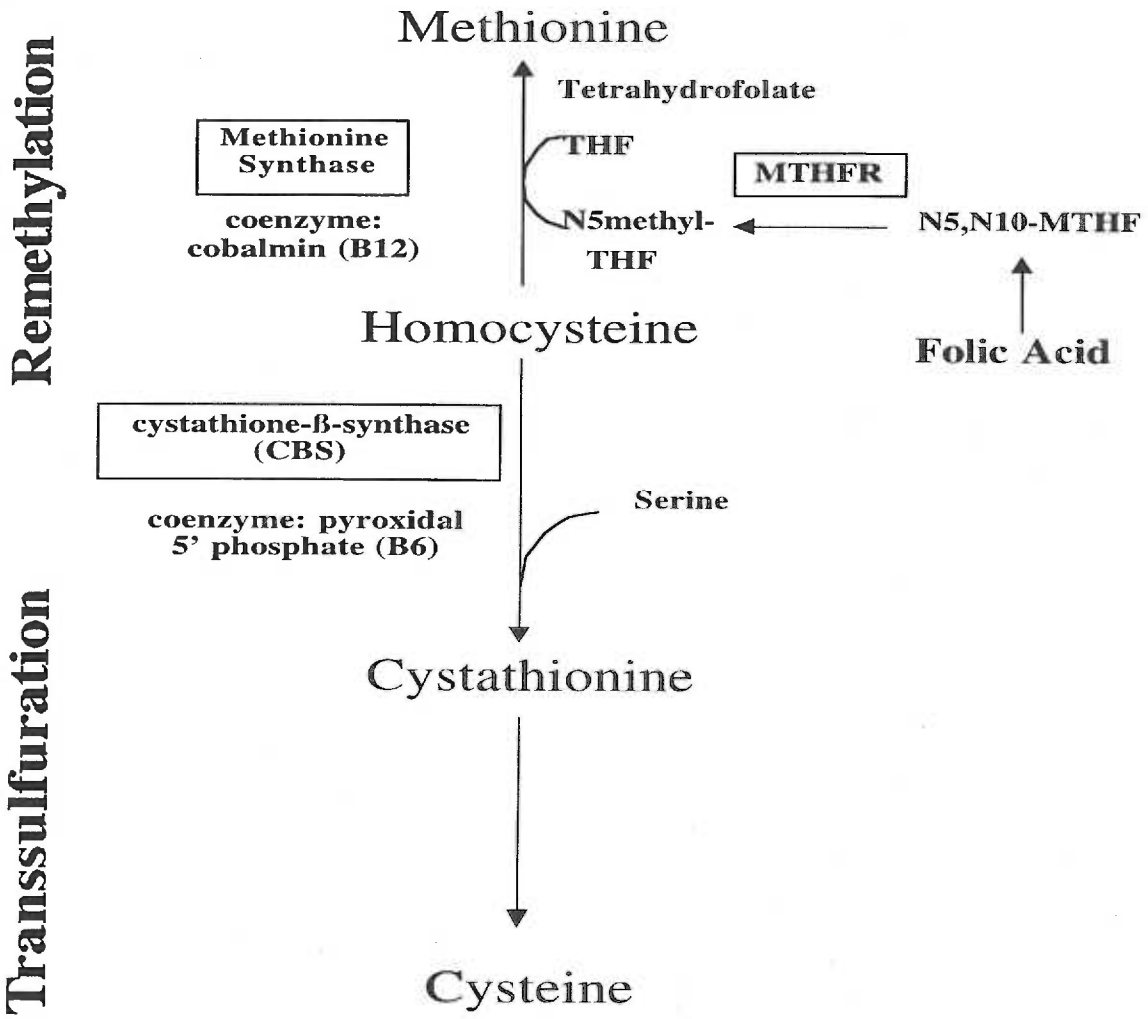
Figure 1. Biochemical Pathway for MTHFR and Homocysteine



Reduction in MTHFR enzyme activity due to the C677T tlMTHFR mutation results in decreased availabilty of N5methylTHF, with subsequent reductions in the remethylation of homocysteine to form methionine.

Figure 2. Relationship Between Serum Folate, Hcy, and MTHFR Genotype

(Adapted from (Deloughery, Evans et al. 1996)*)*

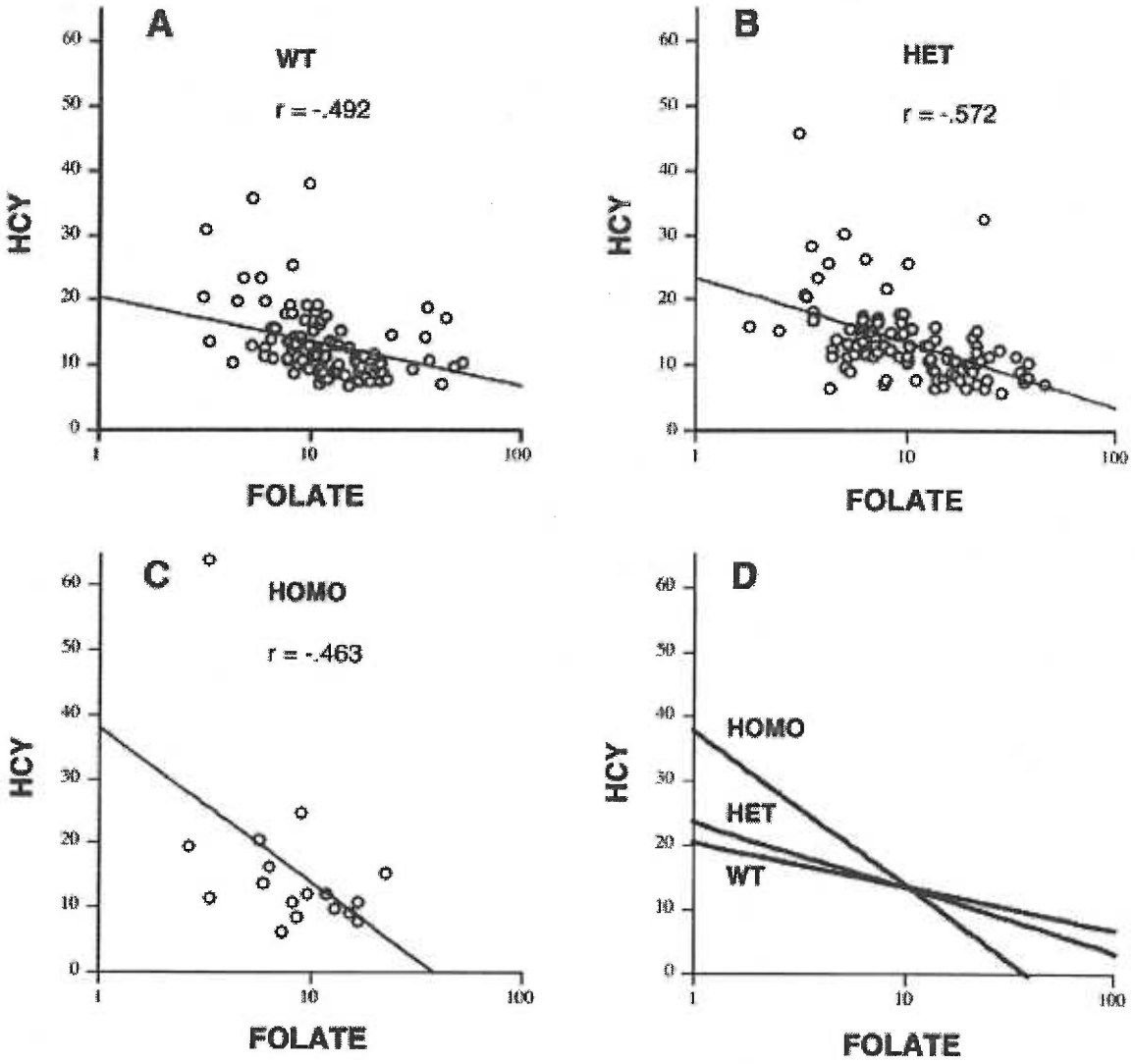


Figure 1.
A,B, and C, Data points and best-fit linear regression lines defining the relationship between homocysteine (HCY) and folate (log scale) levels for the vascular disease subjects in each of the three methylenetetrahydrofolate reductase genotype groups. Spearman's rank correlation coefficient (r) and number of patients analyzed (n) are also shown. D, Three regression lines. The slope of the homozygote regression line is significantly different from each of the other 2 lines. WT (wild type) = Ala/Ala; HET (heterozygote) = Ala/Val; HOMO (homozygote) = Val/Val.

Table 1 Race Distributions For All Genotyped Cases and Controls

	Controls	Combined Cases	Vascular Dx	Venous Thrombosis	Stroke
N	349	530	247	98	185
Race					
Caucasians (%)	272 (87)	236 (96)	236 (96)	-	-
other races (%)	42 (13)	11 (4)	11 (4)	-	-
missing (%)	35 (10)	283 (53)	0	98 (100)	185 (100)

1. Data not collected

Table 2 Demographics: Thesis Groups

	Controls[1]	Combined Cases	Vascular Dx[1]	Venous Thrombosis[2]	Stroke
N	272	519	236	98	185
Age					
mean	53	64	68	49	66
SD	12	12	9	16	8
min-max	24-86	14-88	42-85	14-79	36-88
median	51	66	69	49	67
IQR	14	14	14	24	11
missing (%)	2 (3)	7	0 (0)	7 (7)	0 (0)
Gender					
male (%)	140 (51)	358 (69)	151 (64)	43 (44)	164 (89)
female (%)	132 (49)	161 (31)	85 (36)	55 (56)	21 (11)
missing (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Smoking					
pack years[3] mean (SD)	29 (29)	47 (35)	53 (33)	-	40 (37)
never-smoker (%)	62 (23)	29 (8)	1 (0.5)	-	28 (17)
ever-smoker (%)	208 (77)	351 (92)	211 (99.5)	-	140 (83)
missing (%)	2 (0.7)	139 (27)	24 (10)	98 (100)	17 (9)

1. Note: Both the Control and Vasc Dx studies were selected to include only white subjects.
2. The Venous Thrombosis and Stroke studies include approximately 98% Caucasians (personal communication).
3. Pack Years mean among smokers only.
- Data not collected

Table 3. Genotype Distributions - Black and Caucasian Controls

Genotype	Black	Caucasian	Chi Square[1]	P value[1]
wt (%)	21 (81)	112 (41)	15.6	0.0004
het (%)	5 (19)	123 (45)		
mut (%)	0 (0)	37 (14)		
total	26	272		

1. Overall genotype frequencies between the races was significantly different (X^2=15.6, p=0.0004).

Table 4. Genotype Distributions[1] - Thesis Groups[2]

Genotype	Controls	Combined Cases	Vascular Dx	Venous Thrombosis	Stroke
wt (%)	112 (41)	213 (41)	106 (45)	37 (38)	70 (38)
het (%)	123 (45)	257 (50)	108 (46)	48 (49)	101 (55)
mut (%)	37 (14)	49 (9)	22 (9)	13 (13)	14 (8)
Total[3]	272	519	236	98	185

1. Genotype frequencies for all groups are in Hardy-Weinberg Equilibrium except Stroke (X^2=7.55, p=0.006).
2. Genotype frequencies for Controls and Vasc Dx include only Caucasian subjects. The Venous Thrombosis and Stroke studies include approximately 98% Caucasians (personal communications).
3. Genotype percentages may not total 100% due to rounding.

Table 5. Genotype Distributions - Case Groups

	Case Groups[1]					
	Combined Cases	Vascular Dx	Venous Thrombosis	Stroke	Chi Square[1]	P value[1]
Genotype					5.4	0.25
wt (%)	213 (41)	106 (45)	37 (38)	70 (38)		
het (%)	257 (50)	108 (46)	48 (49)	101 (55)		
mut (%)	49 (9)	22 (9)	13 (13)	14 (8)		
total	519	236	98	185		

Genotype frequencies for case groups are not significantly different (X^2=5.4, p=0.25).

Table 6. Allele Frequencies - Case Groups

	Case Groups					
	Combined Cases	Vascular Dx	Venous Thrombosis	Stroke	Chi Square[1]	P value[1]
Allele					2.0	0.37
ala	683 (66)	320 (68)	122 (62)	241 (65)		
val	355 (34)	152 (32)	74 (38)	129 (35)		
total	1038	472	196	370		

1. Allele frequencies not different across case groups (X^2=2.0, p=0.37).

Table 7. Genotype Distribution - Controls and Combined Cases

	Controls	Combined Cases
Genotype		
wt (%)	112 (41)	213 (41)
het (%)	123 (45)	257 (50)
mut (%)	37 (14)	49 (9)
total	272	519

Table 8. Genotype Association with Vascular Disease- Controls and Combined Cases

	OR	95%CI Lower	Upper	Chi Square[2]	P value[2]
Genotype				3.5	0.17
wt[1]	1.0				
het	1.1	0.8	1.5		
mut	0.7	0.4	1.1		

2. wt (ala/ala) is the referent genotype.
3. The genotype is not associated with vascular disease (X^2=3.5, p=0.17)

Table 9. Genotype Stratified by Age

Genotype	Controls	Combined Cases
Age 60[2]		
wt (%)	90 (42)	61 (38)
het (%)	94 (44)	82 (51)
mut (%)	28 (13)	18 (11)
total	212	161
Age>60[2]		
wt (%)	22 (38)	150 (43)
het (%)	27 (47)	170 (48)
mut (%)	9 (16)	31 (9)
total	58	351
Total both age groups[1]	270	512

1. Total numbers may not match unstratified N due to missing data.
2. Age breakdown at the approximate median age of the cases and controls.

Table 10. Genotype Association with Vascular Disease - Stratified By Age Group

Genotype	OR	95% CI Lower	95% CI Upper	Chi Square[1]	P value
Age 60				1.62	0.45
wt[1]	1.0				
het	1.29	0.83	2		
mut	0.95	0.48	1.86		
Age>60				2.58	0.27
wt[1]	1.0				
het	0.92	0.5	1.69		
mut	0.51	0.21	1.2		

1. wt (ala/ala) is the referent genotype.

Table 11. Genotype Association with Vascular Disease - Stratified by Gender

Genotype	Controls	Combined Cases
Women		
wt (%)	55 (39)	71 (44)
het (%)	64 (46)	72 (45)
mut (%)	21 (15)	18 (11)
total women	140	161
Men		
wt (%)	57 (43)	142 (40)
het (%)	59 (45)	185 (52)
mut (%)	16 (12)	31 (9)
total men	132	358
Total Both Genders	272	519

Table 12. Association Between Genotype and Vascular Disease Stratified By Gender

Genotype	OR	95% CI Lower	95% CI Upper	Chi Square	P value
Women				1.27	0.53
wt[1]	1.0				
het	0.87	0.54	1.42		
mut	0.66	0.32	1.37		
Men				2.44	0.29
wt[1]	1.0				
het	1.26	0.82	1.92		
mut	0.78	0.4	1.53		

1. wt (ala/ala) is the referent genotype.

Table 13. Genotype Stratified by Smoking

Genotype	EPA Controls	Combined Cases
Never Smoker		
wt (%)	19 (31)	7 (24)
het (%)	31 (50)	22 (76)
mut (%)	12 (19)	0 (0)
total never-smokers	62	29
Ever Smoker		
wt (%)	93 (45)	152 (43)
het (%)	90 (43)	167 (48)
mut (%)	25 (12)	32 (9)
total ever-smokers	208	351
<45 Pack Years[1]		
wt (%)	63 (47)	65 (39)
het (%)	55 (41)	88 (52)
mut (%)	16 (12)	15 (9)
total <45	134	168
>=45 Pack Years[1]		
wt (%)	30 (41)	87 (48)
het (%)	35 (47)	79 (43)
mut (%)	9 (12)	17 (9)
total >=45	74	183

1. 45 years was the median pack years for the cases.

Table 14. Association Between Genotype and Vascular Disease - Gene Stratified on Smoking

	OR	95% CI Lower	Upper	Chi Square	P value
Never-smoked				8.18	0.02
wt[1]	1.0				
het	1.93	0.69	5.37		
mut[4]	0.1	.01	1.99		
Ever-smoked					
wt[1]	1.0				
het	1.14	0.79	1.63		
mut	0.78	0.44	1.4		
<45 Pack Years[3]				3.90	0.14
wt[1]	1.0				
het	1.55	0.96	2.51		
mut	0.91	0.41	1.99		
>=45 Pack Years[3]				1.20	0.55
wt[1]	1.0				
het	0.78	0.44	1.38		
mut	0.65	0.26	1.62		

1. wt (ala/ala) is the referent genotype.
2. Cannot compute val/val OR due to zero mutants among never-smoking cases
3. 45 pack years was the median for the cases.
4. OR generated by adding 0.5 to the never-smoking muts, and to all other genotypes.

Table 15. Logistic Regression[1] - Genotype Association With Vascular Disease

		95% CI		
	OR	lower	upper	P-value
MTHFR Genotype				
wt[2]	1.0			
het	1.2	0.8	1.9	0.3527
mut	0.6	0.3	1.3	0.1991

1. Logistic model covariates include age groups (>60 or ≤ 60 years old), gender, and pack years groups (<45 pack years or ≥45 pack years).
2. wt (ala/ala) is the referent genotype.

Table 16. Genotype Association With Vascular disease - Each Case Group Compared to the Control Group

Group Comparisons[2]	OR	95% CI Lower	Upper	Chi Square	P value
Vascular Dx vs. EPA controls				2.41	0.30
wt[1]	1.0				
het	0.93	0.64	1.34		
mut	0.63	0.35	1.13		
Venous Thrombosis vs. EPA controls				0.44	0.80
wt[1]	1.0				
het	1.18	0.72	1.95		
mut	1.06	0.51	2.21		
Stroke Study vs. EPA controls				5.88	0.054[1]
wt[1]	1.0				
het	1.31	0.88	1.96		
mut	0.61	0.31	1.20		

1. wt (ala/ala) is the referent genotype.
2. Test of Homogeneity for each of the case group comparisons p=0.21. Mantel-Haenszel summary OR=0.71 for mut compared to wt (95%CI = 0.49 to 1.05). Mantel-Haenszel test of association p=0.26

Table 17. Studies Associating the C677T tlMTHFR Mutation and Vascular Disease

Coronary Artery Disease and Myocardial Infarction

Adapted from (Fletcher and Kessling 1998)

Coronary Artery Disease and Myocardial Infarction									
Reference	Cases	Controls	Outcome	Exclusions for known risk factors	Plasma homocysteine	Plasma folate	OR	Significance	Comments
(Adams, Smith et al. 1996)	310	222	MI	No	No	No	0.76	P = 0.34	95%CI for age & sex stratified data includes potentially significant ORs.
(Gallagher, Meleady et al. 1996)	111	105	Coronary heart disease, age < 55	No	Yes	No	2.89	P = 0.02	Significant association disease and raised fasting tHcy with homozygosity for the mutation.
(Izumi, Iwai et al. 1996)	250	201	MI/angina pectoris	No	No	No	1.76	P = 0.04	Little experimental detail, used multiple logistic analysis for different risk factors; OR is our calculated OR.

(Ma, Stampfer et al. 1996)	293	290	MI	Excluded history of MI, stroke, TIA, at start of prospective study	Yes	Yes	0.82	P = 0.45	No disease association; for age<60, MTHFR genotype was major determinant of tHcy, for age>60, age, smoking, vitamin use and folate status were important.
(Schmitz, Lindpaintner et al. 1996)	190	188	MI	Age <76	Yes[a]	Sub-group	1.07	P = 0.89	Measured tHcy in 68/190 subjects, tHcy was lower & plasma folate & folate intake were higher in subjects but not significantly

Table 17 (Continued).

Peripheral Vascular Disease Case-Control Studies

Peripheral Vascular Disease									
Reference	Cases	Controls	Outcome	Exclusions for known risk factors	Plasma homocysteine	Plasma folate	OR	Significance	Comments
(Christensen, Frosst et al. 1997)	152	121	Earl onset vascular disease, age <60	No	Yes	Yes	1.4	P = 0.37	Homozygous mutant genotype was higher in patient group (14.5% vs. 10.7%), but not significantly, and assoc/d with higher tHcy in subjects, especially in lower folate group.
(Deloughery, Evans et al. 1996)	247	133	Late onset vascular disease, age 40 - 80	No	Yes	Yes	0.55	P = 0.09	Subjects homozygous for the mutation had higher tHcy, but this was not statistically significant.
(Morita, Taguchi et al. 1997)	362	778	Vascular disease ± MI	Excluded women	Yes[a]	No	1.65	P < 0.01	Subjects divided by disease severity; [a]measured tHcy in

(Wilcken, Wang et al. 1996)	565	225	Vascular disease	Age <66	No	No	1.09	P = 0.80	198/362 subjects; association of genotype with raised tHcy and disease in severe disease group. Analysed subjects at low risk (lipid profile, no smoking, no diabetes, age) separately, no details given, no association

Table 17 (Continued).

Thrombotic Vascular Disease Case-Control Studies

Adapted from (Fletcher and Kessling 1998).

Thrombotic Vascular Disease									
Reference	Cases	Controls	Outcome	Exclusions for known risk factors	Plasma homocysteine	Plasma folate	OR	Significance	Comments
(de Franchis, Mancini et al. 1996)	64	258	Early onset thrombotic vascular disease	NS	Yes	Sub-group	2.37	P = 0.01	29/64 subjects had raised tHcy, and no other known risk factors. Of these, 18 (62.1%) were homozygous for the mutant phenotype. Of the 35 subjects with normal tHcy, only 1 was homozygous for the mutation.

Table 17 (Continued).

Multiple Clinical Endpoints

Adapted from (Fletcher and Kessling 1998).

Combined Vascular Diseases									
Reference	Cases	Controls	Outcome	Exclusions for known risk factors	Plasma homocysteine	Plasma folate	OR	Significance	Comments
(Brugada and Marian 1997)	155	155	MI, stroke, vascular disease	No[a]	No	No	0.82	P = 0.83, P = 0.75	Subjects with known risk factors were separated out, 5/44 subjects vs. 7/89 controls had +/+ genotype, for this group, our calculated OR=1.5.
(Brulhart, Dussoix et al. 1997)	193	456	MI, vascular disease	All subjects had NIDDM	NS	No	0.71	P = 0.19	
(Kluijtmans, van den Heuvel et al. 1996)	60	111	Premature MI, vascular disease, age 13-68	Excluded diabetes, hypertension, hyperlipidemia	Yes	Yes	3.09	P = 0.05	Association of homozygous mutant genotype with disease and elevated fasting tHcy; for homozygotes, there was a negative correlation between tHcy and

									folate.
(Narang, Callaghan et al. 1996)	92	50	Vascular disease ± MI	NS	No	No	0.65	P = 0.52	Letter; little experimental detail, no information on exclusions
(van Bockxmeer, Mamotte et al. 1997)	358, 197	143	Vascular disease[a] ± MI	Age <50 for group 1	No	Sub-group	1.01, 0.86	P = 1.0, P = 0.71	[a]2 subject groups, gp1 assessed by angiography, gp2 previous coronary balloon angioplasty
(Arruda, von Zuben et al. 1997)	191, 127	296	Arterial Disease, venous thrombosis	Yes/no[a]	No	No	5.52, 2.93	P < 0.01 P < 0.01	[a]Separated vascular disease subjects according to known risk factors & analysed data separately.