The spectrum of premature atherosclerosis: from single gene to complex genetic disorder
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The effect of a common methylenetetrahydrofolate reductase mutation on levels of homocysteine, folate, vitamin B12 and on the risk of premature atherosclerosis

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Abstract

An increased total plasma homocysteine level is an established risk factor for atherosclerotic vascular disease. The plasma level of homocysteine is influenced by both environmental and genetic factors. An important genetic determinant of plasma homocysteine is a common amino acid dimorphism (Ala222Val) in the methylenetetrahydrofolate reductase (MTHFR) gene. Individuals homozygous for the Val allele have significantly higher homocysteine levels than those with an Ala/Val or Ala/Ala genotype. Moreover, the Val/Val genotype has been claimed to be a strong genetic risk factor for atherosclerosis.

The aim of the present study is: (1) to determine the risk associated with the MTHFR dimorphism by comparing the genotype distribution in patients with premature atherosclerosis with that in a group of healthy controls; and (2) to investigate the relationship between the MTHFR genotype and parameters of homocysteine metabolism.

The patient group consisted of 257 consecutive referred individuals with angiographically proven premature (< 50 years of age) arterial disease (coronary, and/or peripheral vascular disease). A total of 272 healthy hospital workers without a history of vascular disease were selected as a control group. The MTHFR-genotype was determined by PCR and gel-electrophoresis. A methionine-loading test was performed on 245 patients, and, in addition to homocysteine, levels of folate and vitamin B12 were measured.

We found a strong correlation between MTHFR genotype and plasma homocysteine levels both before and after methionine loading. In addition, the MTHFR genotype seems important for the inverse relationship between homocysteine and folate and vitamin B12 levels. Lastly, the MTHFR genotype distribution was not different between patient and control groups. MTHFR genotype is a strong determinant of plasma homocysteine levels. Moreover, the plasma level of folate, which by itself influences homocysteine levels, is also dependent on the MTHFR genotype. In Val/Val genotypes, low levels of both folate and B12 lead to a relatively large increase in homocysteine levels. Nevertheless, the MTHFR genotype does not increase the risk for premature coronary artery disease.
Introduction

Severe inherited hyperhomocysteinemia was first considered a risk factor for cardiovascular disease in 1969.\(^1\) In recent years, mild hyperhomocysteinemia has also become an established risk factor for atherosclerotic vascular disease, as was demonstrated by numerous epidemiological studies.\(^2\)\(^-\)\(^8\)

Methylenetetrahydrofolate reductase (MTHFR) catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which constitutes the predominant circulating form of folate, and is a methyl donor for the remethylation of homocysteine to methionine. Molecular defects in MTHFR gene, underlying MTHFR deficiency, cause severe hyperhomocysteinemia, premature atherosclerotic vascular disease and neurological disorders.\(^1\)\(^,\)\(^9\)\(^,\)\(^10\) A less severe and common defect results from a C to T mutation at nucleotide position 677 in the cDNA.\(^11\) This mutation predicts replacement of 222Ala by Val which appears to reduce the basal activity of the enzyme by 50% and leads to decreased thermostability in homozygotes.\(^12\)\(^,\)\(^13\) Moreover, homozygosity for this variant allele is associated with elevated fasting and post-methionine loading homocysteine levels.\(^14\)\(^-\)\(^17\)

There is uncertainty about the significance of the MTHFR variant as a risk factor for arterial and thromboembolic disorders. Several recent studies have claimed that homozygous carriers of the thermolabile variant (+/+ or Val/Val genotype) are at an increased risk for these disorders.\(^13\)\(^,\)\(^18\)\(^-\)\(^21\) On the other hand, a variety of studies has been unable to confirm these findings.\(^22\)\(^-\)\(^28\)

Deficiencies of both folate and vitamin B12 increase plasma homocysteine levels, and folate levels have been shown to negatively correlate with plasma homocysteine.\(^29\)\(^-\)\(^32\) Recently it was demonstrated that individuals carrying the +/+ genotype showed a disproportionate increase of plasma homocysteine, suggestive of dependence of folate intake.\(^27\) Another study however showed no significant relation between homocysteine and vitamin B6 or vitamin B12 levels among the different genotypes.\(^13\)

Our first aim was to analyze the distribution of the MTHFR genotype in a carefully selected, large cohort of patients suffering from premature cardiovascular disease (< 50 years of age) and in controls. Our second objective was to determine vitamin status and plasma homocysteine before and after methionine loading in these patients in order to further explore the relationship between MTHFR genotype and homocysteine levels.
Subjects and methods

Patients and controls
A total of 257 consecutive referred patients (both males and females) with premature (< 50 years of age) cardiovascular disease (coronary and/or peripheral vascular disease) proven by angiography or ultrasound were included in our study. These patients were referred to the Academic Medical Centre in Amsterdam for investigation of symptomatic coronary and/or peripheral arterial disease. The diagnosis of atherosclerosis was based on angiography that showed significant arterial stenosis (> 50% obstruction of a major artery). No angiographical evaluation was available for 26 patients with proven myocardial infarction. Controls were healthy volunteers (n = 272) without a history of cardiovascular disease, mostly recruited among the employees of the Academic Medical Center in Amsterdam.

Biochemical analysis
Only patients were subjected to a methionine loading test. Homocysteine concentrations before and after loading were measured according to Ubbink et al. with minor modifications. Folic acid and vitamin B12 concentrations were measured in heparinized plasma (Dualcount, Diagnostic Products, Los Angeles, CA).

Mutation analysis
DNA was obtained from whole blood samples by a standard salting-out procedure. PCR amplification was performed with the following buffer: 67 mM TrisHCl, pH 8.8; 6.7 mM MgCl2; 10 mM β-mercaptoethanol; 6.7 mM EDTA; 16.6 mM (NH4)2SO4; 1 μl DMSO; 5 μg BSA; 6 mM dNTP's (1:1:1:1); 40 ng of forward and reverse primer and 0.2 U Taq polymerase (Perkin Elmer, Branchburg, NJ) in a total volume of 10 μl. The mixture was denatured initially at 95°C for 3 min, followed by 34 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and primer extension at 72°C for 30 s. Blank controls were included with each PCR-set to exclude contamination. PCR fragments were digested with HinfI restriction enzyme according to instructions of the manufacturer (New England Biolabs, Beverly, MA). The digest was analyzed by electrophoresis in a 2% agarose gel in TBE-buffer.
Statistics
A $\chi^2$-test was performed on the results from the DNA analysis. The mean as well as the standard deviation (S.D.) was calculated for homocysteine- and vitamin-concentrations. Means were compared by Students's t-test. Correlation analyses were performed by Spearman's rank correlation test. Associations of plasma homocysteine levels with genotype were further tested by analysis of variance controlling for age, sex, folate and vitamin B12 levels (general linear model procedure). In all statistical analysis, homocysteine and vitamin levels were natural log-transformed but untransformed means are presented in the tables. The statistics were computed with SPSS for Windows (Release 7.5, SPSS, Chicago, IL).

Results
Details of the patient group are listed in Table 1. In women, total cholesterol and LDL cholesterol were significantly lower and HDL cholesterol was significantly higher then in men. In concordance with lower folate levels, postload homocysteine levels in women were increased compared to men. Since gender does not seem to play an important role in our study objectives, and because the trends were similar for both sexes, data were combined for males and females in further statistical analyses.

<table>
<thead>
<tr>
<th>Table 1. Continuous and discrete characteristics of the patient group</th>
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<tbody>
<tr>
<td>Characteristics</td>
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<tr>
<td>Age of onset (years)</td>
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<tr>
<td>BMI (kg/m$^2$)</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
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<td>LDL cholesterol (mmol/l)</td>
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<td>HDL cholesterol (mmol/l)</td>
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<td>Triglycerides (mmol/l)</td>
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<td>Fastening Homocysteine (μmol/l)</td>
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<td>Postload Homocysteine (μmol/l)</td>
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<tr>
<td>Folate (μg/l)</td>
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<tr>
<td>Vitamin B12 (pmol/l)</td>
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</tbody>
</table>

Values are mean ±SD except for triglycerides (median and range)
* Not all individuals were tested for all parameters. (Minimum 193 males, 40 females)
Figure 1. Relation of homocysteine, folate and vitamin B12 in the different genotype groups

Concentrations of homocysteine in μmol/l, folate in μg/l and vitamin B12 in pmol/l. Both intercept and slope of the +/+ genotype are different from other genotypes (fasting: p<0.001 and p=0.001, respectively, postload: p=0.003 and p=0.018, respectively).
MTHFR genotype distribution
Homozygosity for the MTHFR-polymorphism (+/+ genotype) was found in 14.0% of the control subjects compared to 10.5% of the patients (Table 2). There was no significant difference between patients and controls in prevalence of the +/+ genotype (p = 0.23). When the patients with only peripheral disease were excluded the difference in allele frequency between controls and patients increased but remained not significant (p = 0.075, see Table 2, odds ratio (OR = 0.78 (95% CI 0.59-1.03)). The prevalence of the +/+ genotype in the small number of patients with peripheral artery disease is higher than in controls and coronary artery disease patients, but this difference is not significant.

Vitamin and homocysteine concentrations
Substantial differences were found between homocysteine levels of the different genotypes. Patients with the 'thermolabile' MTHFR variant had elevated homocysteine levels before and after methionine loading (Table 3). The OR for elevated homocysteine (exceeding 95% CI according to den Heijer et al.37) between the +/+ genotype group and the combined group of +/− and −/− genotypes is 5.4 (95% CI: 2.0-14.2) before loading and 4.5 (95% CI: 2.0-10.4) after loading.

The folate concentration of the +/+ genotype was significantly lower than in the −/− genotype groups (Table 3). This is in agreement with a previous study.27

Table 2 and Fig 1 examine the relationship between folate and B12 and pre- and postload homocysteine for the different genotypes. A strong correlation was found between folate, vitamin B12 and fasting homocysteine levels (Table 4).

Table 2. MTHFR genotype distribution (n (%))

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
<th>Total</th>
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<tbody>
<tr>
<td>Controls</td>
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<td>Male</td>
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<tr>
<td>Patients</td>
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</tr>
<tr>
<td>Diagnoses*</td>
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<tr>
<td>Coronary</td>
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<tr>
<td>Artery disease</td>
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<tr>
<td>Peripheral vascular disease</td>
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* More than one diagnosis possible.
We also analyzed the relationship between genotype and fasting or postload tHcy in a regression model that also includes age, sex, folate and vitamin B12. This model explains 24% of the variance in plasma homocysteine concentration (p< 0.001). When we dichotomize folate status as ≥ median and < median, and evaluate the relationship between fasting tHcy levels and MTHFR genotype the association was only apparent in the lower than median group. The R-value was 0.28 (p< 0.003) in the < median group and 0.08 (n.s.) in the ≥ median group.

Correlation with homocysteine was significant for all three genotypes when the product of In folate and In vitamin B12 was taken into account. The product of the vitamins was used since both may be rate limiting in the homocysteine metabolic
pathway and therefore may interact. The relationship between pre- and postload plasma homocysteine and the product of ln folate and ln B12 levels is graphically depicted in Fig 1 for the different genotypes. The significantly steeper negative regression line (B = -9.30 vs. -4.61; p<0.01) in the +/+ genotype group compared with the other two genotypes implicates a stronger dependence of homocysteine levels on a combination of folate and vitamin B12 intake. The slope of the regression line in the post-load homocysteine graph was twice as large as in the fasting homocysteine plot, which further strengthens this finding.

**Discussion**

In the present study, we investigated the relationship between MTHFR-genotype, folate and vitamin B12 status, and plasma homocysteine concentrations before and after methionine loading in patients with atherosclerotic vascular disease. The results clearly show that MTHFR genotype is an important determinant of plasma homocysteine levels: +/+ genotype carriers have significantly higher levels than carriers of other genotypes. Moreover, the data show that this difference in plasma homocysteine levels is only pronounced at low folate levels. Given the generally accepted role of increased homocysteine levels as a risk factor one would predict from this finding that the +/+ genotype predisposes carriers to cardiovascular disease. Our results, however, indicate that this is not the case. In fact, the prevalence of the +/+ genotype is several percentage points lower albeit not significantly, in patients than in controls.

Our results add to a series of recent reports on the relationship between MTHFR genotype and cardiovascular or thrombo-embolic disease. These studies differ considerably in size and in the selection of the cohort of patients and controls. The data from these studies were recently summarized and the conclusion based on the then available data was that the +/+ genotype is not an important risk factor for cardiovascular disease. With two exceptions, this conclusion is supported by more recent publications and the present report.

This finding raises doubts on the putative causal relationship between mild increases in plasma homocysteine and cardiovascular disease. One might even argue that these results are contradictory to any causal relationship. Plasma homocysteine may merely reflect atherosclerosis as an 'innocent bystander' and in that case, mild hyperhomocysteinemia could be a marker instead of a cause for disorders of the vessel wall.
The question of causality is all the more important in the light of recent proposals to fortify food products with folate and/or vitamins that have a beneficial effect on homocysteine levels. In view of the finding that +/+ individuals display a stronger negative relationship between homocysteine and vitamin levels (this study), it is clear that +/+ carriers will benefit more from such supplements than individuals with the other genotypes. It even remains possible that the +/+ genotype is only a risk factor for cardiovascular disease in individuals with a poor folate status.

Acknowledgements

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References


Part 2 Chapter 8


