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Thermolabile Methylenetetrahydrofolate Reductase in Coronary Artery Disease

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Background Hyperhomocysteinemia, an independent and graded risk factor for coronary artery disease (CAD), may result from both environmental and hereditary factors. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of methylenetetrahydrofolate to methyltetrahydrofolate, the methyl donor in the remethylation of homocysteine to methionine. A 677C→T mutation in the MTHFR gene has been associated with elevated homocysteine concentrations in homozygous (+/+) individuals.

Methods and Results We assessed the frequency of this common mutation in 735 CAD patients from the Regression Growth Evaluation Statin Study (REGRESS), a lipid-lowering coronary-regression trial, and in 1250 population-based control subjects. Furthermore, the association between the mutation and serum homocysteine concentrations was studied. The frequency of the homozygous (+/+) mutation was 9.5% among patients versus 8.5% among control subjects, resulting in an odds ratio of 1.21 (95% confidence interval [CI], 0.87 to 1.68), relative to the (-/-) genotype. Homocysteine concentrations

were significantly elevated in both (+/+) and (+/-) individuals compared with (-/-) individuals (median homocysteine levels, 15.4, 13.4, and 12.6 $\mu\text{mol/L}$, for (+/+), (+/-), and (-/-) individuals, respectively). For a summary estimation of the risk of the (+/+) genotype for CAD, we performed a meta-analysis on 8 different case-control studies on thermolabile MTHFR in CAD. In the meta-analysis, the homozygous (+/+) genotype was present in 299 of 2476 patients (12.1%) and in 257 (10.4%) of 2481 control subjects, resulting in a significant odds ratio of 1.22 (95% CI, 1.01 to 1.47) relative to the (-/-) genotype.

Conclusions Both the homozygous (+/+) and heterozygous (+/-) genotype result in elevated homocysteine concentrations. From our meta-analysis, we conclude that the homozygous (+/+) genotype is a modest but significant risk factor for CAD. (*Circulation*. 1997;96:2573-2577.)

Key Words • homocysteine • methylenetetrahydrofolate reductase • coronary disease • meta-analysis • risk factors

A recent meta-analysis by Boushey et al¹ of 27 independent studies in which plasma homocysteine concentrations were quantitatively related to atherosclerotic disease demonstrated that mild hyperhomocysteinemia is an independent and graded risk factor for cerebral, peripheral, and CAD. Elevated homocysteine concentrations may originate from nutritional deficiencies in cofactors or cosubstrates of enzymes involved in homocysteine metabolism or from molecular defects in genes coding for enzymes crucial in this metabolism.²⁻⁵

The enzymes pivotal in homocysteine metabolism are CBS, the first enzyme in homocysteine transsulfuration,

and MTHFR, which is involved in the folic acid dependent remethylation of homocysteine to methionine. Both genes have been cloned and characterized,^{6,7} and several mutations have been reported⁸⁻¹² in patients with a homozygous deficient phenotype. Heterozygotes for either CBS or MTHFR deficiency often have elevated homocysteine concentrations. However, the frequency of heterozygosity for CBS and MTHFR deficiency is too low to account for the frequency of mildly elevated homocysteine levels in patients with cardiovascular disease.¹³

MTHFR is a flavoprotein that reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating form of folate. In 1988, Kang et al⁵ described a new MTHFR variant with thermolabile properties. Individuals with this MTHFR variant have decreased specific MTHFR activity in lymphocytes (<50% of the control mean), have increased thermolability after preincubation at 46°C, and may have elevated plasma homocysteine concentrations. In other studies, the same group reported an increased incidence of this MTHFR variant in patients with CAD¹⁴ compared with control subjects (17% versus 5%, respectively), and they were able to correlate the incidence of thermolabile MTHFR to the severity of CAD.¹⁵ In a Dutch study, this thermolabile MTHFR was found to be the cause of abnormal homocysteine metabolism in 11 of 39 hyperhomocysteinemic vascular patients ($\approx 28\%$).¹⁶

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Selected Abbreviations and Acronyms

CAD	= coronary artery disease
CBS	= cystathionine β -synthase
CI	= confidence interval
MTHFR	= 5,10-methylenetetrahydrofolate reductase
REGRESS	= Regression Growth Evaluation Statin Study

Recently, Frosst and coworkers¹⁷ were able to identify a relatively common 677C→T mutation in the MTHFR gene, which substituted a conserved alanine by a valine residue. Individuals who are homozygous for this mutation often have elevated homocysteine concentrations,^{17,18} especially in combination with a low folate status.^{19,20}

In the present study, we investigated the prevalence of the 677C→T mutation in a well-defined population of 735 male CAD patients and in 1250 population-based control subjects and assessed the association of this mutation to serum homocysteine concentration. Several studies have investigated the homozygous 677C→T mutation as a risk factor for CAD but with conflicting results,²¹⁻²⁶ probably because of the relatively small numbers of individuals included in each study separately. We therefore performed a meta-analysis of eight case-control studies reporting data on the MTHFR genotype distribution to estimate the relative risk of the homozygous (+/+) genotype for CAD.

Methods

Study Population

We studied 735 male patients with angiographically assessed CAD enrolled in REGRESS, which was conducted under auspices of the Interuniversity Cardiology Institute The Netherlands. REGRESS is a double-blind, placebo-controlled, multicenter trial designed to assess the effects of lipid-lowering therapy with pravastatin on progression and regression of CAD in 885 male patients with normal to moderately elevated serum cholesterol levels (4 to 8 mmol/L). The overall study design and inclusion criteria of patients have been described extensively.²⁷

A large control group was constructed, consisting of individuals recruited from several published^{28,29} and unpublished Dutch case-control studies (L.A.J.K., H.J.B., Van der Put, Den Heijer, and Rosendaal, unpublished results), which resulted in a control group consisting of 1250 unrelated population-based control subjects. All 677C→T mutation analyses were performed in our laboratory.

For a summary quantitative risk assessment of the 677C→T mutation in CAD, we evaluated eight international case-control studies,^{21-26,29} including the present one. In this analysis, we confined ourselves to case-control studies in which MTHFR genotype distributions among both CAD patients and control subjects either were given or could be calculated from their data. We calculated the MTHFR genotype distribution and odds ratios of the (+/+) genotype for CAD in each study separately and for all studies combined.

MTHFR Genotype Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedure, and mutation analysis was performed essentially as described by Frosst et al.¹⁷ Electrophoresis in a 4% agarose gel followed by ethidium bromide staining and UV illumination allowed detection of mutated alleles.

Homocysteine Determination

After an overnight fast, blood was drawn from the CAD patients for an assessment of fasting homocysteine concentra-

TABLE 1. Distribution of 677C→T MTHFR Variant Among Patients With CAD and Control Subjects

	CAD Patients (n=735), n (%)	Control Subjects (n=1250), n (%)	Odds Ratio (95% CI)
+/+	70 (9.5)	106 (8.5)	1.21 (0.87-1.68)
+/-	328 (44.6)	527 (42.2)	1.14 (0.94-1.38)
-/-	337 (45.9)	617 (49.4)	1.0*

*Reference category: odds ratio=1.0.

tions, and serum was stored at -70°C until analysis. Homocysteine concentrations were determined by high-performance liquid chromatography with use of a 150×4.6-mm Hypersil ODS column in a high-performance liquid chromatography analyzer (Thermo Separation Products) after the thiol groups were bound to a fluorescent label (SBD-F).³⁰

Statistical Analysis

Odds ratios and 95% CIs were calculated as an estimate of the relative risk of the different genotypes in CAD.³¹ Differences in genotype distributions were calculated by χ^2 analysis. To assess the relationship between the 677C→T transition and homocysteine concentrations, we calculated median homocysteine concentrations in different genotype groups. Differences between homocysteine concentrations in these genotype groups were assessed by one-way ANOVA, followed by pairwise *t* tests on log-transformed data. All probability values are two-tailed, and a value of $P < .05$ was considered statistically significant.

Results

MTHFR Genotype Analysis

The overall frequency for the (+) allele was 31.8% among patients and 29.5% among control subjects. The numbers of individuals homozygous for the 677C→T transition were 70 of 735 CAD patients (9.5%) versus 106 of 1250 controls (8.5%; Table 1). The genotype distributions in both groups of individuals are consistent with those calculated from the Hardy-Weinberg equilibrium. The odds ratios as an estimate of the relative risk of the (+/+) and (+/-) genotypes relative to the risk of the (-/-) genotype for CAD were 1.21 and 1.14, respectively (Table 1). We also assessed the relative risk of the (+/+) genotype in relation to the risk of individuals with both other genotypes. In this model, the risk of the homozygous (+/+) genotype in CAD was 1.14 (95% CI, 0.83 to 1.56).

Association Genotype and Total Homocysteine

Homocysteine concentrations were measured in 515 of 735 CAD patients. The numbers of individuals in the three different MTHFR genotype groups in this subset of individuals were 51 (9.9%), 233 (45.2%), and 231 (44.9%) for the (+/+), (+/-) and (-/-) genotype, respectively, which are not substantially different from the MTHFR genotype distribution observed in the entire patient group ($\chi^2=0.137$, $P=NS$). As homocysteine concentrations in the different genotype groups showed a skewed distribution (data not shown), homocysteine concentrations are expressed in median (range) values. Individuals with the homozygous (+/+) genotype have the highest homocysteine concentrations and (-/-) individuals have the lowest, whereas heterozygous (+/-) individuals have intermediate homocysteine levels. Both homozygotes (+/+) and heterozygotes (+/-) have significantly elevated homocysteine concentrations

TABLE 2. Association Between MTHFR Genotype and Fasting Serum Homocysteine Concentrations in CAD Patients

MTHFR Genotype	Fasting Homocysteine Concentration, $\mu\text{mol/L}^*$
+/+ (n=51)	15.4 (8.7-56.9) [†]
+/- (n=233)	13.4 (7.0-42.9) [‡]
-/- (n=231)	12.6 (6.5-30.2)

Homocysteine concentrations are expressed as median values. Range is given in parentheses.

* $P < .001$ (ANOVA with log-transformed data).

[†] $P < .002$ (t test) for +/+ vs +/- and -/- genotypes.

[‡] $P < .05$ (t test) for +/- vs -/- genotype.

compared with (-/-) individuals, demonstrating a significant effect of the homozygous (+/+) as well as heterozygous (+/-) genotype on homocysteine levels (Table 2).

We also assessed the MTHFR genotype distribution in different homocysteine strata (Fig 1). The frequency of the homozygous 677C→T mutation showed a gradual increase from 4% in the lowest homocysteine stratum (homocysteine <10 $\mu\text{mol/L}$) to 24% in the highest stratum (homocysteine >18 $\mu\text{mol/L}$), again indicating the association between homocysteine concentrations and the homozygous (+/+) genotype.

Thermolabile MTHFR in CAD

For a summary estimate of the relative risk of the homozygous (+/+) genotype, we performed a meta-analysis of studies reporting data on the MTHFR genotypes in patients with CAD. In this analysis, we confined ourselves to case-control studies in which MTHFR genotype distributions among CAD patients as well as control subjects either were given or could be calculated from their data. For each study, we calculated odds ratios and 95% CIs for the (+/+) genotype relative to the (-/-) genotype separately (Fig 2). The combination of all studies reported yielded a patient group consisting of 2476 individuals (299 +/+, 1097 +/-, and 1080 -/-) and a control group of 2481 individuals (257 +/+, 1090 +/-, and 1134 -/-). From the MTHFR genotype distribution in this combined study group, we calculated an odds ratio of 1.22 (95% CI, 1.01 to 1.47) for the homozygous (+/+) genotype and 1.06 (95% CI, 0.94 to 1.19) for the heterozygous (+/-) genotype, both relative to the (-/-) genotype. When the heterozygotes (+/-)

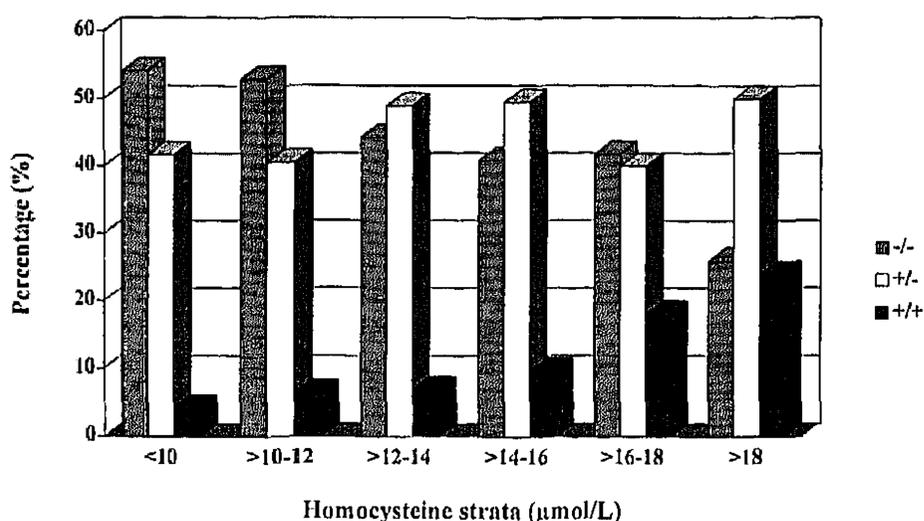


FIG 1. MTHFR genotype distribution in different homocysteine strata. Serum homocysteine concentration is expressed in micromoles per liter. In each stratum, the total number of individuals is set to 100%.

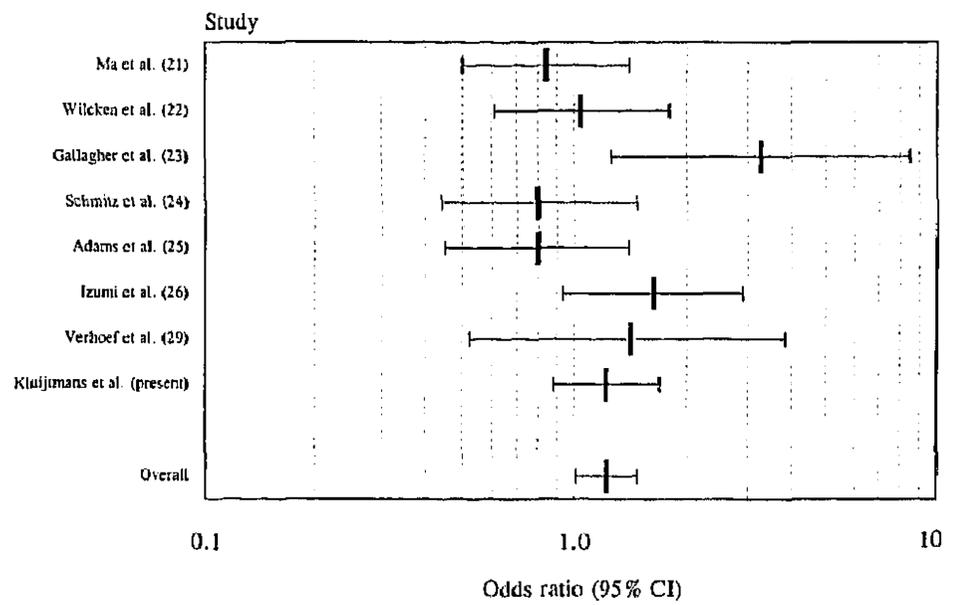


FIG 2. Odds ratios and 95% CIs for CAD associated with the (+/+) genotype relative to the (-/-) genotype. Reference number of each study is given in parentheses.

and (-/-) individuals are combined, the odds ratio for CAD among those with the (+/+) genotype was 1.19 (95% CI, 1.00 to 1.42).

Discussion

In this study, we showed a correlation between the 677C→T mutation in the MTHFR gene and homocysteine concentrations in which homozygous (+/+) and even heterozygous (+/-) individuals exhibited significantly elevated homocysteine levels compared with (-/-) individuals. Furthermore, by combining all previously reported studies, we were able to demonstrate the significance of the homozygous (+/+) genotype as a risk factor for CAD.

Many studies have explored the relationship between elevated homocysteine concentrations and an increased risk for atherosclerotic vascular disease.^{2,3,32-35} Recently, these studies have been summarized in a meta-analysis,¹ which led to the conclusion that elevations in homocysteine concentrations have to be considered as an independent and graded risk factor for different categories of arterial occlusive diseases. Several clinical studies supported this conclusion by establishing a quantitative relationship between coronary occlusion and homocysteine levels.^{35,36} On the basis of a linear relationship between homocysteine and the risk of CAD, Boushey et al¹ calculated an odds ratio for CAD of 1.6 (95% CI, 1.4 to 1.7) for a 5- $\mu\text{mol/L}$ increase in homocysteine concentrations. Accordingly, a risk of 1.12 can be calculated for a 1- $\mu\text{mol/L}$ increase in homocysteine. In our analysis of the association between MTHFR genotype and homocysteine concentrations, we observed an increase in median homocysteine concentrations of 0.8 $\mu\text{mol/L}$ for heterozygous (+/-) and 2.8 $\mu\text{mol/L}$ for homozygous (+/+) individuals relative to (-/-) individuals, which equals a risk for CAD of 1.10 and 1.34 for the heterozygotes (+/-) and homozygotes (+/+), respectively, relative to the risk of (-/-) individuals. These risk estimates calculated are well in line with the odds ratios for the homozygous (+/+) and heterozygous (+/-) genotypes observed in the present study (Table 1).

The frequency of the homozygous (+/+) genotype varies between different populations. The effect of this mutation on homocysteine concentrations depends on study design, inclusion criteria, ethnic background, age, and vitamin intake of the population. Except for the

study of Schmitz et al,²⁴ all recent studies on this MTHFR variant and hyperhomocysteinemia^{18,19,21,29,37,38} showed elevated homocysteine concentrations in homozygous (+/+) individuals. The present study supports these observations and indicates again that the homozygous (+/+) genotype is associated with elevated homocysteine concentrations (Table 2 and Fig 1). Folate status is considered an important environmental modulator of homocysteine levels only in homozygous (+/+) individuals.¹⁸⁻²¹ The effect of the homozygous (+/+) genotype on homocysteine concentrations might therefore differ between separate studies as a result of a different intake of folate. A possible adjustment for plasma folate could not be performed in this study, because blood folate levels were not determined.

In the present study, we were also able to demonstrate a statistically significant effect of the heterozygous (+/-) genotype on homocysteine concentrations. This is in line with the results reported by Harmon et al,³⁸ who observed elevated plasma homocysteine concentrations in heterozygous (+/-) individuals in the top 50% of the homocysteine distribution. On the basis of specific and residual MTHFR activities measured in isolated lymphocytes, this observation was not unexpected, because we have shown that heterozygous (+/-) individuals have significantly decreased specific and residual MTHFR activities compared with nonaffected (-/-) individuals.^{18,20} MTHFR-dependent homocysteine remethylation, in which 5-methyltetrahydrofolate (the product of the reaction catalyzed by MTHFR) serves as methyl donor, is present in nearly every cell of the human body. Therefore, any significantly deleterious effect in MTHFR enzyme activity will be reflected in elevation of homocysteine concentration in these cells. Previous studies were unable to detect an effect of the heterozygous (+/-) genotype on homocysteine concentrations, probably because of the relatively limited number of individuals included in those studies.

Kang et al⁵ were the first to report on a thermolabile MTHFR variant in two patients with CAD and hyperhomocysteinemia. Subsequent studies by the same group^{14,15,39} showed an association between this thermolabile MTHFR and (the severity of) CAD. In a large study among CAD patients and healthy control subjects, Kang et al⁵ detected thermolabile MTHFR in 36 of 212 cases (17%) versus 10 of 202 control subjects (5%).¹⁴ In the present study, we observed a much lower frequency of the thermolabile (+/+) genotype among Dutch CAD patients and a higher frequency among Dutch population-based control subjects. Several possible explanations for this phenomenon should be considered. In the study by Kang et al,⁵ the frequency of thermolabile MTHFR was assessed biochemically and was not based on genotyping of the 677C→T mutation. Because of the wide range in MTHFR activities in homozygous (+/+) and heterozygous (+/-) individuals,^{18,20} some individuals with a biochemically determined thermolabile MTHFR might not have been homozygotes (+/+), but heterozygotes (+/-) for the thermolabile allele or carriers for other mildly defective MTHFR alleles. In addition, Kang et al used in their studies a control group consisting of healthy controls with no history or clinical evidence of arterial occlusive disease. In our study, control subjects were recruited from the general population, possibly including individuals with a positive

history of CAD, which may dilute an eventual effect of the homozygous (+/+) genotype.

For a summary estimation of the relative risk of the homozygous (+/+) genotype, we analyzed eight different case-control studies presenting data on the MTHFR genotype distribution in CAD patients. From these studies,^{21-26,29} only an Irish study²³ observed a significant odds ratio for the homozygous (+/+) genotype in CAD. In all other studies, the odds ratios for the homozygous (+/+) genotype were not significantly increased. By combining all studies, we were able to calculate a significant odds ratio of 1.22 (95% CI, 1.01 to 1.47) for the homozygous (+/+) genotype relative to the (-/-) genotype in CAD, an odds ratio comparable to that obtained in the present study on the risk of thermolabile MTHFR in REGRESS. This overall result indicates that the thermolabile (+/+) genotype itself is a modest but significant genetic risk factor for CAD, a risk that is likely modulated by environmental factors, especially folate status.¹⁸⁻²¹

In conclusion, we demonstrated for the first time, that both homozygotes (+/+) and heterozygotes (+/-) for the 677C→T mutation in the MTHFR gene have significantly elevated homocysteine concentrations relative to (-/-) individuals. The odds ratios observed for both (+/+) and (+/-) genotypes for CAD are graded and in concordance with the risk calculated from a large quantitative study on homocysteine as a risk factor for CAD. By performing a meta-analysis, we were able to show that the homozygous (+/+) genotype is a genetic risk factor for CAD.

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