

Thermolabile Methylenetetrahydrofolate Reductase and Factor V Leiden in the Risk of Deep-Vein Thrombosis

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Summary

Mild hyperhomocysteinemia is an established risk factor for both atherosclerosis and thrombosis, and may be caused by genetic and environmental factors. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the cofactor for the methylation of homocysteine to methionine. Individuals with the thermolabile variant of MTHFR have decreased MTHFR activities, resulting in elevated plasma homocysteine concentrations. A homozygous 677C→T transition in the MTHFR gene has recently been identified as the cause of reduced enzyme activity and thermolability of the protein. We studied the frequency of the homozygous mutant (+/+) genotype in 471 patients with deep-vein thrombosis and 474 healthy controls enrolled in The Leiden Thrombophilia Study (LETS), its interaction with factor V Leiden, and assessed the association between the MTHFR genotypes and plasma homocysteine concentration. Homozygosity for the 677C→T polymorphism was observed in 47 (10%) patients, and in 47 (9.9%) controls (OR 1.01 [95% CI 0.7-1.5]). No modified risk of the (+/+) genotype was observed in carriers of factor V Leiden. Our data suggest that, although the homozygous mutant genotype is associated with elevated plasma homocysteine concentrations, this homozygous mutation itself is not a genetic risk factor for deep-vein thrombosis, irrespective of factor V Leiden genotype.

Introduction

Mild hyperhomocysteinemia, a disorder of methionine metabolism, is an established risk factor for arterial cardiovascular disease (1). Although vascular accidents in homocystinuria due to cystathionine β -synthase deficiency are of venous origin in 51% of the cases (2), only a few studies have explored the relation between mild hyperhomocysteinemia and venous thrombosis. Blomstrom *et al* were the first who examined a possible relationship between mild hyperhomocysteinemia and venous thrombosis in a sex- and age-matched case-control study (3). They observed mild hyperhomocysteinemia in 14% of the patients versus 5% of the controls after methionine loading; however, no differences were found in mean plasma homocysteine concentrations. Amundsen *et al* (4) did not report a significant difference in mean plasma homocysteine concentrations between young adults (<56 y) with venous thrombosis and control subjects. However, from their data, we

were able to calculate an odds ratio of 1.7 [95% CI 0.5-5.9] for a post-load homocysteine concentration above 50 μ mol/l (~90th percentile of their control group). In both studies, no differences were observed, probably due to the low number of patients and controls included in both studies. Falcon *et al* (5) found a high prevalence of hyperhomocysteinemia in patients with juvenile venous thrombosis. In their study, only mean post-methionine load increase in plasma homocysteine in patients appeared to be different from controls, and post-load hyperhomocysteinemia was observed in almost 20% of the cases. In subsequent studies, Den Heijer *et al* found similar results on mild hyperhomocysteinemia in ~25% of the patients with recurrent venous thrombosis, both in fasting state and after methionine loading (6). In the Leiden Thrombophilia Study (LETS), fasting homocysteine concentrations were elevated in patients with deep-vein thrombosis compared with age- and sex-matched healthy controls, suggesting that hyperhomocysteinemia is a risk factor for deep-vein thrombosis (7).

In 1988, Kang *et al* (8) described a new variant of methylenetetrahydrofolate reductase (MTHFR) with thermolabile properties and reduced enzyme activity, resulting in mildly elevated plasma homocysteine concentrations. Previously, we have shown that this thermolabile MTHFR is a cause of abnormal homocysteine metabolism in ~28% of cardiovascular disease patients with mild hyperhomocysteinemia (9). We elucidated the genetic basis underlying this thermolability to be a 677C→T transition, resulting in a conserved amino acid change from alanine to valine (10). This mutation in homozygous form appeared to be in high agreement with thermolability of the protein, reduced specific enzyme activity, and increased plasma homocysteine concentrations (10-12), especially in circumstances of low folate status (13, 14).

In the present study, we investigated the 677C→T mutation as a risk factor for deep-vein thrombosis in 471 patients and in 474 controls enrolled in the Leiden Thrombophilia Study (LETS), and its interaction with factor V Leiden (FVL), the most common heritable cause of deep-vein thrombosis is studied (15). Furthermore, we assessed the association of the mutation to fasting total plasma homocysteine concentration in a subset of 269 matched case-control pairs.

Patients and Methods

Patients The Leiden Thrombophilia Study (LETS) is a population based case-control study in three Dutch Anticoagulation Clinics, including 474 patients with a first episode of deep-vein thrombosis and 474 age- and sex-matched healthy control subjects, designed to clarify the contribution of several risk factors to deep-vein thrombosis, which has been described in detail elsewhere (16).

Methods Genomic DNA was extracted from peripheral blood lymphocytes by standard techniques and the MTHFR mutation analysis was performed essentially according to Frosst *et al* (10), by a technician unaware of the status of

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the DNA sample. The restriction fragments (198 bp for the 677C allele, and 175 bp and 23 bp for the 677T allele) were separated in 4% agarose gels and visualized after ethidium bromide staining. Mutation screening for FVL has been performed earlier (15), in which 92 cases and 14 controls were found to be carriers of the FVL mutation.

Total plasma homocysteine concentrations were determined in the 269 matched case-control pairs who attended the Leiden Anticoagulation Clinic (7) by reverse phase HPLC and fluorescent detection, as described by Te Poele-Pothoff *et al.* (17).

Statistics. Unmatched odds ratios and 95% confidence intervals as an estimate of the relative risk of the homozygous (+/+) genotype were calculated in the entire study population, in the Leiden subgroup, and in different age and sex groups. The 95% Confidence Intervals were calculated from a conditional logistic-regression algorithm by the maximum likelihood method, with Egret software. To assess the relation between the mutation and homocysteine level, we calculated homocysteine concentrations in different genotype groups, and prevalences of the three genotypes in different strata of homocysteine levels. The possible effect modification by FVL genotype, i.e. whether the MTHFR variant exerted a different effect in FVL carriers than in non-carriers, was examined by stratified analysis. We subdivided cases and controls in those with neither, one, or both of the variant genotypes, and calculated unmatched odds ratios as estimates of the relative risk of thrombosis for each group relative to those with double wildtype genotype.

Results

The mean age for the entire study group was 44 years (range 16-70 in patients, and 16-71 in controls). The male to female ratio was 1:1.3 for cases and controls alike (7).

Fasting plasma homocysteine concentrations were determined in 269 out of 474 matched case-control pairs. Median plasma homocysteine concentration was elevated in cases compared to controls (12.9 $\mu\text{mol/L}$; range 4.8-60.2 $\mu\text{mol/L}$, versus 12.3 $\mu\text{mol/L}$; range 6.4-37.5 $\mu\text{mol/L}$, respectively) (7). Mild hyperhomocysteinemia, defined as a fasting total plasma homocysteine concentration >18.5 $\mu\text{mol/L}$ (i.e. above the 95th percentile of the control group), resulted in a matched odds ratio of 2.5 (95% Confidence Interval: 1.2-5.2) (7).

The prevalence of the 677C→T transition was examined in 471 patients with deep-vein thrombosis and in 474 healthy controls. We observed a prevalence of the homozygous mutant genotype (+/+) in 47 (10%) out of 471 patients, versus 47 (9.9%) out of 474 healthy controls (Table 1). The unmatched odds ratio, as an estimate of relative risk of deep-vein thrombosis, for homozygous mutant (+/+) individuals relative to heterozygous (+/-) and homozygous normal (-/-) individuals was 1.01 (95% Confidence Interval: 0.7-1.5).

The prevalence of the homozygous mutant genotype (+/+) in a subset of 269 matched case-control pairs for whom plasma homocysteine measurements were performed, was 10.4% (28 out of 269) in cases, and 8.6% (23 out of 269) in controls, which is not substantially different from the overall result. In this subgroup, we associated the MTHFR genotype to homocysteine concentrations in plasma. As shown in Table 2, homocysteine concentrations were higher among individuals with the homozygous mutant genotype (+/+) compared with heterozygous (+/-) and (-/-) individuals. We then subdivided this population in different homocysteine strata and calculated the MTHFR genotype distribution, Fig. 1. In the lowest three homocysteine strata, the frequency of the (+/+) genotype was <10%, whereas in the higher strata the frequency increased from 12-15% to more than 50% in the highest stratum. These results indicate that this mutation is a significant contributor to elevated homocysteine concentrations.

In 11 out of 28 (+/+) cases (39%), mild hyperhomocysteinemia was observed compared with 4 out of 23 (+/+) controls (17.4%). The latter

Table 1 MTHFR genotype distribution among patients with a first episode of thrombosis and controls

Genotype	Thrombosis Patients (n=471)	Controls (n=474)	Odds Ratio (95% CI)
+/+ (%)	47 (10.0)	47 (9.9)	1.05 (0.7 - 1.6)
+/- (%)	213 (45.2)	203 (42.8)	1.10 (0.8 - 1.4)
-/- (%)	211 (44.8)	224 (47.3)	1.0*

* Reference category, odds ratio = 1

Table 2 Relationship between MTHFR genotype and fasting plasma homocysteine concentration

	+/+	+/-	-/-
Controls	15.3 $\pm$ 5.8 (n=23)	13.1 $\pm$ 4.0 (n=112)	12.3 $\pm$ 2.7 (n=134)
Patients	19.6 $\pm$ 13.0 (n=28)	13.5 $\pm$ 4.1 (n=117)	12.9 $\pm$ 4.4 (n=124)

Plasma homocysteine concentrations are expressed in $\mu\text{mol/L}$ as mean $\pm$ SD.

indicates that (+/+) cases are more prone to develop a mild hyperhomocysteinemia than controls from the same genotype group. In men we found an odds ratio for the (+/+) genotype slightly below one, and for women slightly above one; both these estimates had wide confidence limits (men: OR 0.76 [95% Confidence Interval: 0.40-1.44]; women: OR 1.33 [95% Confidence Interval: 0.69-2.61]). No differences were observed in different age groups (Table 3).

We also studied the interaction of thermolabile MTHFR with the most common inherited cause of deep-vein thrombosis: APC resistance due to FVL. In these analyses, we calculated unmatched odds ratios for deep-vein thrombosis due to either MTHFR, FVL or both risk genotypes, relative to those individuals with neither of these risk genotypes (Table 4). Although the number of individuals with both risk factors are

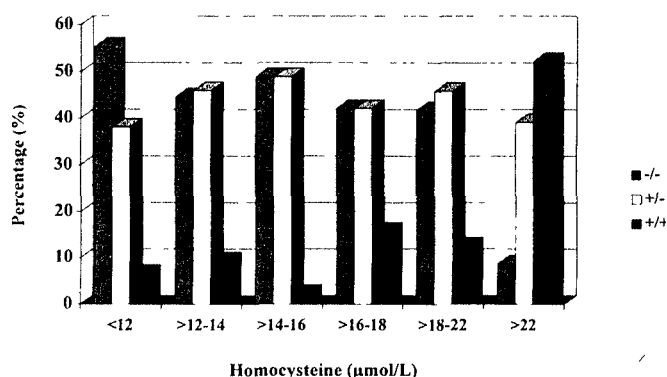


Fig. 1 MTHFR genotype distribution in different homocysteine strata. Homocysteine concentrations are expressed in $\mu\text{mol/L}$. In each homocysteine stratum, the number of individuals is set to 100%

relatively small, we observed no modified risk of the MTHFR (+/+) genotype by concomitant FVL. For a summary estimate of the relative risk of venous thrombosis in jointly affected individuals, we combined three studies recently presented at the XVIth Congress of the International Society on Thrombosis and Haemostasis, Florence, Italy, in which we were able to calculate the genotype distributions of both mutations in cases and controls (18, 20), and combined them with the present study. Overall, the homozygous mutant (+/+) genotype in the MTHFR gene was observed in 117 (14.4%) out of 810 cases, and in 121 (13.9%) out of 870 controls (OR 1.0 [95% CI 0.8-1.4]). FVL was detected in 164 (20.2%) cases and in 26 (3.0%) controls (OR 8.2 [95% CI 5.4-12.6]), whereas the coexistence of both mutations was observed in 26 (3.2%) cases and in 4 (0.5%) controls (OR 7.2 [95% CI 2.5-20.7]). These summary estimates indicate that the MTHFR 677C→T variant itself is not a risk factor for venous thrombosis, and does not modify the risk in individuals with FVL genotype.

Discussion

Homozygosity for the 677C→T mutation in the MTHFR gene is associated with elevated plasma homocysteine concentrations. However, this homozygous mutation is not associated with an increased risk of deep-vein thrombosis, whereas mild hyperhomocysteinemia is (7).

Den Heijer *et al.* (7) found a 2.5-fold increased risk [95% CI 1.7-3.7] for a homocysteine concentration above 18.5 mmol/L (95th percentile of the control group) in the general population. This result and those from other studies (3, 5, 6) all suggested that mild hyperhomocysteinemia is a risk factor for occurrence and recurrence of venous thrombosis, which resembles the association observed in arterial cardiovascular disease (21).

Elevated plasma homocysteine concentrations may originate from nutritional deficiencies (low folate, vitamin B-12 or vitamin B-6 status) or from genetic aberrations in enzymes involved in homocysteine metabolism. The recently detected homozygous 677C→T transition in the MTHFR gene has been shown to be associated with elevated mean plasma homocysteine concentrations (10, 11). By combining eight case-control studies in a meta-analysis, we were able to show that this mutation is a rather modest but significant risk factor conferring a 22% higher risk for coronary artery disease (22). In the present study, mild hyperhomocysteinemia, defined as a homocysteine concentration exceeding the 95th percentile of the control group (>18.5 μmol/L), was observed in 28 (10%) out of 269 patients. We observed that the homozygous mutation is a contributor to elevated plasma homocysteine concentrations, which is in agreement with several other studies (10-12). However, we found no difference in prevalence of the 677C→T transition in patients with a first episode of venous thrombosis, indicating that disturbed homocysteine remethylation due to this genetic defect alone seems not a risk factor for deep vein thrombosis.

Several explanations may be offered.

(1) Recent studies by Jacques *et al.* (13) and Van der Put *et al.* (14), demonstrated a strong interaction between this genetic predisposition and folate status on the plasma homocysteine concentration. In individuals with inadequate folate status, the homozygous (+/+) genotype led to elevated homocysteine levels, contrary to (+/+) individuals with an adequate folate status, in whom plasma homocysteine was not elevated in comparison with both other genotypes. The fact that 39% of the cases versus only 17% of the controls with the (+/+) genotype were hyperhomocysteinemic, certainly implies the interaction with and the existence of another factor determining plasma homocysteine levels, more often found among cases than controls. Whether this can be

Table 3 Thrombosis risk due to +/- genotype in different age groups

Age (years)	Odds ratios (95% Confidence Interval)
<30	2.00 (0.42-12.4)
30-50	0.96 (0.50-1.82)
>50	0.89 (0.42-1.85)
Overall	1.05 (0.7-1.6)

Table 4 Relationship between thermolabile MTHFR, factor V Leiden, and the risk of deep-vein thrombosis

FVLeiden	MTHFR	Cases	Controls	OR	95% CI
		342	416	1*	
+	-	82	11	9.1	4.8-17.3
	+	37	44	1.0	0.6-1.6
+	+	10	3	4.1	1.1-14.8

* Reference category: OR = 1, FV Leiden heterozygotes are referred to as "+", wildtype is referred to as "-", MTHFR homozygotes (+/+) are referred to as "+", heterozygotes (+/-) and wildtype (-/-) are referred to as "-".

attributed to an inadequate folate status in patients could not be assessed, because the vitamin status was not determined in our study population. In a study on hyperhomocysteinemia in recurrent venous thrombosis, Den Heijer *et al.* did not find differences in vitamin B12 and folate status in patients compared with population-based controls (6, 23). This could also be the case in our study on deep-vein thrombosis, although a change in dietary intake of vitamin nutrients due to a first episode of thrombosis cannot be ignored.

(2) In the LETS-study, as we reported previously (7), the risk of venous thrombosis did not increase gradually, the odds ratios only increased substantially with homocysteine concentrations above 22 μmol/L, indicating that there might be a threshold above which homocysteine has thrombogenic repercussions. The MTHFR mutation itself may not be sufficient to lead to such pronounced homocysteine elevation, and will only in combination with an environmental or another genetic factor lead to such a hyperhomocysteinemia.

(3) The homozygous (+/+) genotype causes a redistribution of folate derivatives (12, 14) leading to a higher availability of one-carbon moieties for thymidine synthesis. The latter may have a concomitant beneficial effect on the cardiovascular system.

(4) There could be a, yet undetermined, stronger determinant of elevated homocysteine levels contributing to the risk of deep vein thrombosis, a contributor not associated to this genetic defect in MTHFR. This stronger determinant may mask a possible effect of the MTHFR mutation.

(5) The observation of an equally distribution of the 677C→T transition over both controls and patients even raises the question whether homocysteine itself is the prime compound causing venous thrombosis and not another metabolite related to homocysteine metabolism but not associated to thermolabile MTHFR. The thrombogenic effect of mildly elevated homocysteine concentrations could be dependent of the primary underlying cause of mild hyperhomocysteinemia. Obviously, this

sheds serious doubts to the status of mild hyperhomocysteinemia as an independent cause of deep-vein thrombosis, and opens the possibility that hyperhomocysteinemia is only a marker of another defect, which is the actual cause of an increased risk for deep-vein thrombosis (23).

Activated protein C resistance (APC-resistance) due to FVL is the most common inherited cause of venous thrombosis (15) in Europeans (24). The findings of Mandel et al. (25) in highly consanguineous homocystinuric families, suggested that concomitant FVL is an absolute prerequisite for thrombosis in severe hyperhomocysteinemic patients. These results challenged the observation of Den Heijer et al. (7), who observed mildly elevated homocysteine concentrations as a risk factor for thrombosis, independent from coexisting abnormalities in blood coagulation, such as protein S-, protein C-, antithrombin deficiency and APC-resistance. In the Physician's Health Study, an interaction was reported between mild hyperhomocysteinemia and concomitant FVL in the risk of venous thromboembolism (26).

In the present study, we did not observe an interaction between thermolabile MTHFR, a major determinant of elevated homocysteine concentrations, and FVL, indicating that the presence of both genetic variants do not lead to an additional risk compared with FVL alone. A number of studies have addressed the MTHFR variant and concomitant FVL in the risk of venous thrombosis, however with contradictory results (18-20, 27-29). Zighetti (20) and Gaustadness (27) and their co-workers observed an overrepresentation of the frequency of joint abnormalities in thrombosis patients, indicative of an interaction between the MTHFR variant and FVL in the risk of venous thrombosis. On the other hand, several other studies did not report an increased thrombotic risk in FVL carriers due to thermolabile MTHFR (18, 19, 28, 29). In a summary estimate, we did not observe an increased risk in jointly affected individuals compared with the risk in individuals with isolated FVL.

The mechanism by which homocysteine may cause venous thrombosis is obscure. Several *in vitro* studies suggest that homocysteine interferes with the anticoagulant and fibrinolytic system (30, 31), although in most studies the homocysteine concentrations applied were almost ten times higher than those observed in homocystinuria patients. Furthermore, homocysteine concentrations were added in its free sulfhydryl (reduced) form, leading to tremendous shift in redox status, whereas in blood almost 99% is present in an oxidated state.

In conclusion, we have demonstrated that the prevalence of the homozygous 677C→T mutation in the MTHFR gene is not increased in patients with deep-vein thrombosis compared to healthy age- and sex-matched controls, indicating that this mutation itself is not a risk factor for deep-vein thrombosis. A small risk however, as previously shown in coronary artery disease (22), cannot be excluded. A possible interaction between the 677C→T mutation in the MTHFR gene and FVL was not observed. Further studies are warranted to elucidate the relationship between abnormal homocysteine metabolism and its contribution to the risk of deep-vein thrombosis.

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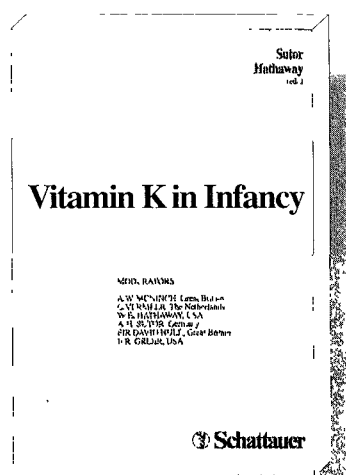
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