Genetic variation and susceptibility to venous thrombosis : Etiology and risk assessment

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No Association Between The Common MTHFR 677C>T Polymorphism And Venous Thrombosis

Chapter 4

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ABSTRACT

Background Increased homocysteine levels are related to the occurrence of venous thrombosis, but whether this relation is causal is unclear. The T-variant of the common methylenetetrahydrofolate reductase (MTHFR) 677CT polymorphism mildly increases homocysteine levels. Meta-analyses have demonstrated a weak effect of the MTHFR 677TT genotype on risk but are sensitive to selective publication of positive results. The aim of the present study was to evaluate the effect of the MTHFR genotype on the risk of venous thrombosis, overall and in subgroups of known risk factors, in a single large study.

Methods In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA Study), a population-based case-control study, we collected DNA from 4375 patients with a first deep vein thrombosis of the leg or pulmonary embolism and from 4856 control subjects. Information about risk factors for venous thrombosis was obtained from questionnaires.

Results MTHFR 677CT was not associated with the risk of venous thrombosis (odds ratio [95% confidence interval], 0.99 [0.91-1.08] for the CT genotype and 0.94 [0.81-1.08] for the TT genotype). Stratification by known risk factors for venous thrombosis provided no evidence of an association in specific groups.

Conclusions In a single large study, MTHFR 677CT was not associated with the risk of venous thrombosis, and the narrow confidence interval excludes even a small effect. Therefore, mildly elevated homocysteine levels as a result of MTHFR 677TT do not seem to cause venous thrombosis. There is no rationale for measuring the MTHFR 677CT variant for clinical purposes.

INTRODUCTION

Venous thrombosis is a common disease with an annual incidence of 1 to 3 in 1000 individuals and is caused by the joint effect of environmental and genetic risk factors [135]. One of the genetic factors that have been extensively studied over the past decade is a polymorphism in the gene encoding 5,10-methylenetetrahydrofolate reductase (*MTHFR*). MTHFR is an enzyme involved in homocysteine metabolism by converting folate, a cofactor for homocysteine conversion, into its major circulating form 5-methyltetrahydrofolate. A common C>T substitution at nucleotide 677 converts an alanine to a valine residue [136] and causes thermolability of the enzyme at 37°C. Homozygotes have more than 50% reduced enzyme activities, but the effect of reduced *MTHFR* activity on homocysteine levels is dependent on folate intake. Homocysteine levels are about 25% higher in homozygous carriers only when plasma folate concentration is low [137].

Hyperhomocysteinemia is associated with venous thrombosis [138] and therefore *MTHFR* 677C>T has been one of the candidate genetic risk factors for venous thrombosis. However, most case-control and cohort studies that assessed the association between *MTHFR* 677C>T and venous thrombosis reported either a weak association or no relationship at all. Because these studies were small and often underpowered to detect weak effects, several meta-analyses have been performed [123,139,140]. The most recent and largest meta-analysis, including 8364 cases and 12 468 controls, found a small increase in risk for *MTHFR* 677TT carriers (odds ratio [OR], 1.20; 95% confidence interval [CI], 1.08-1.32) [123]. This risk increase is in line with the expected risk based on the association of the variant with homocysteine levels and the effects of hyperhomocysteinemia. The major disadvantage of meta-analyses, however, compared with a single large study, is the possibility of publication bias. Meta-analyses are based on published studies and rely on the quality of the collected studies. Publication bias is present when studies with "positive" results have a higher probability of being published than studies with "negative" results. It has been shown repeatedly that

publication bias is common in the medical literature [141]. Publication bias leads to an overestimate of the risk. Although no evidence of publication bias was found in the meta-analysis mentioned previously [123], it cannot be ruled out that studies that found no association, irrespective of sample size, were underrepresented. The alternative is a single large study, in which publication bias obviously can play no role.

Several studies have suggested that the effect of *MTHFR* 677C>T on venous thrombosis is only visible in specific subgroups with other predisposing genetic or environmental factors or, on the contrary, in subgroups in which conventional risk factors for venous thrombosis are absent [142-150]. To adequately investigate an effect in subgroups, a large study is needed because only a relatively small proportion of study subjects will carry both the risk factor and the *MTHFR* 677TT genotype.

In this article, we report on the association between *MTHFR* 677C>T and venous thrombosis in a single large study. The analysis included 4375 patients with a first venous thrombotic event, either deep vein thrombosis of the leg or pulmonary embolism, and 4856 control subjects from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA Study).

METHODS

Study Population

Between March 1, 1999, and August 31, 2004, consecutive patients aged 18 to 70 years with a first venous thrombosis of the leg or arm or a pulmonary embolism were recruited from 6 anticoagulation clinics in the Netherlands. Partners of patients were invited as control subjects. An additional control group was recruited between January 1, 2002, and December 1, 2004, using a random digit dialing (RDD) method [33]. The random control group was age- and sex frequency matched to the group of patients that provided a

blood sample. For practical reasons individuals with severe psychiatric problems or individuals who did not speak Dutch were excluded.

All participants were asked to fill in a questionnaire on acquired risk factors for venous thrombosis, family history of venous thrombosis, and vitamin B supplementation. The date of diagnosis as reported by the patient in the questionnaire or, when missing, the date of the first visit at the anticoagulation clinic served as the index date for patients and their partners. The index date for RDD control subjects was the date on which the questionnaire was completed or, when missing, the date on which the completed questionnaire was returned.
A blood sample was taken approximately 3 months after discontinuation of anticoagulant therapy. If patients continued their anticoagulant therapy, blood was drawn 1 year after the index date. Partner controls were invited for a blood draw along with their partner; RDD controls were invited after returning their questionnaire. Blood samples were taken from patients who were diagnosed before June 1, 2002, and their partners. Patients who were diagnosed from June 1, 2002, onwards and their partners received a cotton swab along with their questionnaire for collecting buccal cells. In the RDD group, blood samples were collected throughout the entire study period. Participants who refused to or were unable to provide a blood sample were offered the option of providing a buccal swab sample. Ideally, participants filled in a full questionnaire and provided a DNA sample, but a minor proportion provided only DNA. When the full questionnaire was not returned, we attempted to collect information about acquired risk factors (but not family history and vitamin supplementation) through a miniquestionnaire by telephone.

Of 6333 eligible patients with deep vein thrombosis of the leg or a pulmonary embolism, 358 died before inclusion and 271 could not be contacted. Of the remaining 5704 patients, 5053 (89%) participated. A DNA sample was donated by 4379 patients, among whom 4257 full questionnaires and 99 miniquestionnaires were collected.

Partners of participating patients were invited as control subjects. Of 3655 eligible partners, 1 died before inclusion and 10 could not be contacted. Of the remaining 3644 partners, 2984 (82%) participated. A DNA sample was donated by 2602 partners. In addition, we collected DNA from 240 controls whose partner had venous thrombosis of the arm (n = 104), eventually refused to participate (n = 10), or was excluded (n = 126). Among these 2850 partners, 2800 full questionnaires and 34 miniquestionnaires were collected.

The RDD method yielded 4350 eligible control subjects, but 4 died before inclusion and 88 control subjects could no longer be contacted despite repeated efforts. Of the remaining 4258, 3000 (70%) participated. A DNA sample was provided by 2023 RDD controls, among whom 2011 full questionnaires and 11 miniquestionnaires were collected.

To study the association between *MTHFR* 677C>T and venous thrombosis in subgroups, participants were stratified according to acquired risk factors as reported in the questionnaire. Participants were also stratified according to 2 common genetic risk factors, factor V Leiden, and prothrombin 20210G>A. Complete genetic data on *MTHFR* 677C>T, factor V Leiden, and prothrombin 20210G>A was available from 4375 patients and 4856 control subjects, and these were included in the present analysis.

Laboratory Analysis

Collection and processing of blood samples and buccal swabs and subsequent DNA isolation has been described previously [19]. Assessment of *MTHFR* 677C>T (rs1801133), factor V Leiden (rs6025), and prothrombin 20210G>A (rs1799963) in DNA retrieved from whole blood or DNA from buccal swabs was initially performed by restriction fragment length polymorphism analysis after conventional polymerase chain reaction (PCR). The presence of the *MTHFR* 677T allele was assessed by incubation with the restriction enzyme *Hinf*I. Factor V Leiden and prothrombin 20210G>A were analyzed in a combined method using *Mnl*I and *Hind*III restriction enzymes. Later, all 3 polymorphisms were determined by a 5' nuclease (Taqman;

Applied Biosystems, Foster City, Calif) assay using a standard PCR reaction mix (Eurogentec, Seraing, Belgium) and allele-specific fluorescent probes equipped with a minor groove binding moiety (Applied Biosystems).

Statistical Analysis

Odds ratios and 95% CIs were computed as an estimate of the risk of venous thrombosis associated with *MTHFR* 677CT and TT genotypes relative to CC. Adjustment for age and sex was performed by logistic regression.

The association between *MTHFR* genotype and venous thrombosis was further explored through stratification by known risk factors and computing ORs for the *MTHFR* 677TT genotype in strata of the risk factor under study, relative to the combined 677CT or CC genotype. Strata were made for the factor V Leiden, prothrombin 20210G>A, vitamin B supplementation, age group, family history of venous thrombosis, and the presence of predisposing factors for venous thrombosis.

Age was categorized as younger than 50 years (18-49 years) or 50 years and older (50-70 years). Family history was positive if at least 1 parent or sibling had venous thrombosis or a pulmonary embolism before the age of 50 years. For 2212 (24%) of 9055 participants with a full questionnaire, family history status could not be determined because of incomplete data on 1 or more family members. Predisposing factors were surgery, immobilization, pregnancy or puerperium within the year preceding the index date, and diagnosis of malignancy before or within 6 months after the index date. Participants who did not have complete information on these variables (239 [3%] of 9199 miniquestionnaires) were not included in the subgroup analysis. Vitamin B supplementation was defined as the self-reported use of vitamin supplementation that contained pyridoxine hydrochloride (vitamin B_6), folic acid (vitamin B_{11}), or cyanocobalamin (vitamin B_{12}), which are all cofactors for homocysteine conversion. Among 383 (4%) of 9055 participants, no information on vitamin B use was available. All statistical analyses were performed with SPSS for Windows, release 12.0.1 (SPSS Inc, Chicago, Ill).

RESULTS

Patients included in the analysis were diagnosed as having a first deep vein thrombosis of the leg (n = 2519), a first pulmonary embolism (n = 1315), or both (n = 541). In total, 4375 patients and 4856 control subjects were included. Median age (5th-95th percentile) at the index date was 50 years (26-68 years) for patients and 49 years (27-67 years) for control subjects. Slightly more women than men were included in both groups (54% of patients and 53% of control subjects).

The MTHFR 677TT genotype was present in 440 patients (10%) and 517 control subjects (11%), and the 677CT genotype in 1891 patients (43%) and in 2094 control subjects (43%).
Since genotype distributions did not differ between these 2 groups, there was no excess risk associated with the T allele: ORs (95% CIs) of venous thrombosis when carrying the 677T allele were 0.99 (0.91-1.08) for heterozygous and 0.94 (0.81-1.08) for homozygous carriers, relative to 677CC (Table 1).

Table 1. MTHFR Genotype Distribution Among Patients With Venous Thrombosis and Control Subjects

MTHFR 677 C>T	Cases, No (%) (n=4375)	Control subjects, No (%) (n=4856)	Odds Ratio (95% Confidence Interval)
CC	2044 (47)	2245 (46)	1 [Reference]
CT	1891 (43)	2094 (43)	0.99 (0.91-1.08)
TT	440 (10)	517 (11)	0.94 (0.81-1.08)

Factor V Leiden was present in 685 cases (16%) and 256 control subjects (5%) (Table 2). The association between MTHFR 677C>T and venous thrombosis did not differ between strata of factor V Leiden. Among patients, 224 individuals (5%) carried the prothrombin 20210A mutation and 94 controls (2%) were carrier. In prothrombin 20210A carriers,

the risk associated with MTHFR 677TT was somewhat higher than in noncarriers (OR, 1.63) but the 95% CI was wide (0.75-3.56).

Table 2. Association of Venous Thrombosis With MTHFR 677TT Relative to 677CC/677CT in Subgroups of Coexisting Risk Factors

	No.*	Odds Ratio (95% Confidence Interval)		No.*	Odds Ratio (95% Confidence Interval)
Factor V Leiden GG	8290	0.91 (0.79-1.06)	Factor V Leiden GA/AA	941	0.94 (0.61-1.45)
Prothrombin 20210 GG	8913	0.91 (0.79-1.05)	Prothrombin 20210 GA/AA	318	1.63 (0.75-3.56)
Age 18-49	4775	1.04 (0.87-1.26)	Age 50-70	4456	0.84 (0.69-1.02)
Negative family history	5999	0.97 (0.82-1.15)	Positive family history	844	0.98 (0.61-1.57)
No predisposing factors	5956	0.91 (0.76-1.08)	Predisposing factors	3004	1.05 (0.81-1.35)
Vitamin B supplementation	2441	0.96 (0.74-1.25)	No vitamin B supplementation	6407	0.93 (0.79-1.10)

* Total number of subjects in indicated group

The association between MTHFR 677C>T was also studied in a subgroup of participants who did not take vitamin supplements containing folic acid (vitamin B11), vitamin B6, or vitamin B12. The use of these vitamin supplements was more frequently reported by control subjects than by patients (29% vs 26%). In the subgroup without vitamin B supplementation, no effect of MTHFR genotype was observed (OR, 0.93; 95% CI, 0.79-1.10).

The association between MTHFR 677C>T and venous thrombosis was further explored by stratifying patients and control subjects according to age at index date (age 50 or <50 years), family history of venous thrombosis, and

the presence of predisposing factors for venous thrombosis. In none of these strata was an effect of MTHFR 677TT observed.

Adjustment for age or sex, stratifying patients according to diagnosis (thrombus in the leg, pulmonary embolism, or both), excluding study subjects with cancer, or restricting the control group to either partners of patients or RDD control subjects did not change these observations.

COMMENT

In the MEGA Study, a very large population-based case-control study, *MTHFR* 677C>T was not associated with the risk of venous thrombosis. Stratification by factor V Leiden, prothrombin 20210G>A, family history, age, presence of predisposing factors, and vitamin B supplementation did not provide evidence of an association in specific groups.

These results should be seen in the light of many conflicting study results. As summarized in 3 meta-analyses, the majority of previous single studies found no association. From these meta-analyses mildly elevated ORs of 1.29 (95% CI, 1.08-1.54) [140], 1.2 (95% CI, 1.1-1.4) [139], and 1.20 (95% CI, 1.08-1.32) were calculated [123]. These 3 meta-analyses mostly included the same single studies. The most recent one was the largest and included 20 832 study subjects from 53 studies [123]. When these single studies were grouped by population, a slightly increased risk of venous thrombosis was associated with the *MTHFR* 677TT genotype in European populations (OR, 1.15; 95% CI, 1.02-1.28). In total, 30 European case-control studies were included, with 1280 subjects in the largest single study [151]. If this meta-analysis [123] were repeated to include the MEGA Study, the risk estimate for European studies would decrease to 1.06 (95% CI, 0.96-1.16). The absence of any association in the present analysis, which is many times larger than any previously published case-control study, suggests that there may have been an overrepresentation in the literature of studies with positive results.

MTHFR 677C>T has been a candidate genetic risk factor for venous thrombosis because its phenotype, elevated serum homocysteine level, is associated with venous thrombosis. In the present study, homocysteine levels were not measured, but elevated levels were observed in homozygous carriers of the T variant in many studies, including studies in the Dutch population [143]. The mechanism by which homocysteine would affect thrombotic risk is unknown, and therefore it is still a matter of debate whether the relation is causal or whether homocysteine is a marker of other causal risk factors or the consequence of venous thrombosis. The study of the *MTHFR* genotype offers the possibility to investigate these various hypotheses, since a genotype cannot be a marker of another risk factor or a post hoc phenomenon. So, when elevated levels of homocysteine cause thrombosis, *MTHFR* 677TT is expected to be related to thrombotic risk. According to this reasoning, the absence of an association between *MTHFR* genotype and venous thrombosis suggests that the association between elevated homocysteine levels and venous thrombosis is not causal.

Another way to disentangle causal and noncausal effects is to perform an experiment. If hyperhomocysteinemia causes thrombosis, lowering homocysteine level is expected to protect those with the *MTHFR* 677TT genotype from developing thrombosis. Several randomized trials in which homocysteine level was lowered by vitamin B supplementation have been performed or are still ongoing, both in arterial and venous disease. Three trials on the effect of lowering homocysteine level on arterial thrombosis have been completed [152-154], and 1 trial examined the effect in venous thrombosis [155]. Despite a decrease in homocysteine levels in the vitamin treatment group, none of these trials showed a beneficial effect on disease outcome. Thus, the results of these trials do not support the hypothesis that high levels of homocysteine cause thrombosis. It should be noted, however, that these trials examined the effect of lowering homocysteine level on recurrent thrombosis, not on a first event.

Alternatively, the effect of thermolabile MTHFR on homocysteine levels may be too small to cause thrombosis on its own. In the Leiden Thrombophilia Study [138], the risk of thrombosis was only increased when homocysteine

concentrations were above 2.43 mg/L (>18 µmol/L), compared with a reference level below 1.62 mg/L (<12 µmol/L), which corresponds to at least 50% higher homocysteine concentrations. The 25% increase in homocysteine concentrations that is generally observed in individuals with the *MTHFR* 677TT genotype may therefore not be enough in most cases to cause thrombosis.

The *MTHFR* 677TT genotype increases homocysteine levels only when combined with low vitamin B levels. Sufficient intake of folic acid (vitamin B_{11}), vitamin B_6, or vitamin B_{12} normalizes serum homocysteine in *MTHFR* 677TT carriers [156,157]. Therefore, the use of vitamin supplements could mask a possible association between *MTHFR* genotype and venous thrombosis. In the MEGA Study, the association between *MTHFR* genotype and venous thrombosis did not depend on vitamin B supplementation as reported in the questionnaire.

If *MTHFR* 677C>T is only a weak risk factor for venous thrombosis, it may only be discernible in individuals with a specific predisposition for developing venous thrombosis. A number of studies have evaluated the joint effect of *MTHFR* 677C>T and predisposing genetic factors, mainly factor V Leiden and prothrombin 20210G>A. Factor V Leiden was most frequently studied, but owing to the small numbers in strata of the combined factors, risk estimates in these studies had wide confidence intervals [142,144,145,148,149]. Only 1 study showed an association between *MTHFR* 677C>T in carriers of factor V Leiden [142]. None of the studies that focused on the coexistence of *MTHFR* 677C>T and prothrombin 20210G>A found evidence of effect modification [144,146]. The MEGA Study confirms these negative results.

Other factors previously studied in relation to the *MTHFR* genotype and venous thrombosis were age, family history of venous thrombosis, and presence of acquired risk factors such as recent surgery, immobilization, malignancy, and pregnancy. In previous studies, different results were reported about specific subgroups in which an effect of *MTHFR* genotype

was observed. Some studies found an association in individuals in whom other known risk factors were absent [147], while others suggested that *MTHFR* 677C>T mainly affects the risk of venous thrombosis in cooperation with genetic or acquired risk factors [142,143,150]. Again, small study sizes might account for these conflicting results. The MEGA Study was large enough to make sufficiently large strata. No association between *MTHFR* genotype and venous thrombosis was observed within any of these strata.

Taken together, no evidence was found for an association between *MTHFR* 677C>T and the risk of venous thrombosis. There is no rationale for measuring the *MTHFR* 677C>T variant for clinical purposes.

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