Venous Thromboembolic Disease and the Prothrombin, Methylene Tetrahydrofolate Reductase and Factor V Genes

Martine Alhenc-Gelas, Emmanuel Amaud, Viviane Nicaud, Marie-Laurence Aubry, Jean-Noël Fiessinger, Martine Alach, Joseph Emmerich

From Unité INSERM 428, Unité INSERM 258, and Centre Claude Bernard de Recherche sur les Maladies Vasculaires, Hôpital Broussais – AP-HP, Paris, France

Summary

The prevalence of the A20210 allele of the prothrombin (PT) gene and the T677 allele of the methylene tetrahydrofolate reductase (MTHFR) gene was determined in 205 patients with venous thromboembolism (VTE) and in 398 healthy subjects of similar age and sex distribution. We also determined the frequency of these two candidate risk alleles in subjects carrying the factor V (FV) Q506 allele, to identify a possible interaction. Forty patients (19.5%) and 14 control subjects (3.5%) were heterozygous for the FV R506Q mutation. Twenty-one patients (10.2%) and 11 controls (2.8%) were heterozygous for the PT A20210 allele (odds ratio (OR) 4.02, 95% confidence interval (CI): 1.90-8.50, p < 0.001). This confirmed that the PT A20210 allele was a risk factor for VTE in our population. Among the FV Q506 allele carriers, 9 patients (22.5%) and no control also had the PT gene G20210A mutation. The absence of the combined abnormality in the control group made it impossible to calculate the relevant ORs but the lower bound of the 95% CI was 3.94, suggesting that individuals bearing the two mutations have a higher risk than those with a single mutation. Twenty-six patients (12.7%) and 49 controls (12.3%) were heterozygous for the MTHFR T677 allele (OR 1.04, 95% CI: 0.62-1.72, not significant). Four patients and 1 control were also heterozygous for the FV R506Q mutation (OR 9.33, 95% CI: 1.03-84.23). However, the ORs for carriers of the FV R506Q mutation were not significantly influenced by MTHFR gene C677T homozygosity.

Introduction

Venous thromboembolism (VTE) is a multifactorial disease in which both genetic and acquired risk factors are involved. The most frequent known genetic risk factor for venous thrombosis is the factor V (FV) R506Q mutation which leads to resistance to activated protein C (for review, see 1). Recently, a mutation in the 3' untranslated part of the prothrombin (PT) gene was associated with VTE in a series of Dutch patients (2). The mutation – a G to A transition at position 20210 of the nucleotide sequence – was associated with higher circulating plasma folate status is low (for review, see 5).

In addition to the FV R506Q mutation, the PT A20210 gene variant and the MTHFR T677 gene variant might thus be genetic risk factors for VTE. This study focused on the role of these two genetic risk factors in a population of unselected patients with VTE.

Subjects and Methods

Subjects

Two-hundred and five patients, 92 men and 113 women with VTE were recruited in a university hospital vascular medicine department (Hôpital Broussais, Paris) between November 1995 and November 1997 and were included in the study if they were younger than 61 and had had at least one objectively diagnosed episode of deep venous thrombosis (compression ultrasonography or venography) and/or pulmonary embolism (perfusion and ventilation lung scan, conventional pulmonary angiography or computed tomographic angiography). A complete clinical summary with emphasis on personal and family history for thromboembolic disease, and acquired risk factors (surgery or trauma within the past three months, immobilization (> 72 h), pregnancy, post-partum, treatment with oestrogens, varicose veins and malignancy) was obtained from all patients. Thirty-seven women were on oral contraception at the time of the thrombotic event. Fifty-six patients (27.1%) had at least one previous thrombotic event. The mean age [standard deviation (SD)] of the patients was 42.2 (11.1) years. The mean age (SD) at the first thrombotic event was 38.1 (12.0) years. The presenting thrombotic episode was deep venous thrombosis of the lower limb in 166 patients, and of the upper limb in 19 patients. Isolated symptomatic pulmonary embolism occurred in 20 patients. VTE was idiopathic in 79 patients (38.5%). Among the cases, six had also a history of arterial thrombosis (5 myocardial infarction and one thrombosis of a popliteal aneurysm), and only two had a history of malignancy (breast and gastric tumors).

Three hundred ninety eight healthy subjects (199 men, 199 women) aged from 20 to 60 years, without a history of VTE, arterial disease (stroke, myocardial infarction, angina or peripheral vascular disease), or malignancy, were recruited between May and September 1996 from a health center specializing in the prevention of cardiovascular disease, to which they had been referred for a routine check-up. The mean (SD) age of the controls was 42.9 (9.5) years. Thirty-seven women were on oral contraception. The case and control groups did not differ significantly according to age (Student t-test: p = 0.46) or sex [Chi-square test with one degree of freedom (DF), p = 0.23]. Women on oral contraception were more frequent among the cases than the controls (32.7% as compared to 18.6% ; Chi-square test: 1 DF, p < 0.01).

The birthplace of cases and controls was recorded, but there was no geographic or ethnic criterion for eligibility. However, most of the subjects (85% of controls and 88% of cases) were born in Europe, and non-European subjects were similarly distributed in both groups.

The study was approved by the local ethics committee and all the subjects gave their informed consent.

Correspondence to: Dr. Martine Alhenc-Gelas, Laboratoire d’Hémostase, Hôpital Broussais, F-75674 Paris Cedex 14, France – FAX Number: 33 1 45 41 35 13; Tel.: 33 1 43 95 99 22; Email: martine.alhenc-gelas@bhs.ap-hop-paris.fr
Laboratory Investigations

Venous blood was collected on 0.129 M trisodium citrate (1:10) and two steps of centrifugation at 2,000 \( \times \) g for 15 min were performed in order to obtain platelet poor plasma. Plasma was frozen and stored in small aliquots at \(-40^\circ \)C until tested. Coagulation tests included antithrombin, protein C and protein S measurements and lupus anticoagulant screening (6). Lower limits of normal for antithrombin and protein C activities were 0.80 IU/ml and 0.65 IU/ml respectively. The lower limit of normal for free protein S was 0.70 IU/ml in men and in women older than 50 years, 0.60 IU/ml in women younger than 50.

DNA was isolated from leukocytes by standard methods and stored at 4°C. DNA was screened for the FV gene R506Q mutation after polymerase chain reaction (PCR), amplification of exon 10 of the FV gene and digestion by restriction enzymes (7). The MTHFR gene C677T mutation was detected as described elsewhere (4). The PT gene G20210A transition was identified after amplification with primers A (5'-TTACAAGCTGTGATGAGGGA-3') and B (5'-CCATGAATGCTGGAGCATTGAGGC-3'). Primer B was designed with a nucleotide substitution (C to A) at position 20210 which allows for enzymatic detection of the G20210A, factor V gene A4070G, prothrombin gene C3062A, factor V gene R506Q and MTHFR gene C677T mutation.

Results

There were no deviations from Hardy Weinberg equilibrium in the study population. Of the 205 patients with VTE, 21 (10.2%) were heterozygous for the PT gene G20210A mutation, 40 (19.5%) were heterozygous for the FV R506Q mutation and 26 (12.7%) were homozygous for the MTHFR gene C677T polymorphism. Of the 398 controls, 11 (2.8%) were heterozygous for the PT gene G20210A mutation, 14 (3.5%) were heterozygous for the FV R506Q mutation, and 49 (12%) were homozygous for the MTHFR gene C677T polymorphism (Table 1). Thus, the PT A20210 and FV Q506 allele frequencies were significantly more frequent in the patients with VTE than in the healthy controls (0.015 as compared to 0.009 and 0.098 as compared to 0.018 respectively; \( p < 0.01 \)), whereas the frequency of the MTHFR T776 allele at the homozygous state was not significantly different (0.342 as compared to 0.366). None of the cases or controls was homozygous for the PT gene G20210A mutation or the FV R506Q mutation. Thus, the relative risk of venous thrombosis was about 4 when the PT A20210 allele was present (OR 4.02, 95% CI: 1.90-8.50, \( p < 0.001 \)) and about 7 when the FV R506Q mutation was present (OR 6.53; 95% CI: 3.51-12.52, \( p < 0.001 \)). The relative risk of thrombosis was not influenced by the MTHFR T776 genotype.

Among the patients, 150 had suffered from a first thrombosis and 55 had recurrent thrombosis. The ORs computed after excluding recurrent cases are shown in Table 1. The relative risk of venous thrombosis was 3.34 (95% CI: 1.46-7.63, \( p = 0.004 \)) when the PT A20210 allele was present and 4.70 (95% CI: 2.34-9.46, \( p < 0.001 \)) when the FV R506Q mutation was present. The MTHFR T776 genotype was not a risk factor for thrombosis in these patients.

None of the polymorphisms was significantly associated to the circumstances of thrombosis (spontaneous or secondary to an acquired

---

Table 1: Genotype and allele frequency (f) of prothrombin gene G20210A, factor V gene G1691A and factor V gene A4070G mutations in cases and controls, and odds ratios (95% confidence interval [95% CI]) associated with rare allele carrying

<table>
<thead>
<tr>
<th>Genotype and allele</th>
<th>Prothrombin G20210A</th>
<th>MTHFR C677T</th>
<th>Factor V G1691A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>Cases n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole pop</td>
<td>184 (89.8)</td>
<td>21 (10.2)</td>
<td>0</td>
</tr>
<tr>
<td>First thrombosis</td>
<td>137 (91.3)</td>
<td>13 (8.7)</td>
<td>0</td>
</tr>
<tr>
<td>Controls n (%)</td>
<td>397 (97.2)</td>
<td>11 (2.8)</td>
<td>0</td>
</tr>
</tbody>
</table>

* OR associated with MTHFR T776 genotype by reference to CC + CT.

---

Age, sex and potential risk factors (inherited: low antithrombin, protein C, protein S levels; or circumstantial) were studied as possible effect modifiers. The heterogeneity of the ORs associated with PT A20210, FV A1691 and MTHFR T677T was compared between males and females, between subjects below and above the median age of 45 years and among subjects free from potential risk factors versus those exhibiting at least one of them, by entering the corresponding interaction term (1 DF) in the logistic regression procedure.

The frequencies of spontaneous and recurrent thrombosis according to genotype were compared by a Chi-square test (1 DF). The difference in age at first VTE according to the PT G20210A, FV gene G1691A mutations and MTHFR T677T homozgyosity was tested by a non-parametric Wilcoxon rank-sum test.

Differences with \( p \) value <0.05 were considered significant.
risk factor) or with age at first thrombosis and no significant heterogeneity of the ORs calculated for males and females, above and below the median age (45 years) and among subjects free from potential risk factors (inherited or circumstantial) versus those exhibiting at least one of them could be demonstrated (data not shown). When subjects with isolated low antithrombin, protein C or protein S levels and patients on oral anticoagulants were excluded, leaving 142 patients and 372 controls, thrombosis was still associated with the PT G20210A and FV R506Q genotypes, with ORs of 3.04 (95% CI: 1.26-7.32, p = 0.013) and 6.53 (95% CI: 3.33-12.78, p < 0.001), respectively.

As homozygosity for the MTHFR T677 allele was observed in 26 cases, we studied the interaction of the MTHFR variant with the FV R506Q mutation. As shown in Table 2, 4 cases and 1 control had both abnormalities, and the OR was 9.33 (95% CI: 1.03-84.23). However, there was no significant heterogeneity in the ORs associated with the FV mutation alone and those associated with both the FV mutation and the MTHFR homozygous mutation.

The PT A20210 allele was present in 9 of 40 (22.5%) patients heterozygous for the FV R506Q mutation and 12 of 165 (7.3%) patients without the FV R506Q mutation (p = 0.004), pointing to an increased risk in patients carrying both mutations. However, as the PT A20210 allele was not found in controls homozygous for the FV gene R506Q mutation we could not evaluate the OR for carriers of both mutations. The lower bound of the 95% CI was 3.94.

No cases or controls were both homozygous for the MTHFR T677 allele and heterozygous for the PT A20210 allele.

We also searched for an association between the studied genotypes and recurrence of thrombosis in patients (Table 3). Forty-five per cent of FV R506Q heterozygotes had recurrent thrombosis, compared to 22.7% of non carriers (p = 0.004). The OR for recurrence was 2.79 (95% CI: 1.35-5.74, p = 0.005). Thrombosis was recurrent in 38.1% of carriers of the PT A20210 allele and 25.8% of non carriers, but the difference did not reach statistical significance.

The results did not change when we excluded from cases the six patients with a history of arterial thrombosis and the two patients with known malignancy.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Odds ratio (OR) for venous thromboembolism associated with combined factor V and methylene tetrahydrofolate reductase (MTHFR) polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V</td>
<td>MTHFR</td>
</tr>
<tr>
<td>A1691</td>
<td>T677*</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* a = TT  
Tests of heterogeneity of the odds ratios: not significant (p = 0.80)

![Discussion](https://www.thrombosis-online.com)

**Discussion**

The prevalence of the FV R506Q mutation, the PT gene G20210A mutation and the MTHFR gene C677T homozygous mutation, and the relative risks of thrombosis associated with these genotypes considering the whole patients' group or the patients with a single thrombotic episode, were similar in this study to values reported in other European populations. The relative risk of recurrent thrombosis associated with the FV R506Q mutation [2.79 (95% CI: 1.35-5.74, p = 0.005)] also compares well to the values reported by Ridker (incidence rate ratio 4.1, p = 0.04) (8) and Simioni (2.4, 95% CI: 1.3-4.5, p ≤0.01) (9).

The PT gene G20210A mutation was recently identified by Poort et al. as a risk factor for venous thrombosis in subjects with familial thrombophilia, and also in a large case-control study (the LETS study) (2). This has since been confirmed in four other case-control studies of European subjects (10-13) and in a study of Brazilian subjects (14). Combined analysis of our data and the results of the previous studies allows a comparison between almost 2,000 patients with a thrombotic phenotype and around 2,000 healthy controls. As shown in Fig. 1, these studies consistently show a prothrombotic effect of the PT A20210 allele, with ORs between 2 and 6.6.

Moderate hyperhomocysteinemia is believed to be frequent in patients with venous thrombosis. Individuals homozygous for the MTHFR thermolabile variant (due to the MTHFR gene C677T mutation) have significantly elevated plasma homocysteine levels. MTHFR T677T homozygosity might therefore be a risk factor for VTE. This possibility has been tested in 6 case-control studies (3 in Italy (15-17), 1 in England (18), 1 in the Netherlands (19) and 1 in Brazil (20)). Although MTHFR T677T homozygosity was a risk factor in the
controls. Eighteen patients and no control had both mutations. Once again, the OR for carriers of both mutations could not be calculated. The lower bound of the 95% CI was 6.02, suggesting a higher risk associated with the combination of both mutations.

Homozygosity for the MTHFR gene C677T mutation might also increase the thrombotic risk in patients with the FV R506Q mutation: Cattaneo et al (16) studied 77 patients with VTE and 154 age- and sex-matched healthy controls and found that 10 patients and 3 controls bore the FV Q506 allele, and that 6 patients and 1 control were also homozygous for the MTHFR T677 allele. The OR in subjects with both mutations was 65% to 75% higher than the expected joint effect calculated with an additive or multiplicative model. The synergistic effect was particularly evident in patients with spontaneous deep vein thrombosis. These results extended Mandel’s observation that coexistence of severe hyperhomocysteinemia due to homozygous homocystinuria and the FV Q506 allele increased the thrombotic risk (9). However, in the LETS study (19), the homozygous MTHFR T677T genotype had no effect on the thrombotic risk in carriers of the FV R506Q mutation. Homozygosity for the MTHFR T677 allele was associated with a thrombotic risk by Margaglione et al, only in the subgroup of patients with inherited, acquired or circumstantial risk factors (17). In our study, 4 patients and 1 control were both homozygous for the MTHFR T677 allele and heterozygous for the FV R506Q mutation. The small number of combined defects ruled out a precise assessment of the subsequent risk, and this issue must therefore be addressed in a larger series of patients and controls.

Acknowledgments

This work was supported by a grant from Programme Hospitalier de Recherche Clinique n’AO94031 “Evaluation clinique et biologique du risque thromboïétique”.

We wish to thank Dr. Louis Guize from Institut de Pathologie Cardio-Vasculaire (Paris) for providing healthy subjects.

References


