ORIGINAL ARTICLE

Prevalence of thrombophilic mutations in patients with unprovoked thromboembolic disease. A comparative analysis regarding arterial and venous disease

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Abstract

Background: Thromboembolic disease (TED) represents one of the main reasons of morbitity and mortality in Western World. Venous and arterial thrombotic disorders have long been viewed as separate pathophysiological entities. However, in recent times the separate nature of arterial and venous thrombotic events has been challenged. Although inherited thrombophilia's predominant clinical manifestation is venous thrombosis, its contribution to arterial thrombosis remains controversial. Purpose of the study was to evaluate the prevalence of the most common thrombophilic mutations, FV Leiden G1691A-FVL and FII G20210A-PTM and to assess the differences between venous, arterial and mixed thrombotic events. Testing for polymorphism MTHFR C677T and antithrombin, protein C and protein S was also performed. Correlations with dyslipidemia, smoking, obesity, homocysteine and antiphospholipid antibodies were made.

Methods: 515 patients with unprovoked TED, 263 males, median age 44 years, were studied. Patients were divided into three groups: 258 with venous thrombosis (group A), 239 with arterial (group B) and 18 with mixed episodes (group C). All patients were interviewed regarding family history of TED, origin, smoking and dyslipidemia. Body mass index (BMI) had been calculated. Molecular assessment of the FVL, PTM and MTHFR C677T was performed. Antithrombin, protein C, protein S, APCR, homocysteine, antiphospholipid antibodies and lipid profile were also measured.

Results: The population studied was homogenous among three groups as regards age (p=0.943), lipid profile (p=0.271), BMI (p=0.506), homocysteine (p=0.177), antiphospholipid antibodies (p=0.576), and positive family history (p=0.099). There was no difference in the prevalence of FVL between venous and arterial disease (p=0.440). Significant correlation of PTM with venous TED was found (p=0.001). The number of positive and negative for MTHFR presented statistically significant difference with a support in arterial disease (p=0.05). Moreover, a 2-fold increase in the risk of venous thrombosis in FVL positive patients (odds ratio: 2.153) and a positive correlation of homocysteine levels with MTHFR C677T (p<0.001) was found.

Conclusions: Correlation of PTM with venous thrombosis was established. Analysis showed no difference in prevalence of FVL between venous and arterial thrombosis, indicating that FVL might be a predisposing factor for arterial disease. A significant increase in MTHFR C677T prevalence in arterial disease was found. In conclusion, young patients with unprovoked arterial disease should undergo evaluation for thrombophilic genes. Identification of these mutations is important in the overall assessment and management of patients at high risk. Findings will influence the decisions of stratified approaches for antithrombotic therapy either primary or secondary thromboprophylaxis, the duration of therapy, the potential for avoiding clinical thrombosis by risk factor modification and the genetic counselling of family members. However, further studies are needed to clarify the nature of the association regarding venous and arterial thrombotic events. Hippokratia 2012; 16 (3): 250-255

Key words: thrombophilic mutations, unprovoked thromboembolic disease, arterial thromboembolic disease, venous thromboembolic disease

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Introduction

Thromboembolic disease (TED) is multifactorial and represents one of the major causes of morbitity and mortality in developed countries. Thrombosis can occur in the arterial or the venous circulation and has a major medical impact. The most frequent clinical manifestations include either pulmonary embolism and/or deep venous thrombosis (venous TED) or acute coronary syndromes and ischemic cerebrovascular disease (arterial TED), together the leading causes of death in Western World[1,2].

Thrombophilia is a hypercoagulable state predisposing to thrombosis[3-6]. Thrombosis is a multifactorial disease resulting from the dynamic interaction between genetic and acquired risk factors[1]. Despite considerable progress in identifying important genetic risk factors underlying predisposition to venous thrombosis, the genetic factors contributing to the risk for arterial thrombosis remain largely unknown. Although inherited thrombophilia's predominant clinical manifestation is venous thrombosis, its contribution to arterial thrombosis still remains controversial[7].

The pathogenic changes that occur in the blood vessel wall and in the blood itself resulting in thrombosis are not fully understood. Venous and arterial thrombotic disorders have traditionally been considered as separate pathophysiological entities, partly as a result of the obvious anatomical differences as well as their distinct clinical presentations[8]. However, in recent times the separate nature of arterial and venous thrombotic events has been challenged. The two clinical conditions may be simultaneously triggered by biological stimuli responsible for endothelial injury, platelet activation, elevated levels of intrinsic clotting factors and inflammatory markers, increased fibrinogen, and impaired fibrinolysis, in both the arterial and venous system[9]. In addition, older age, obesity, dyslipidemia and smoking predispose to both venous and arterial thrombosis. Furthermore, there are a few disorders that are characterized by having both venous and arterial thromboses: the antiphospholipid antibody syndrome, heparin-induced thrombocytopenia, myeloproliferative disorders, and hyperhomocysteinemia. Recent studies indicate that arterial cardiovascular diseases and venous thromboembolism share common risk factors, and there is evidence that persons with venous thrombosis may be at greater risk for arterial events[10-14].

Purpose of this study was to evaluate the prevalence of the most common thrombophilic mutations, FV Leiden G1691A-FVL and FII G20210A-PTM and to assess the differences among patients presenting with venous disease, arterial disease or both. Testing for homocysteine metabolism polymorphism MTHFR C677T and deficiencies in the anticoagulant proteins antithrombin, protein C and protein S was also performed. Correlations with acquired risk factors such as dyslipidemia, smoking, obesity, homocysteine and antiphospholipid antibodies were made.

Methods

Cases with TED were classified as unprovoked, when no overt risk factors were recognized. Particularly, thrombosis without cancer or hospitalization within preceding 6 months for venous TED and absence of classical risk factors for arterial TED (familial dyslipidemia, hypertension, diabetes, smoking and chronic atrial fibrillation). After protocol exclusions, 515 patients, 263 males, 15-68 years old (median age 44 years), were studied during the last decade. Our study concerned the population of Northern Greece. Cases were either referred to the outpatients' haematology clinic for thrombophilia work-up after documented venous TED and/or arterial TED without a readily identifiable risk factor, or were hospitalized in the Internal Medicine Department for venous TED or ischemic cerebrovascular disease.

Diagnosis of TED was documented by objective diagnostic techniques comprised compression ultrasonography and/or nuclear venography for deep venous thrombosis (DVT), ventilation/perfusion scintigraphy or contrast enhanced spiral computed tomography imaging for pulmonary embolism (PE), coronary angiography for acute coronary syndromes (ACS, a number of patients had undergone percutaneous coronary angioplasty or coronary by pass grafting) and magnetic resonance and/or computed tomography imaging for cerebrovascular events. Transoesophageal heart ultrasonography had been performed in patients with ischemic cerebral stroke only in cases with suspected cardiac embolic events according to clinical evaluation at transthoracic echocardiographic study. All patients were interviewed regarding family history of TED, origin, smoking habits and dyslipidemia. The calculation of body mass index (BMI) had also been used in the definition of obesity.

Patients were divided into three groups: 258 (50.1%) with venous thrombosis (Group A), 118 males, median age 38 years (range 15-68 years) and 140 females, median age 42 years (range 21-67). Two hundred and thirty nine (46.4%) with arterial thrombosis (Group B), 133 males, median age 48 years (range 18-66) and 106 females, median age 38 years (range 16-64). Finally 18 patients (3.5%), 12 males, median age 43 years (range 41-54) and 6 females, median age 49 years (range 42-56) with mixed episodes (ACS or ischemic CVD and DVT and/or PE) comprised Group C.

The study was conducted in accordance with the principles of the Helsinki declaration and was approved by the Institutional Review Board. All subjects gave informed consent and the procedures followed were in accordance with institutional guidelines.

Laboratory assays

Blood samples were collected by venipuncture into plastic Vacutainer®, Becton Dickinson, Franklin Lakes, New Jersey, USA, tubes, one containing 1/10 volume of 0.5 mol/l sodium EDTA for DNA extraction and the other containing 1/10 volume 3.8% sodium citrate for coagulation assays.

Genomic DNA was extracted from fractionated peripheral blood with the QIAamp DNA Blood Midi Kit (Qiagen Corporation; cat. no. 51185). FV Leiden, FII G20210A and MTHFR C677T genotyping were performed by multiplexed allele-specific PCR methodology, as previously described[15-17].

Antithrombin activity was measured by Chromogenix Coamatic® Antithrombin chromogenic kit, Chromogenix, Instrumentation Laboratory Company - Lexington, MA 2421-3125, USA. A chromogenic assay for the determination of protein C activity in human plasma was

Table 1: FV Leiden positive (+) and negative (-) patients with unprovoked venous and arterial TED. The number of positive and negative for the FVL mutation patients did not show any statistically significant difference between groups A and B (chi square 0.097; p=0.440.

	FV Leiden (-)	*FV Leiden (+)*
Venous disease	60.1%	39.9%
Arterial disease	62.8%	37.2%

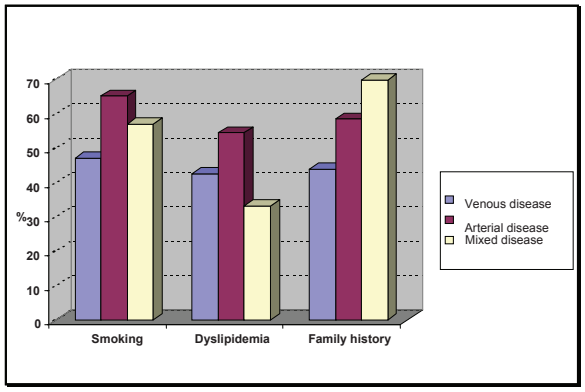

Figure 1: Smoking, dyslipidemia, and positive family history for TED in patients with venous disease (group A), arterial disease (group B), and mixed (group C).

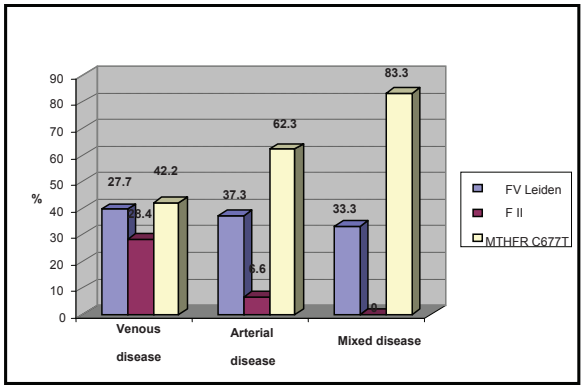

Figure 2: Prevalence of thrombophilic mutations (FV Leiden G1691A-FVL, FII G20210A-PTM), and MTHFR polymorphism C677T in patients with unprovoked TED.

also used, Berichrom® Protein C, Dade Behring Marburg GmbH Germany. We used the Chromogenix Comatic® Protein S-free kit, Milan, Italy, in an automated Dade Behring instrument. Activated protein C resistance (APCR) was evaluated using Chromogenix Coatest® APCTM Resistance kit Chromogenix, Instrumentation Laboratory Company - Lexington, MA 2421-3125, USA, performed on the Behring Coagulation System Analyzer, Dade Behring.

Fasting serum homocysteine concentrations were measured with Ready pack Advia Centaur/Siemens Medical Solution Diagnostic, Europe Ltd., Chapele Lain, Swords Co., Dublin Ireland. Blood samples were collected in Venoject® plain silicone coated tubes.

Antiphospholipid antibodies, namely lupus anticoagulant, anticardiolipin and anti-beta2 glycoprotein I (GPI) IgM and IgG antibodies were also measured. The Lupus Anticoagulant tests, chosen to comply with the strategy defined by the International Society of Thrombosis and Hemostasis[18], were performed in a Dade Behring automatic coagulation analyzer using Dade Behring reagents: LA1 Screening Reagent/LA2 Confirmation Reagent. LA1 Screening Reagent is a simplified Dilute Russell's Viper Venom Reagent that screens for the presence of lupus anticoagulants. LA2 Confirmation Reagent is a phospholipids-rich reagent for the specific correction of lupus anticoagulants. An ELISA method was performed to determine anticardiolipin and anti-beta2 GPI IgG and IgM antibodies using Varelisa CL IgG antibodies and Varelisa CL IgM antibodies, Pharmacia & Upjohn Diagnostics GmbH & Co KG, Freiburg, Germany.

Lipid profile was measured in an Olympus UA 2700 analyser after an overnight fast including levels of cholesterol, triglycerides, and lipoprotein cholesterol levels (LDL-C, HDL-C). Blood samples were collected in Venoject® plain silicone coated tubes.

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 13.0 for windows XP (SPSS Inc., Chicago, IL, USA). Data for continuous variables are presented as mean values ± standard deviation (SD) or median values and range, depending on the normality of the distribution, which was assessed with the Kolmogorov-Smirnov test. The Pearson's Chi-Square test was used for categorical variables and one way ANOVA for comparisons between groups. The associations were also examined using multivariate regression stepwise analysis. A p value level <0.05 was considered statistically significant.

Results

From the total population studied, 296 (57.47%) were smokers, 248 (48.15%) had dyslipidemia, and 269

patients (52.23%) had positive family history for TED. Particularly, 122 (47.28%) in Group A were smokers, 156 (65.27%) in Group B and 10 (55.55%) in Group C, 110 (42.63%) had dyslipidemia in Group A, 130 (54.39%) in Group B and 6 (33.33%) in Group C. As regards family history, it was positive in 113 (43.79%) of patients in Group A, 140 (58.57%) in Group B and 12 (66.66%) in Group C (Figure 1). BMI was increased in 118 patients (45.73%) in Group A, 126 (52.72%) in Group B and 9 (50%) in Group C.

Positive for FVL in all Groups were found 197 patients (38.3%), 191 (37.1%) heterozygous and 6 (1.2%) homozygous, 83 (16.1%) were positive for the PTM, 79 (15.4%) heterozygous and 4 (0.7%) homozygous, respectively and 317 (61.5%) had the MTHFR C677T polymorphism, 228 (44.3%) heterozygous and 89 patients (17.3 %) were homozygous, respectively (Figure 2). Particularly, 103/258 patients with venous TED were positive for FVL in 39.9% (Table 1), 96 (37.2%) heterozygous and 7 (2.7%) homozygous, 73/258 (28.3%) were positive for the PTM, 69 (26.8%) and 4 (1.5%), respectively and 109/258 (42.2%) had the MTHFR C677T polymorphism, 90 (34.9%) heterozygous and 19 (7.3%) homozygous, respectively. Combined genetic defects were found in 24/258 (9.3%) of Group A. More specific, 6 patients were PTM heterozygous and MTHFR C677T homozygous, 12 were FVL heterozygous and PTM heterozygous, 6 patients FVL heterozygous and MTHFR C677T homozygous.

Patients with arterial TED were heterozygous for FVL in 89/239 (37.2%) (Table 1), 16/239 (6.7%) were heterozygous for the PTM and 149/239 patients (62.3%) had the MTHFR C677T (48% heterozygous and 14.3% homozygous). Combined genetic defects, FVL heterozygous and PTM heterozygous were found in 6/239 (2.5%) of patients in Group B.

Patients with mixed TED were heterozygous for FVL in 6/18 (33.3%), no one had the PTM and 15/18 (83.3%) had the MTHFR C677T (50% heterozygous and 33.3% homozygous). Combined genotypic for FVL and PTM was not detected in this group.

The levels of antithrombin, protein C and protein S were within the normal range, except for two in 515 patients (0.38%) with severe congenital protein S deficiency (protein S activity 15-22%) and recurrent severe thromboembolic events. Homocysteine levels were increased in 43.8% of patients in group A (13.9 ± 5.4 μmol/l), 50.2% in group B (16.6 ± 6.6 μmol/l) and 83.3% in group C (22.3 ± 10.0 μmol/l). Positive antiphospholipid antibodies were detected in 12.8% of total patients studied.

One way ANOVA analysis revealed that the population studied was homogenous among the three groups, as regards the age (p=0.943). Statistically significant differences among smokers in both groups A and B were found, with a support in arterial disease (chi square 6.635; p=0.036). No statistically significant differences among the three groups were found regarding the lipid profile (chi square 2.611; p=0.271), BMI (chi square 1.361; p=0.506), homocysteine levels (chi square 3.458; p=0.177), antiphospholipid antibodies (chi square 1.104; p=0.576), and positive family history (chi square 4.490; p=0.099).

The number of positive and negative for the FVL mutation patients did not show any statistically significant difference between groups A and B (chi square 0.097; p=0.440). The number of positive and negative patients for the PTM in the three groups presented statistically significant difference, with a statistically significant increase in venous disease (chi square 12.009; p=0.001). The number of positive and negative for the MTHFR C677T polymorphism patients presented a statistically significant difference between groups A and B, with a support in arterial disease (chi square 3.410; p=0.05). Moreover, a 2-fold increase in the risk of venous thrombosis in FVL positive patients (odds ratio: 2.153) and a positive correlation of homocysteine levels with the MTHFR C677T polymorphism (regression stepwise analysis, p<0.001) and the male gender (regression stepwise analysis, p=0.005) was found.

Discussion

The FVL and PTM mutations are the most frequent causes of inherited thrombophilia[5,19,20]. In spite of the progress in identifying important genetic factors predisposing to venous thrombosis, the corresponding factors concerning arterial thrombosis remain still unknown. Arterial thrombosis underlies the central pathophysiologic mechanism in acute coronary syndromes and ischemic cerebrovascular disease, which represent one of the main causes of morbitity and mortality in the Western World. It appears that there is an overlap between known genetic factors for venous thrombosis and that predisposing to arterial thrombosis[21-23].

Multiple studies demonstrate a major genetic component to the risk for arterial disease. For example, a twin study estimated a heritability of ~50% for mortality from coronary heart disease[24]. How to address complex diseases like arterial thrombosis is a critical problem at the forefront of modern genetics. The disappointing and frustrating results of previous linkage studies suggest that genetic determinants of this risk are complex, likely to involve multiple genes, and may also be dependent on interactions between these genes. Such small and complex effects may be very difficult to detect by studying complex human populations.

There are clearly also major environmental and acquired risk factors of great importance that also interact with the genetic factors, making detection even more difficult. For arterial thrombosis, these include obvious factors such as diet, cholesterol and cigarette smoking. In our study, the patients with documented unprovoked TED evaluated were young, as evidenced by their median age. The population studied was homogenous among the three groups as regards the age, lipid profile, BMI, homocysteine levels and antiphospholipid antibodies (acquired risk factors). Even if it is widely accepted that antiphospholipid syndrome is a common cause of arterial throm-

bosis, especially in ischemic arterial cerebral stroke in young individuals, statistical analysis revealed no difference among the three groups, regarding antiphospholipid antibodies (p=0.576). Actually, we found very few cases completing the classification criteria for a definite antiphospholipid syndrome among the population studied[25]. Probably, this is due to the fact that many cases were referred to our outpatients' haematology clinic retrospectively and the laboratory examination was performed a long time after the acute episode.

Statistically significantly correlation of PTM with venous thrombosis was established. Additionally, a statistically significant increase in the MTHFR C677T polymorphism prevalence in arterial disease was found. However, MTHFR is not a risk factor for thrombosis, as it is shown by its prevalence in the population studied. FVL is the leading cause of inherited thrombophilia predisposing to venous thrombosis[26-28]. Statistical analysis of our results showed no difference in the prevalence of FVL between venous and arterial thrombosis, indicating that FVL might be a predisposing factor for arterial disease too. Consequently, it is concluded that young patients with clinical manifestations of unprovoked arterial TED should also be tested for the FVL variant.

Testing for deficiencies of intrinsic anticoagulants antithrombin, protein C and protein S activities was negative except for two patients (0.38%) with severe congenital protein S deficiency. While inherited deficiencies of intrinsic anticoagulants are well established as risk factors for a first thromboembolic event, they are rarely found[29]. Additionally, the magnitude of risk of recurrent thromboembolic events in these settings is less clearly defined[30]. As a conclusion, after validating our negative results and taking into account the above mentioned and the financial cost, our estimation is that natural anticoagulation testing could be excluded from the routine laboratory thrombophilia assessment.

In the literature, epidemiological studies indicate differences in the etiological association of thrombophilia genes in thromboembolic disease manifestations, possibly due to the different impact among the populations studied (demographic distribution) [31-33]. Our study is concerning the population of Northern Greece.

According to our results and the fact that the majority of our patients studied (52.23%) had a positive family history, with no statistically significant differences among groups, indicates that young patients with arterial disease and positive family history should undergo the appropriate laboratory evaluation for thrombophilic mutations. On the other hand, family history of myocardial infarction is a risk factor for both myocardial infarction and venous disease and provides further evidence of a link between venous and arterial thrombosis [21]. Findings from this assessment will influence the decisions of stratified approaches for antithrombotic therapy either primary or secondary thromboprophylaxis, the duration of therapy, the potential for avoiding clinical thrombosis by risk factor modification and the genetic counselling of family members[34,35].

With improvement of laboratory methods for thrombophilia investigation and selection of population being tested, combined (multi-trait) thrombophilia is more frequently found, in 5.82% of our cases. The synergistic effect of combined gene abnormalities has been demonstrated[36]. Identification of combined FVL and prothrombin mutations is important in the overall assessment and management of patients with thrombophilia, as detection of these mutations can identify patients at high risk and help evaluate the interaction of genetic and acquired risk factors[34-36].

Knowledge of the biochemistry of hemostasis has rapidly expanded in recent years with details of interaction of the proteins involved. This knowledge permits design of targeted drugs and developing more effective antithrombotic therapies such as direct thrombin inhibitors, representing a new therapeutic concept. The availability of multiple drugs targeting different enzymes raises the possibility of combined therapy using several agents in individualized doses, based on the characteristics of a patient's hemostatic system or pattern of vascular disease and thrombosis.

Thromboembolic disease is potentially fatal. In conclusion, clinicians should be aware of the epidemiology of thromboembolic disease and familiar with the diagnostic methodology for determining high risk groups, to select the appropriate therapeutic and prophylactic antithrombotic therapy. However, further studies are needed in the future to clarify the nature of the association regarding venous and arterial thrombotic events, to assess its extent and to evaluate its implications in clinical practice.

Conflict of interest

The authors declare no conflict of interest

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