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Abstract

Venous thromboembolism (VTE) is a multifactorial disease determined by a combination of inherited and acquired factors. Inherited factors include mutations in the genes coding for coagulation factors, some of which seem to exert a differential influence on the risk of developing deep vein thrombosis (DVT) and pulmonary embolism (PE). In post- mortem studies of subjects who have died from pulmonary embolism (PE), the analysis of the factors that may have aug- mented the VTE risk is often limited to acquired factors. This is due to the complexity—and sometimes the unfeasibility— of analyzing genetic factors and to insufficient knowledge of their individual roles in PE development. The present study used formalin-fixed paraffin-embedded (FFPE) tissue to investigate a panel of 12 polymorphisms—the largest ever studied—that affect the VTE risk. Tissue samples came from post- mortem examinations performed by the specialists of the Section of Legal Medicine of the Department of Pathology of Marche's Polytechnic University, and by the specialists of Health Care District Hospital of Imola, on 44 subjects who died from PE in the period 1997–2014. All individuals were found to have at least one mutation affecting the VTE risk. The present study demonstrates that genetic analysis can be performed post-mortem and the results are useful for forensic investigations, especially from MTHFR C677T and PAI-1 4G/5G polymorphisms. Broader studies using the techniques described herein are needed to determine the relative influence of the individual polymorphisms and their interaction in PE deaths.

Keywords Venous thromboembolism, Pulmonary embolism, Deep vein thrombosis, Hereditary thrombophilia

Introduction

Pulmonary embolism (PE) and deep vein thrombosis (DVT) are manifestations of venous thromboembolism (VTE).

Venous thromboembolic disease is the result of gene-gene and gene-environment interactions, i.e., a multicausal disease where one or more genetic abnormalities, like hereditary thrombophilia, combined with acquired risk factors such as trauma, immobilization, malignancy, inflammation, pregnancy, use of oral contraceptives, autoimmune disease, hyperten- sion, diabetes, and obesity.

Hereditary thrombophilia involves a genetically deter- mined increased likelihood of thrombosis. It is most often due to changes in physiological coagulation inhibitors or to mutations in the genes encoding coagulation factors. A single individual may have none, one, or multiple polymorphisms that involve a predisposition to develop VTE.

DVT and PE are generally considered as facets of the same disease. However, a different prevalence of some inherited risk factors has been documented in DVT and PE patients [1]. For instance, factor V Leiden mutation seems to involve a lower risk of PE than of DVT (factor V Leiden paradox) [2], since a similar proportion of individuals carrying it has been found among subjects who have died of PE and in the general population [3–5]. Further insights into the genetic factors that increase the risk of PE are required to elucidate the multifactorial pathophysiological mechanisms underlying some other- wise unexplained or only partially explained deaths from this disorder.

To the best of our knowledge, there are no systematic in- vestigations involving a broad panel of polymorphisms related to the VTE risk in individuals who have died from PE. An earlier Italian study has explored exclusively the mutations within the factor V Leiden gene (G1691A) and the factor II gene (G201210) [6].

In this study, a panel of 12 polymorphisms that influence the VTE risk was investigated to improve the assessment of the risk related to hereditary thrombophilic factors. Formalin- fixed paraffin-embedded (FFPE) tissue samples collected at autopsy were used to establish the ability of FFPE tissue to be used in these tests.

The following polymorphisms were investigated: C677T and A1298C in the MTHFR (methylene tetrahydrofolate re- ductase) gene; G1691A and A4070G in factor V gene; G20210A in the prothrombin gene; the PAI1 4G/5G gene polymorphism; the β-fibrinogen -455G/A and -854G/A gene polymorphisms; Factor XIII Val34Leu and HPA-1 T196C (Leu33Pro) polymorphisms; −33T → C and −287T → C of the tissue factor pathway inhibitor (TFPI) gene polymor- phisms−33T (Table 1).

The MTHFR enzyme catalyzes the conversion of 5,10- methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulatory form of folate, and a cosubstrate for ho- mocysteine remethylation to methionine. Methionine is sub- sequently converted to S-adenosylmethionine, which serves as an essential methyl donor in reactions involving nucleic acids, proteins, and many other biological compounds. C677T and A1298C polymorphisms cause a substitution of an alanine to a valine in the protein and a strong reduction in the activity of the enzyme MTHFR resulting in elevated levels of homocysteine in the blood and, consequently, in an increased risk of blood clots.

Factor V is a protein of the coagulation system. In contrast to most other coagulation factors, it is not enzymatically active but functions as a cofactor. Factor V Leiden is a specific gene mutation that increases tendency to form abnormal blood clots that can block blood vessels and the risk of developing deep venous thrombosis (DVT). The A4070G polymorphism in exon 13 of the factor V (FV) gene, which replaces His by Arg at position 1299 of the B domain influences circulating FV levels and contributes to the activated protein C (APC) resistance phenotype.

The G20210A polymorphism in the prothrombin gene is a single nucleotide substitution in the Factor II molecule pro- moter region that increases the normal levels of prothrombin in the blood and consequently the risk of blood clots,

The 4G/5G polymorphism in the promoter region of the PAI-1 influences the levels of PAI-1. Homozygosity for the deletion genotype (4G/4G) has been associated with PAI-1 concentrations higher than those associated with the insertion genotype (5G/5G) and, hence, with reduced fibrinolytic activ- ity. Fibrinogen plays a central role in the coagulation cascade with a critical impact on the formation of fibrin clots. The 2455G/A and 2854G/A polymorphisms of the β-fibrinogen gene cause an increase in β-fibrinogen transcription and lead- ing to increased plasma fibrinogen levels.

Val34Leu polymorphism of the A subunit of coagulation factor XIII (FXIII-A) is located in the activation peptide (AP) just three amino acids away from the thrombin cleavage site. This mutation has a protective effect against occlusive arterial diseases and venous thrombosis.

The HPA-1b platelet antigen polymorphism is associated with increased platelet thrombogenecity.

Tissue factor pathway inhibitor (TFPI) is an important reg- ulator of the tissue factor-mediated blood coagulation path- way. Polymorphisms within this gene may increase the risk of venous thromboembolism (VTE).

Not all of these polymorphisms appear to be associated with a risk increment. According to a comprehensive meta-analysis, factor XIII Val34Leu and β-fibrinogen -455G/A seem to actu- ally exert a protective role against VTE [7]. As regards −33T → C and −287T → C of the regulatory regions of the TFPI gene, combination of −287 TT and −33TT in intron 7 has been reported to be associated with low plasma TFPI and a greater risk of recurrent VTE, whereas it has been postulated that the −33 CC and −287 CC polymorphisms involve higher plasma TFPI and decreased coagulation activity [8, 9].

Defects of antithrombin III, protein C and S, and plasmin- ogen, dysfibrinogenemia, hyperhomocysteinemia, and antiphospholipid antibodies could not be tested because some abnormalities require investigation by functional and serolog- ical diagnostic assays in the blood from living subjects.

Materials and methods

The records of autopsies performed by the pathologists of the Section of Legal Medicine of Polytechnic University of Marches and the Department of Pathology, Health Care District Hospital of Imola from 1997 to 2014 were reviewed. Caseworks where the summary of the cause of death in the autopsy report mentioned PE were retrieved. Forty-nine cases were found. Medical records, clinical data, and autopsy and pathology reports were examined for cases where PE was described as the main cause of death and for those where evidence of thrombosis had been found in the main pulmo- nary trunk, right or left branches, and their distal portions. In most cases, the origin of the thrombus was described. Forty- four cases met these criteria. Four cases were excluded be- cause VTE was considered to be related to the agony stage. Since it has been suggested that the biological effect of a specific genetic variant may differ depending on ancestral origin [7], we decided to exclude one further death because the deceased was a native of northern Africa.

FFPE tissue specimens were available for all 44 cases and were used for genetic testing. The results were compared with those obtained from genetic tests on blood of 102 healthy Italian living subjects and with the data recovered from previ- ous studies described in literature.

Genetic analysis

The panel included 12 mutation sites that are known to affect the thrombophilic risk (Table 1).

Genetic screening for identification of genotypes listed above was conducted on DNA purified from FFPE samples by QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's instructions. Heart tissue was preferentially used when available. Extracted DNA was analyzed by 1% agarose gel electrophoresis and real-time PCR using the Plexor® HY System (Promega). For healthy controls, DNA was purified from peripheral whole blood using a lysis buffer, PCR buffer with nonionic detergent, and proteinase K.

PCR was performed on a GeneAmp PCR System 9700 (PE Applied Biosystems). The amplification reaction mix (50 μL) consisted of 2.5 U Taq DNA polymerase in 10 mmol/L Tris- HCl, pH 8.3 (Bioaesis); 50 mmol/L KCl, pH 9.0 buffer con- taining 1.5 mmol/L MgCl2, 200 μmol/L of each dNTP (Bioaesis); 50 pmol/L of each primer (forward and reverse); and extracted DNA (up to 50 μL). Oligonucleotides were synthesized by M-Medical. Primers and PCR conditions are summarized in Table 1. PCR products were digested by re- striction enzymes as described in Table 1. Digested amplifica- tion products were analyzed by gel electrophoresis on 2% agarose gel in a single gel lane and stained with Gel Red (Biotium). For each gene, subjects were classified as normal (wild type), heterozygous, and homozygous based on the number and size of the bands.

The −33T → C and −287T → C polymorphisms of the TFPI gene were assayed by PCR and sequencing analysis. The amplification reaction contained 16 mM (NH4)2SO4; 67 mM Tris-HCL pH 8.8; 0.01% Tween-20; 1.5 mM MgCl2; 200 pM of each primer; 0.2 mM of each dNTP; 2.5 units of Taq DNA polymerase (Euroclone), and 10 μL DNA. Primers and PCR conditions are listed in Table 1.Ten nanograms of amplified product was added to each sequenc- ing reaction using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and the PCR primers according to manufacturer's instructions. Sequencing products were purified with the DyeEx Spin kit (Qiagen). Capillary electrophoresis was carried out on an ABI Prism3130 Genetic Analyzer (Applied Biosystems). Data were analyzed with Seqscape software v2.5.

PubMed search

PubMed was searched for papers assessing the prevalence of the 12 polymorphism in the general population or where their prevalence could be inferred from case-control comparisons. The polymorphisms were used as the search terms.

Statistical analysis

BGenotype^ frequencies were estimated as punctual and 95% confidence intervals (95%CI), for cases and controls, using the binomial distribution. Genotype frequencies of case group

observed in the present study were compared with genotype frequencies from case control and literature. Comparisons be- tween cases and controls were evaluated using the exact test for proportions. Since multiple testing on the same data set was performed, the Benjamini and Hochberg procedure was applied to adjust p values. Comparison with frequencies from literature was performed analyzing 95%CI: the two frequen- cies were considered as significantly different when the 95%CI of a frequency evaluated in our study did not include the one estimated in other published studies.

Quantitative variables were summarized using quartiles (median as measure of centrality, 1st–3rd quartiles as measures of variability) and comparison between cases and controls was evaluated by means of the Wilcoxon rank sum test.

A multiple logistic regression analysis was performed to analyze the association between genotypes (independent var- iables) and presence of deep venous thrombosis (dependent variables) adjusted for age and gender. Genotypes included in the model were chosen according to their observed frequen- cies: MTHFR C677T, MTHFR A1298C, PAI-1 4G/5G, HPA- 1 (T196C, Leu33Pro), TFPI −33T → C, TFPI −287 T → C;

for the first four genotypes, wild type was considered as ref- erence category; for the last two genotypes, CC was the refer- ence category. HPA-1 (T196C, Leu33Pro) was dichotomized WT versus HE + HO. Results were expressed as odds ratios (OR) and 95%CIs.

Results

Forty-four cases and 102 controls were evaluated; median age of case and control groups was, respectively, 70 (1st–3rd: quartiles 58–78 years) and 46 (1st–3rd: 36–63 years), with cases significantly older than controls ($p < 0.001$). Cases were more frequently males, 26 (59.1%), than controls, 21 (20.6%). All were native Italians. The main characteristics of the sub- jects involved in the study and gene test results are reported in Tables 2 and 3.

According to the predisposing factors provided by 2014 ESC guidelines on the diagnosis and management of acute pulmonary embolism [10], analysis of clinical history disclosed that all subjects in the case group had one or more clinical risk factors for thrombophilia: fracture of lower limb (n = 5); hip replacement (n = 2); myocardial infarction (n = 3); previous venous thromboembolism (n = 2); major trauma (n = 1); cancer (n = 5); myelodysplastic syndrome (n = 1, refractory cytopenia); oral contraceptives therapy (n = 2); pro- tein S deficiency, anticardiolipin antibodies (n = 1); infection (pneumonia) (n = 4); and inflammatory bowel disease (ascites) (n = 1). Other risk factors classified as Bweak risk factors, including increasing age (≥ 65 years), hypertension, bed rest >3 days, obesity, and diabetes mellitus were also present in the sample, as shown in Table 4.

Genetic analysis in the case group showed all subjects had at least one mutation affecting the VTE risk, up to a maximum of eight mutations for subject n. 43 died from lung cancer (Table 4). The mutation sites more representative were heterozygosity for the TFPI-33 (n = 23), the C677T (n = 19) and the A1298C mutation (n = 17) on the MTHFR gene; heterozygosity for the PAI-1 4G/5G (n = 13) and HPA-1 (n = 9) polymorphisms; homozigosity for TFPI gene −33 T/ T (n = 7). With respect to the polymorphisms involving a reduced risk of thrombosis, 5 individuals bore the Val34Leu Factor XIII polymorphism, 5 were heterozygous for the −455 G/A β-fibrinogen mutation, 14 carried the TFPI gene −33 C/C, and 37 carried the −287 C/C genotype. No subject bore mutations of G1691A (Leiden), A4070G of factor V, G20210A of factor II, −854 G/A of β-fibrinogen, or the TFPI gene −287 C/C polymorphism (Table 2).

Genetic analysis in the control group showed all subjects had at least one mutation affecting the VTE risk, up to a maximum of 6 mutations for a single subject (Table 3). The mutation sites more representative were heterozygosity for the polymorphism PAI-1 4G/5G (n = 60); heterozygosity for the C644T (n = 58) and A1298C polymorphism (n = 49) at the MTHFR gene. Homozigosity for TFPI -33 T/T polymorphism was observed in 12 subjects. The majority of the subjects (n = 100) were wild type for the HPA-1 polymorphism. With respect to the polymorphisms involving a reduced risk of thrombosis, 4 individuals bore the heterozygous Val34Leu Factor XIII polymorphism and 1 was homozygous, 5 were heterozygous for the −455 G/A β-fibrinogen mutation, 53 carried the TFPI gene −33 C/C polymorphism and 77 the TFPI gene −287 C/C. Seven individuals bore mutations for G1691A (Leiden) and A4070G factor V; 7 individuals more were heterozygous for the G20210A of factor II and 3 for the −854 G/A polymorphism of β-fibrinogen (Table 3). Table 5 summarizes the frequencies of the wild and mutational geno- types and their 95%CI according to presence of deep venous thrombosis. Wild-type genotype frequency in PAI-1 4G/5G polymorphism was significantly higher among cases than controls, whereas heterozygosity status was significantly less frequent in cases than in controls. Wild-type genotype fre- quency in HPA-1 (T196C, Leu33Pro) polymorphism was sig- nificantly lower in cases than controls, whereas heterozygous frequency in cases was significantly higher than in controls.

Since the prevalence of each mutation (and apparently the expected VTE risk) is affected by ethnicity [7], findings were preferentially compared with those described in studies assessing polymorphism frequency in the Italian population. In the absence of Italian studies for the TFPI −33 T → C and −287 T → C polymorphisms, data were taken from a Norwegian paper, in which the control group was Caucasian (Table 5). It should be taken into account that the controls investigated in the various case series in the literature were

living at the time of the study, and even though PE is infre- quent, no data are available on whether any of them eventually developed it. Furthermore, none of the individuals in the con- trol group had developed PE until the moment of the study.

Homozygous MTHFR A1298C, heterozygous Factor V HR2 A4070G, heterozygous and homozygous PAI-1 4G/ 5G, heterozygous β-fibrinogen -455G/A, heterozygous β- fibrinogen -854G/A, heterozygous Factor XIII Val34Leu, TT TFPI -33 T- > C and TFPI -287 T- > C genotypes were found significantly lower than frequencies found in previous studies. Factor V HR2 A4070G, PAI-1 4G/5G, β-fibrinogen -455G/A, β-fibrinogen -854G/A, Factor XIII Val34Leu wild type were found significantly higher than the frequencies found in other study, as well as CC of TFPI −33T → C and CC of TFPI −287T → C genotypes (Table 5).

Table 6 shows the results of multiple regression analysis. MTHFR C677T and PAI-1 4G/5G were found significantly associated with deep venous thrombosis. Heterozygous con- dition for the first genotype and homozygous and heterozy- gous conditions for the second one significantly reduced the probability of suffering of deep venous thrombosis. On the other hand, the probability of having deep venous thrombosis was significantly higher in homozygotes or heterozygotes at HPA-1 (T196C, Leu33Pro) genotype than in wild types

Discussion and conclusion

In post-mortem studies of subjects who have died from PE, the search for the factors that involve an augmented VTE risk is often confined to acquired factors. This is due to the com- plexity—and sometimes the unfeasibility—of analyzing the genetic factors, to the large number of polymorphisms, and to the insufficient knowledge of their role in PE development. A panel approach was thus devised to shed some light on the risk related to hereditary factors. Our series of 44 cases is satisfactory for a comparative study and allow to draw some conclusions on the prevalence of the mutations investigated. Comparison with the control group and with studies reported in the general population (represented by case controls from similar studies in literature) demonstrates significant differ- ences in mutation frequency for the factors responsible for hereditary thrombophilia.

Genetic testing may be discriminatory in cases of malprac- tice. In such cases the analysis of genetic factors and the knowledge of their role in DVT and PE development—be- sides investigation of other thrombophilic factors—may help for establishing the role that questionable prophylaxis could have played in PE development. Investigations establishing the absence of inherited thrombophilic risk factors would in- dicate a greater role of acquired factors, which might have been insufficiently explored or kept into account by the med- ical team. In contrast, severe genetic factors would suggest a strong likelihood of death despite appropriate prophylaxis. Our findings therefore indicate that genetic testing, for some polymorphisms, has a strong potential in assessing individuals who have died of PE, both for clinical and medico-legal purposes.

The two major genetic factors predisposing to DVT (factor V Leiden mutation, in combination or not with the H1299R polymorphism, and prothrombin G20210A mutation) were never observed in our samples. The factor V Leiden mutation is quite frequent in the northern European population and it is uncommon in Italians. Its complete absence in our sample is, however, in line with the Bfactor V Leiden paradox,^ whereby the mutation would exert a much weaker effect on the risk of developing PE than DVT. Concerning the prothrombin G20210A mutation, its prevalence has been shown to differ between PE and DVT patients, but no clear paradox as for FVL has been described yet. The presence of the wild-type genotype of G1691A, A4070G (factor V gene), and G20210A (factor II gene) should be considered with regard to the pos- sibility of developing pulmonary embolism.

Polymorphisms C677T (MTHFR) and PAI-1 4G/5G are informative as predictive factors for developing PE. Heterozygosity for C677T polymorphisms in MTHFR gene and homozygosity and heterozygosity for PAI-1 polymor- phism could have a protective role and reduces the probability to develop PE, unlike what reported in controls and data from literature (Table 6) On the contrary, the probability of devel- oping PE was significantly higher in homo- and heterozygous of HPA-1 (T196C, Leu33Pro) genotype than in wild type. The

odds ratios and the interval of confidence associated with the HPA-1 genotype (T196C, Leu33Pro) are depending on the small frequency of heterozygous and homozygous genotypes observed in our study, especially among controls. Therefore, the study was not able to accurately estimate the linkage (since the confidence interval is very wide), although a statistically significant association is already conceivable.

In our samples β-fibrinogen -455G/A and β-fibrinogen - 854G/A polymorphisms are not informative with regard to the prediction of developing pulmonary embolism. This would suggest that a mutation in these genes increasing the risk of hereditary thrombophilia is not a critical factor in PE develop- ment, and that its search may be not absolutely necessary when investigating the cause of death determined by this condition.

Since only some polymorphisms are informative, a multi- plex PCR reaction could be set up to carry out a preliminary, rapid, and cheap screening in those subjects forced to immo- bilization even for a few days.

Finally, the present study demonstrates that FFPE tissue may be used for post-mortem testing of inherited thrombophilic risk factors. Further investigation of larger sam- ples is expected to provide useful data on the role of the indi- vidual mutations and their interaction in increasing the risk of dying from PE.

Compliance with ethical standards

Ethical approval For this type of study, formal consent is not required.

References

1. Mäkelburg AB, Veeger NJ, Middeldorp S, Hamulyák K, Prins MH, Büller HR, Lijfering WM (2010) Different risk of deep vein throm- bosis and pulmonary embolism in carriers with factor V Leiden compared with non-carriers, but not in other thrombophilic defects. Results from a large retrospective family cohort study. Haematologica 95(6):1030–1033

2. BounameauxH (2000) Factor V Leidenparadox: riskofdeep-veinthrom- bosis but not of pulmonary embolism. Lancet 356(9225):182–183

3. Kuismanen K, Savontaus ML, Kozlov A, Vuorio AF, Sajantila A (1999) Coagulation factor V Leiden mutation in sudden fatal pul- monary embolism and in a general northern European population sample. Forensic Sci Int 106(2):71–75

4. Vandenbroucke JP, Bertina RM, Holmes ZR, Spaargaren C, van Krieken JH, Manten B, Reitsma PH (1998) Factor V Leiden and fatal pulmonary embolism. Thromb Haemost 79(3):511–516

5. Ameziane N, Seguin C, Borgel D, Fumeron F, Moatti D, Alhenc- Gelas M, Grandchamp B, Aiach M, Emmerich J, de Prost D (2002) The −33T → C polymorphism in intron 7 of the TFPI gene influences the risk of venous thromboembolism, independently of the factor V Leiden and prothrombin mutations. Thromb Haemost 88(2):195–199

6. Fineschi V, Bafunno V, Bello S, De Stefano F, Margaglione M, Neri M, Riezzo I, Turillazzi E, Bonsignore A, Vecchione G, Ventura F,

Grandone E (2012) Fatal pulmonary thromboembolism. A retro- spective autopsy study: searching for genetic thrombophilias (factor V Leiden (G1691A) and FII (G20210A) gene variants) and dating the thrombus. Forensic Sci Int 214(1–3):152–158

7. Gohil R, Peck G, Sharma P (2009) The genetics of venous throm- boembolism. A meta-analysis involving approximately 120, 000 cases and 180, 000 controls. Thromb Haemost 102(2):360–370

8. Luyendyk JP, Tilley RE, Mackman N (2006) Genetic susceptibility to thrombosis. Curr Atheroscler Rep 8(3):193–197

9. Skretting G, Stavik B, Landvik NE, Myklebust CF, Iversen N, Zienolddiny S, Sandset PM (2010) Functional characterization of polymorphisms in the human TFPI gene. Biochem Biophys Res Commun 397(1):106–111

10. Konstantinides SV, Torbicki A, Agnelli G, Danchin N, Fitzmaurice D, Galiè N, Gibbs JS, Huisman MV, Humbert M, Kucher N, Lang I, Lankeit M, Lekakis J, Maack C, Mayer E, Meneveau N, Perrier A, Pruszczyk P, Rasmussen LH, Schindler TH, Svitil P, Vonk Noordegraaf A, Zamorano JL, Zompatori M, Task Force for the Diagnosis and Management of Acute Pulmonary Embolism of the European Society of Cardiology (ESC) (2014) 2014 ESC guide- lines on the diagnosis and management of acute pulmonary embo- lism. Eur Heart J 35(43):3033–3069 3069a-3069k

11. Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group (2003) No evidence of association between prothrombotic gene polymorphisms and the development of acute myocardial in- farction at a young age. Circulation 107(8):1117–1122

12. Zappacosta B, Romano L, Persichilli S, Cutrone LA, Graziano M, Vitrani A, Di Castelnuovo A, Giardina B, Musumeci S, Mastroiacovo P (2009) Genotype prevalence and allele frequencies of 5, 10-methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms in Italian newborns. Lab Medicine 40(12): 732–736

13. Faioni EM, Castaman G, Asti D, Lussana F, Rodeghiero F (2004) Association of factor V deficiency with factor V HR2. Haematologica 89(2):195–200

14. Menegatti M, Asselta R, Duga S, Malcovati M, Bucciarelli P, Mannucci PM, Tenchini ML (2001) Identification of four novel polymorphisms in the Aalpha and gamma fibrinogen genes and analysis of association with plasma levels of the protein. Thromb Res 103(4):299–307

15. Saibeni S, Vecchi M, Faioni EM, Franchi F, Rondonotti E, Borsi G, de Franchis R (2003) Val34Leu factor XIII polymorphism in Italian patients with inflammatory bowel disease. Dig Liver Dis 35(1):32– 36

16. Abbate R, Marcucci R, Camacho-Vanegas O, Pepe G, Gori AM, Capanni M, Simonetti I, Prisco D, Gensini GF (1998) Role of platelet glycoprotein PL(A1/A2) polymorphism in restenosis after percutaneous transluminal coronary angioplasty. Am J Cardiol 82(4):524–525

17. Opstad TB, Pettersen AA, Weiss T, Arnesen H, Seljeflot I (2010) Gender differences of polymorphisms in the TF and TFPI genes, as related to phenotypes in patients with coronary heart disease and type-2 diabetes. Thromb J 8:7

Table 1 – Mutation sites included in the panel, primers, PCR conditions and restriction enzymes where used.

Polymorphism	**Primers (5'→3')**	**PCR conditions**	**Restriction enzyme**
C677T (MTHFR gene)	Fw: TGAAGGAGAAGGTGTGCGGGA Rv: AGGACGGTGCGGTGAGAGTG	30 s at 94 °C followed by 30 cycles (30 s at 94 °C, 30 s at 61 °C and 1 min at 72 °C) and final extension of 7 min at 72 °C.	Hinf I
A1298C (MTHFR gene)	Fw : CTTTGGGGAGCTGAAGGACTACTAC Rv: CACTTTGTGACCATTCCGGTTTG	2 min at 92 °C followed by 35 cycles (1 min at 92 °C, 1 min at 51 °C and 30 s at 72 °C) and final extension of 7 min at 72 °C	Mbo II
G1691A (factor V gene)	Fw: TGCCCAGTGCTTAACAAGACCA Rv: TGTTATCACACTGGTGCTAA	3 min at 95 °C followed by 33 cycles (30 s at 95 °C, 30 s at 63 °C and 1 min at 72 °C) and final extension of 5 min at 72 °C	Mnl I
A4070G (factor V gene)	Fw: CAAGCTCTTCCCCACAGATATA Rv: AGATCTGCAAAGAGGGGCAT	5 min at 93 °C followed by 30 cycles (20 s at 93 °C, 25 s at 57 °C and 1 min at 72 °C) and final extension of 5 min at 72 °C	Rsa I
G20210A (factor II gene)	Fw: CCATGAATAGCACTGGGAGCATTGAAGC Rv: TTACAAGCCTGATGAAGGGA	5 min at 94°C followed by 35 cycles (1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C) and final extension of 10 min at 72°C	Hind III
4g/5g (PAI1 gene)	Fw: CACAGAGAGAGTCTGGCCACGT Rv: CCAACAGAGGACTCTTGGTCT	5 min at 94 °C followed by 30 cycles (1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C) and final extension of 5 min at 72°C	Bsl I
-455G/A (β-fibrinogen)	Fw: GAACATTTTACCTTATGTGAATTAAGG Rv: GAAGCTCCAAGAAACCATCC	5 min at 94 °C followed by 35 cycles (1 min at 94 °C, 1 min at 58 °C and 2 min at 72 °C) and final extension of 5 min at 72°C	Hae III
-854G/A (β-fibrinogen)	Fw: GGTGTTCCTATTGATTCTTGTAGG Rv: AATGAGGCCCATTTTCCTTGAATT	4 min at 94 °C followed by 35 cycles (30 s at 94 °C, 30 s at 51 °C and 30 s at 72 °C) and final extension of 5 min at 72 °C	EcoRI
Val34Leu (factor XIII gene)	Fw: CATGCCTTTTCTGTTGTCTTC Rv: TACCTTGCAGGTTGACGCCCCGGGGCACTA	3 min at 94 °C followed by 35 cycles (1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C) and final extension of 5 min at 72°C	Dde I
T196C (HPA-1 gene)	Fw: TGGGACTTCTCTTTGGGCTCCTGACTTAC Rv: CCTTCAGCAGATTCTCCTTCAGGTCAC	3 min at 96 °C followed by 39 cycles (20 s at 96 °C, 40 s at 56 °C and 30 s at 72 °C) and final extension of 5 min at 72 °C	MspI
-33T→C (TFPI gene)	Fw: CAATATACAATACGAAAACCTGAAATCC Rv: CGTGAAATTCTAAAAACAATCAGGAA	1 min at 94 °C followed by 30 cycles (1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C) and final extension of 7 min at 72°C	
-287T→C (TFPI gene)	Fw: ACTTGAAGAAGAAACAAACTGCAAAA Rv: CCCCCTAGAGTTAGGGTTTAAAAAA	1 min at 94 °C followed by 30 cycles (1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C) and final extension of 7 min at 72°C	

Table 2 – Subject characteristics and gene test results

Case number	Thrombus site of origin	Age/sex	C677T – MTHFR gene	A1298C – MTHFR gene	G1691A – Factor V gene	A4070G – Factor V gene	G20210A – Factor II gene	4g/5g – PAI1 gene	-455G/A – β-fibrinogen	-854G/A – β-fibrinogen	Val34Leu – factor XIII gene	T196C – HPA-1 gene	-33T→C – TFPI gene	-287T→C – TFPI gene
1	Right femoral vein	75/M	2	1	1	1	1	1	2	1	1	1	T/T	T/T
2	Deep veins of right leg	61/F	2	2	1	1	1	1	1	1	1	1	T/T	C/T
3	not reported	74/F	2	1	1	1	1	2	1	1	1	1	T/T	T/T
4	right femoral vein	82/F	1	1	1	1	1	1	2	1	1	2	T/T	C/T
5	left popliteal vein	31/F	2	2	1	1	1	2	1	1	1	1	T/T	T/T
6	not reported	63/M	1	1	1	1	1	1	1	1	1	1	T/T	C/T
7	not reported	21/F	2	2	1	1	1	1	1	1	1	1	C/T	T/T
8	right femoral vein	53/F	1	2	1	1	1	2	1	1	1	2	C/T	T/T
9	not reported	82/F	2	1	1	1	1	2	1	1	2	1	C/T	T/T
10	left femoral vein	77/F	2	2	1	1	1	1	1	1	1	1	C/C	T/T
11	not reported	86/F	2	1	1	1	1	1	1	1	2	1	T/T	T/T
12	not reported	67/M	3	1	1	1	1	2	1	1	1	1	C/C	T/T
13	right popliteal vein	66/M	1	1	1	1	1	1	1	1	1	1	T/T	T/T
14	not reported	79/M	2	2	1	1	1	1	1	1	1	1	C/T	T/T
15	left femoral vein	74/F	2	2	1	1	1	1	1	1	1	1	C/T	T/T
16	right femoral vein	48/M	1	1	1	1	1	2	1	1	1	1	C/C	T/T
17	not reported	41/F	1	1	1	1	1	1	1	1	1	1	C/C	T/T
18	right femoral vein	65/M	3	1	1	1	1	1	1	1	1	1	C/T	T/T
19	right femoral vein	78/M	2	2	1	1	1	2	1	1	1	1	T/T	T/T
20	not reported	42/F	1	1	1	1	1	1	1	1	1	1	C/T	T/T
21	not reported	76/M	2	2	1	1	1	1	1	1	1	2	C/T	T/T
22	not reported	70/M	1	1	1	1	1	1	1	1	1	1	C/T	T/T
23	not reported	84/F	1	3	1	1	1	2	2	1	2	2	C/T	T/T
24	not reported	75/M	3	1	1	1	1	1	1	1	1	1	C/T	T/T
25	left femoral vein	74/M	2	2	1	1	1	1	1	1	1	1	C/T	T/T
26	not reported	80/M	1	1	1	1	1	1	1	1	1	1	C/T	T/T
27	not reported	50/M	1	1	1	1	1	1	1	1	1	1	T/T	C/T
28	not reported	80/M	1	1	1	1	1	3	1	1	1	1	C/T	T/T
29	not reported	83/ F	1	3	1	1	1	1	1	1	1	1	T/T	T/T
30	left femoral vein	78/M	2	2	1	1	1	1	1	1	1	1	C/T	C/T
31	not reported	59/M	3	1	1	1	1	1	1	1	1	1	T/T	T/T
32	not reported	82/M	1	1	1	1	1	1	1	1	1	1	C/T	T/T
33	not reported	67/M	2	2	1	1	1	1	1	1	1	2	C/T	T/T
34	not reported	91/M	1	1	1	1	1	2	1	1	1	2	C/T	T/T
35	right femoral vein	42/F	1	1	1	1	1	2	1	1	1	2	C/T	T/T
36	not reported	73/M	3	1	1	1	1	1	1	1	1	1	T/T	C/T
37	not reported	69/M	1	1	1	1	1	1	1	1	1	1	C/T	T/T
38	not reported	40/M	2	2	1	1	1	1	1	1	1	1	C/T	T/T
39	not reported	67/M	2	2	1	1	1	1	1	1	1	1	C/C	T/T
40	not reported	46/F	2	2	1	1	1	2	1	1	1	1	C/T	C/T
41	not reported	68/M	3	1	1	1	1	2	2	1	2	2	C/T	T/T
42	not reported	37/M	1	2	1	1	1	1	1	1	1	2	C/C	T/T
43	left femoral vein	63/F	2	2	1	1	1	2	2	1	2	3	T/T	T/T
44	right femoral vein	88/F	1	1	1	1	1	3	1	1	1	1	C/C	T/T

Legend: 1 = wild type; 2 = heterozygous; 3 = homozygous; C = protector; T = promoter

Table 3 – Summary of clinical and genetic risk factors for VTE found in all patients

Case number	Age/sex	n. mutations able to promote	n. mutations enable prevention	Strong risk factors	Moderate risk factors	Weak risk factors
1	75/M	4				increasing age, bed rest > 3 days
2	61/F	3			breast cancer, chemotherapy, glioblastoma multiforme	
3	74/F	4			myelodysplastic syndrome	increasing age, hypertension, bed rest > 3 days
4	82/F	3		femur fracture		obesity, increasing age
5	31/F	5			arthroscopic Knee surgery, oral contraceptive therapy	
6	63/M	1		femur fracture		
7	21/F	3			deficit S protein, Ac anticardiolipin, MTHFR + (C677T)	
8	53/F	4				varicose veins, obesity
9	82/F	4			breast cancer	chest trauma, increasing age
10	77/F	2	1			increasing age, hypertension, bed rest > 3 days, chest trauma
11	86/F	4				increasing age, hypertension, bed rest > 3 days
12	67/M	3	1			increasing age, bed rest > 3 days
13	66/M	2		previous venous thromboembolism		increasing age
14	79/M	3		myocardial infarction		increasing age
15	74/F	3		previous venous thromboembolism		laparoscopic surgery, increasing age
16	48/M	1	1			hypertension
17	41/F	1	1		oral contraceptive therapy	hypertension
18	65/M	2		myocardial infarction		increasing age
19	78/M	5				increasing age, laparoscopic surgery
20	42/F	1			infection (ab ingestis pneumonia)	
21	76/M	4				increasing age, bed rest > 3 days
22	70/M	1				increasing age, hypertension, diabetes mellitus
23	84/F	6		fracture of left femur	cancer	increasing age
24	75/M	2				increasing age, hypertension
25	74/M	3				increasing age, hypertension
26	80/M	1				increasing age, hypertension
27	50/M	1		myocardial infarction		obesity
28	80/M	2				increasing age, hypertension
29	83/ F	3				increasing age, hypertension
30	78/M	2		hip replacement	infection (pneumonia)	increasing age
31	59/M	3				
32	82/M	1				increasing age, hypertension
33	67/M	4		hip replacement		increasing age
34	91/M	3		fracture of left femur		increasing age, hypertension
35	42/F	3				obesity
36	73/M	2				increasing age
37	69/M	1				increasing age
38	40/M	3			inflammatory bowel disease (ascites)	
39	67/M	3	1		infection (pneumonia)	diabetes mellitus, hypertension, increasing age
40	46/F	3			cancer (AML)	
41	68/M	6		fracture of hip		increasing age
42	37/M	3	1		cancer (linfoma)	
43	63/F	8			cancer (lung adenocarcinoma)	
44	88/F	2	1	major trauma	infection (pneumonia)	increasing age

Table 4 – Comparison between the results of the present study and the assessed polymorphism frequency of the general population

Genoype		Data from our study			Data from literature		
		Absolute frequency	**Percent frequency**	**95%CI**	**n**	**Percent frequency**	**95%CI**
MTHFR C677T	WT	19	**43.2**	28.3-59	363	**30**	27.4 – 32.7
	HE	19	**43.2**	28.3-59	620	**51.2**	48.3 – 54.1
	Ho	6	**13.6**	5.2-27.4	227	**18.8** [11]	16.6 – 21.1
MTHFR A1298C	WT	25	**56.8**	41-71.7	49	**47.1**	37.5 – 56.7
	HE	17	**38.6**	24.4-54.5	42	**40.4**	31.0 – 49.8
	Ho	2	**4.5**	0.6-15.5	13	**12.5** [12]	6.1 – 18.9
Factor V Leiden G1691A	WT	44	**100.0**	92-100	1166	**96.4**	95.2 – 97.4
	HE	0	**0.0**	0-8	44	**3.6**	2.6 – 4.8
	Ho	0	**0.0**	0-8	0	**0** [11]	
Factor V HR2 A4070G	WT	44	**100.0**	92-100	445	**89.4**	86.4 – 92.0
	HE	0	**0.0**	0-8	52	**10.4**	7.9 – 13.4
	Ho	0	**0.0**	0-8	1	**0.2** [13]	0.0 – 1.2
Prothrombin G20210A	WT	44	**100.0**	92-100	1171	**96.8**	95.6 – 97.7
	HE	0	**0.0**	0-8	38	**3.1**	2.2 – 4.2
	Ho	0	**0.0**	0-8	1	**0.1** [11]	0.0 – 0.5
PAI-1 4G/5G	WT	29	**65.9**	50.1-79.5	342	**28.3**	25.8 – 30.9
	HE	13	**29.5**	16.8-45.2	588	**48.6**	45.7 – 51.5
	Ho	2	**4.5**	0.6-15.5	280	**23.1** [11]	20.8 – 25.6
β-fibrinogen -455G/A	WT	39	**88.6**	75.4-96.2	744	**61.5**	58.7 – 64.3
	HE	5	**11.4**	3.8-24.6	398	**32.9**	30.3 – 35.6
	Ho	0	**0.0**	0-8	68	**5.6** [11]	4.4 – 7.1
β-fibrinogen -854G/A	WT	44	**100.0**	92-100	120	**60.3**	53.1 – 67.2
	HE	0	**0.0**	0-8	70	**35.2**	28.6 – 42.2
	Ho	0	**0.0**	0-8	9	**4.5** [14]	2.1 – 8.4
Factor XIII Val34Leu	WT	39	**88.6**	75.4-96.2	86	**66.1**	87.3 – 74.2
	HE	5	**11.4**	3.8-24.6	37	**28.5**	20.9 – 37.1
	Ho	0	**0.0**	0-8	7	**5.4** [15]	2.2 – 10.8
HPA-1 (T196C, Leu33Pro)	WT	34	**77.3**	62.2-88.5	51	**69.9**	58.0 – 80.2
	HE	9	**20.5**	9.8-35.3	19	**26**	16.3 – 37.7

	Ho	1	**2.3**	0.1-12	3	**4.1** [16]	0.7 – 11.9
TFPI -33T→C	CC	7	**15.9**	6.6-30.1	102	**50.3**	43.2 – 57.4
	CT	23	**52.3**	36.7-67.5	87	**42.9**	36.0 – 50.0
	TT	14	**31.8**	18.6-47.6	14	**6.8** [17]	3.7 – 11.2
TFPI -287T→C	CC	0	**0.0**	0-8	147	**72.6**	65.9 – 78.6
	CT	7	**15.9**	6.6-30.1	55	**27**	21.0 – 33.7
	TT	37	**84.1**	69.9-93.4	1	**0.4** [17]	0.0 – 2.7

TABLE HEADINGS

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