

PREDISPOSITION TO THROMBOPHILIA AND HYPOFIBRINOLYSIS IN PULMONARY EMBOLISM: ANALYSIS OF INHERITED FACTORS

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Summary

Pulmonary embolism (PE) is a relatively common cardiovascular emergency, though its exact incidence is difficult to assess. Accurate diagnosis is critical because of the high 30-day mortality in patients in whom the diagnosis is missed on admission. Doubt for PE is often raised by the presence of risk factors for venous thromboembolism (VTE), which are categorized into inherited and acquired. Among these, the importance of inherited/genetic thrombophilic factors is increasingly recognized. The most frequent markers of inherited thrombophilia are Factor V Leiden (FVL) and G20210A prothrombin gene mutation. Among the inherited factors causal to thrombophilia, the C677T variant in methylenetetrahydrofolate reductase (MTHFR) gene as well as factors like PIA1/PIA2 polymorphism in platelet glycoprotein IIb/IIIa (PIA2) and hypofibrinolytic polymorphism 4G/4G in PAI-1 gene are discussed with controversial results. In our study, thrombophilic and hypofibrinolytic genetic variants were identified in 54.2% of 115 patients with PE. The most common significant genetic defects were FVL – 16.5% in patients versus 6.2% in controls (OR=3.102; p=0.05), G20210A PT 5.7% versus 2.1% (OR=2.983; p>0.05). PIA2 was found in 27.3% patients versus 19.9% in controls (OR=1.523, p>0.05) and PAI-1 27.8% versus 22.6% (OR = 1.501 p>0.05). MTHFR C677T carriage was inverse: 6.7% in patients versus 13.4% in controls. (OR=0.461 p=0.05). Of all the patients studied, 15.65% had a history of recurrent embolic incidents. The risk of recurrence was higher for the carriers of FVL and G20210A prothrombin gene mutation. The association between carriage of thrombophilic genetic factor and the early onset of the first embolic episode was found in the patients with PE. The awareness of risk factors and risk stratification is a critical issue in treatment and prevention policy. Preventive measures should be taken in particular medical conditions.

Key words: thrombophilia, hypofibrinolysis, FVL, PTA G20210A, MTHFR, GLPR IIb/IIIa, PAI-1, pulmonary embolism

Introduction

Pulmonary embolism (PE) is a relatively common cardiovascular life-threatening disease. Accurate diagnosis is critical because of the high 30-day mortality in patients in whom the diagnosis is missed

on admission. Guidelines propose the methodology and approaches for the best diagnostic procedures [1]. Previous studies have shown that, if left untreated, its mortality could reach approximately 20-30% while appropriate treatment decreases it to 5% [2, 3]. Despite the great number of publications, there is still no consensus regarding the optimal diagnostic approach, as the event is often "atypical", and signs and symptoms are usually vague and nonspecific [4]. Table 1 lists the most common risk factors associated with VTE. Previous studies on VTE have stated that at least one risk factor was found in 80%-96% of patients with proven VTE disease [5].

The proportion of patients with idiopathic or unprovoked PE was about 20% in the International Cooperative Pulmonary Embolism Registry (ICOPER) [6]. However, in younger

patients this proportion was as low as 28% [7]. VTE is considered to be a multifactorial disease caused by environmental and genetic risk factors, as well as by some medical conditions that interact dynamically.

Recently, gene polymorphisms have received an increasing attention. An inherited hypercoagulability should be suspected in patients who have had a VTE event (documented) at a younger age (<40) or have a family history of thrombosis.

The impact of genetic factors in PE development is recognised in Pulmonary British Guidelines [8]. However, there is no recognized role, nor are there clear guidelines for the DNA testing in subjects with suspected PE. This impedes accurate diagnosis and treatment strategy.

Table 1. Risk factors for Venous Thromboembolic Disease

Non-modifiable factors:			Modifiable factors:	
	Inheritance	Lifestyle	Clinical parameters	Medical conditions
Age	Factor V Leiden	Tobacco smoking	Malignancy	Congestive heart failure
Personal history of venous thromboembolism	Prothrombin 20210A mutation	Weight gain/obesity	Trauma	Nephrotic syndrome
Family history	Protein C deficiency	Sedentary life/long flights	Surgery	Myocardial infarction
	Protein S deficiency		Pregnancy	Stroke
	Antithrombin III deficiency		Central venous catheters	Crohn's disease
	Plasminogen deficiency		Immobilization	
	PAI 1 4G/4G		Hormone replacement therapy	
	Factor XII deficiency		Lupus anticoagulant/anticardiolipin antibody	
	Hyperhomocysteinemia			
	Dysfibrinogenemia			

Hereditary thrombophilia

Factor V Leiden is the most common thrombophilic inherited disorder. It has been reported with a prevalence as high as 20% in patients with VTE [9]. Its contribution is up to 50% in selected patients with a family history of thrombophilia [10]. A transition in guanine to adenine at 1691 position (G1691A) leads to production of factor V protein (FVL) resistant to the action of activated protein C (APC). FVa is suggested as a cofactor of protein S contributing to degradation of FVIIIa by APC. Lack of FVa decreases the activity of APC. FVL is a common mutation that is present in 4% to 6% of the general population.

Prothrombin G20210A mutation (PTM) represents the second most important

prothrombotic mutation. In subjects with a history of venous thromboembolism it ranges from about 5% to 15%, reaching up to 20% in selected patients with familial thrombophilia [11, 12]. In Caucasians the prevalence of the G20210A allele is about 2.3% among healthy carriers, and about 6.2% among VTE patients.

Heterozygous carriers have 30 percent higher plasma prothrombin levels as compared to controls. Prothrombin (factor II) possesses procoagulant, anticoagulant and antifibrinolytic activities [13]. That is why this polymorphism results in multiple imbalances in hemostasis. Heterozygotes have a 2 to 5-fold higher risk of thrombosis [14].

The homozygous form (TT) of C677T point mutation in the methylentetrahydrofolate reductase (MTHFR) gene and its impact on

hyperhomocystinemia related to PE development have been reported with conflicting results [14, 15]. The genetic variants C677T in MTHFR produce a thermostable and less active enzyme, which leads to a slower metabolism of methionine, related mutilations and homocysteine (Hcy) [16]. TT homozygotes for C677T have approximately a 70% reduction of normal MTHFR enzyme activity and heterozygotes have approximately a 40% reduction of normal enzyme activity.

Recently, hyperhomocystinemia related to homozygous form of mutation C677T in the gene for MTHFR has been recognized as an independent risk factor for venous thrombosis, particularly in combination with deficiency of folic acid, vitamin B12 and B6. Variant C677T of MTHFR is very common and is present in the homozygous form in 5–15% of the population [17, 18].

Two other genetic variants - PIA1/PIA2 polymorphism in platelet glycoprotein IIb/IIIa (PIA2) and hypofibrilic PAI-1 4G/4G have also been associated with an incidence of venous thrombosis, as they change blood clotting/fibrinolysis process.

PIA2 polymorphism in platelet glycoprotein GP IIb/IIIa, being a critical element of the clot formation process was discussed as a possible inherited factor of prothrombotic tendency. The prevalence ranges from 14.5% [19] to 31.6% [20] in VTE patients. In the general population, most frequent values of polymorphism carriage vary between 10 and 25%. Nonetheless, a controversy exists as to whether this genetic variation can contribute to the susceptibility of PIA2 allele carriers to thrombotic events.

Plasminogen activator inhibitor-1 (PAI-1) is an important regulatory molecule of the fibrinolytic pathway. PAI-1 binds to tissue plasminogen activator (tPA), and by inhibiting conversion of plasminogen to plasmin leads to decreased fibrinolysis. High levels of PAI-1 may be associated with a risk of thrombosis due to the inhibition of fibrinolysis [21].

Although several studies have suggested a contribution of the 675 4G/5G variant in myocardial infarction development, very few data are available on the connection between PAI-1 polymorphisms and the occurrence of VT [22].

Thrombophilia is considered as a multifactorial disease. It could be caused by several dynamically interacting inherited and/or

acquired factors, including a coinheritance of two or more gene defects.

The set of environmental risk factors and coexisting factors was published in International Cooperative Pulmonary Embolism Registry (ICOPER) study. The key acquired risk factors for PE were as follows: DVT (49.3%), obesity (29.2%), surgery (28.9%), bed rest (28.1%) and previous PE (24.9%).

Regardless of the progress in the investigation of the role of these factors as single and/or as co-segregated in the development of thrombosis and related diseases, their impact on the progression of venous thromboembolism and of PE in particular, as well as on the recurrence of episodes remains debatable.

We present our investigation on the incidence of the variant alleles of FVL, G20210A PTM, C677T MTHFR and PIA2, (PAI-1) 4G/5G; and their association with the onset and recurrence of episodes in PE patients.

Materials and Methods

Selection of Patients and Study protocol

A study was conducted at Pulmonology Department of University Hospital (Pleven, Bulgaria) from 2008 to 2013. A total of 115 unrelated patients with PE and 94 control subjects (age and sex adjusted) were investigated. The median age of the patients was 47.51 years (range 14 to 79 years).

The selection criterion was a history of one or more episodes of VTE and/or PE. PE was diagnosed by ventilation-perfusion lung scan, according to criteria set by British Thoracic Society. Deep venous thrombosis (DVT) was diagnosed by compression ultrasonography and Doppler ultrasonography. There were no patients with malignant or myeloproliferative diseases or systemic infections in the study group. Six out of a total of 53 investigated women had a history of obstetric complications: recurrent spontaneous abortions in the first and/or second trimester.

Data Collection

A specific questionnaire was used to interview patients with embolic incidence. The survey listed number and type of vascular incidents, age at onset of the first episode, number of recurrent episodes, family history, previous and present anticoagulation therapy, presence of risk factors such as surgery, trauma, immobilization, oral

contraceptive use, pregnancy or postpartum period within the last 3 months.

Both patients and control groups were of similar demographic data and from the same geographical area. The study protocol was approved by the ethics commission of Medical University-Pleven, Bulgaria. All investigated subjects gave written informed consent for the investigation.

Sample collection and DNA analysis

Venous blood was collected in vacutainers with 0.084 ml 15% EDTA (Becton, Dickinson and Company). DNA was isolated following the procedure of GFTTM Genomic Blood DNA Purification Kit (Amersham Pharmacy Biotech Inc) and quantified using the agarose gel procedure.

Polymerase Chain Reaction (PCR) was performed in a total volume of 20 µl containing the following: 1 µl (100 ng/µl) genomic DNA, 0.4 µl (20 pmol/µl) of respective forward and reverse primer, 1.8 µl (5 mmol/µl) deoxynucleotide triphosphates, MgCl₂ 25 mmol/µl, 2.0 µl, 2.0 µl Buffer for Taq polymerase and Taq polymerase 1 U per sample (AB gene).

The amplification was carried out in a thermal cycler (Techne, version 11.04). The primers and reaction conditions for the investigated genetic defects have been described in earlier publications [23].

PCR products were fractionated by electrophoresis through 2.5% agarose (AppliChem) and visualized in UV light by ethidium bromide staining (10 mg/ml, 10 µl).

The amplified samples (10 µl) were incubated at 37°C for 12 hours with a specific enzyme-restrictase (3U per sample), in the presence of 0.2 µl Bovine serum albumin (Purified BSA 10 mg/ml – New England BioLabs Inc) and 2 µl NEB Buffer 2 (New England BioLabs Inc) in a total volume of 20 µl.

A specific restrictase was used for each mutation:

FVL – Mnl I (5000 U/ml); PTM G20210 – Hind III (20 000 U/ml); MTHFR C677T – Hinf I (10 000 U/ml); PIA2 in GLPR IIb-IIIa – Msp I (20 000 U/ml); (New England BioLabs Inc).

The products of restrictase reaction were separated by electrophoresis over 3.5% agarose gel and visualized in UV light by ethidium bromide staining (10 mg/ml, 10 µl).

Allele specific PCR was used to detect 4G and 5G genotypes. Amplification was performed in

two separate series, with the two constitutive primers and two inner primers corresponding to sequences 4G and 5G [24], and 270 bp and 170 bp products were separated on 3% agarose gel.

Visualisation with UV light was performed in ethidium bromide staining. PCR guidelines were strictly followed to avoid contamination. Sample collection, DNA isolation and quantitation were performed in the laboratory room, separated from the laboratory where sample amplification and analysis were performed [25].

Statistical analysis

Allele frequencies were calculated for each subgroup of patients and controls. Statistical Package for Social Sciences (SPSS) version 19.0 was used. Statistical significance was taken at $p < 0.05$.

Due to the heterogeneity in the group of the patients with pulmonary embolism, the construction of the control group was not a simple process and there was a risk of mistake in the selection. To verify compliances between the groups in age and gender, an appropriate statistical methods was necessary. The control group included 94 unrelated healthy individuals (53 men and 41 women) with no family history of venous thrombosis and/or embolism. There was no statistically significant difference between the groups by gender, as evaluated by Fisher exact test $p = 0.92$. There was no statistically significant difference between the groups in age – Student t-test, $p = 0.293$.

Results

A total of 115 patients with VTE and 94 control subjects were tested for the variant alleles of FVL, G20210A PTM, C677T MTHFR, PIA2 IIb/IIIa and PAI-1 4G/5G. The characteristics of the patients are presented in Table 2. Respectively, the clinical parameters and life style factors were: surgery and trauma (37.39%), immobilisation (18.26%), obesity (25.21%) and tobacco smoking (26.95%). The results of DNA testing are presented in Table 3. The overall number of mutation carriers was 62 (53.91%) and double carriers were 14 (12.17%).

Onset of VTE

The median age at first embolic incident was 44.8 years. Eighteen (15.65%) of all patients had a history of recurrent embolic incidents. The clinical presentation of the VTE depended

largely on the underlying cause. In the absence of inherited risk factors, thrombosis occurred in the older subjects: 49.7% versus 42.5% in mutation carriers (shown already in a previous pilot study [26]), mainly in the context of modifiable factors, such as surgery and trauma, immobilisation,

obesity and tobacco smoking. The average age of the clinical manifestation in the subjects with extrinsic factors only was around 47 years. After adjusting for mutation carriage and life style factors it reached 57 years.

Table 2. Characteristics of the patients (n=115)

Characteristics	Value numbers and percentages in brackets
Age (years): Mean	47.48 45.19 male, 50.11 female
Range (years)	14-79
Men	62 (53.9%)
Women	53 (46.1%)
Age of the first thrombotic incidence	44.8 43.0 (males), 47.6 (females)
Diagnosis of DVT	33 (28.7%)
Unexplained cough	64 (55.75%)
Previous pulmonary embolism/deep vein thrombosis	18 (15.65 %)
Trauma or surgery	43 (37.39%)
Immobilisation	21 (18.26%)
Obesity	31 (26.69 %)
Tobacco smoking	31 (26. 69%)
Diabetes	9 (7.41%)
Early pregnancy loss (female patients)	6 of 53 (11.32%)

Prothrombotic mutations

The prevalence of thrombotic mutations, as well as the percentage within group, Pearson Chi-

Square, Fisher's Exact Test, Odds Ratio and 95% confidence interval are presented in Table 3.

Table 3. Prevalence of thrombophilic factors FVL, prothrombin G20210A (PTM), MTHFR C677T, PIA2 IIb/IIIa, and PAI-1 4G/4G in patients as compared to controls

Statistical values	Prevalence in patients %	Prevalence in controls %	Percentage within group %	Pearson Chi-Square	Fisher's Exact Test	Odds Ratio	95%Confidence Interval
Mutation							
FVL	16.5	6.2	69.0	7.770 ^a	0.005	3.102	1.357- 7.094
PTM	5.7	2.0	70.0	2.654 ^a	0.096	2.983	0.755- 11.789
PIA2	27.3	19.9	53.2	2.038 ^a	0.100	1.523	0.855-2.676
PAI 1	27.8	22.6	52.1	1.934	0.110	1.501	0.942-3.657
MTHFR	6.7	13.4	28.6	-3.253 ^a	0.050	0.461	0.195 -1.087
Total	53.91	35.3	60.7	3.129 ^a	0.050	1.553	0.953- 2.532

Modifiable risk factors

Numerous modifiable factors trigger VTE. Modifiable /reversible factors, such as surgery or trauma (37.39%), obesity (26.69%), immobilization (18.26%), smoking (26.69 %),

estrogen therapy (7%) and pregnancy-related problems (11.32%) were associated with a significant risk of a pulmonary event in our study.

Discussion

The contribution of FVL was significant. About 16.5% of the patients carried FVL as compared to the controls (6.2%) with an odds ratio 3.1 (95% CI; 1.357-7.094). In our investigation, 25% of mutation carriers had FVL mutation. Recent investigations have presented similar data on Factor V Leiden: carriage of FVL is associated with 3- to 7-fold increase of risk of a first episode of VTE [27, 28] with about 15-20% prevalence of FVL reported [29, 30].

The patients carriers of FVL were at higher risk of recurrent thrombotic episode RR=1.879 (95% CI; 0.643-4.446; $p>0.5$). Conflicting reports have been published in the medical literature, whether this polymorphism increase the risk of recurrent VTE. However, a recent review on reports from 10 studies [31], involving 3104 patients with first VTE revealed that FVL was present in 21.4% of patients and associated with an increased odds of recurrent VTE of 1.41 (95% CI; 1.14-1.75; $p=0.08$ for heterogeneity).

The penetrance of homozygous carriage of FVL was stronger, the carriers had both – recurrent episodes and DVT at unusual location. Of the FVL carriers, 75% had an additional precipitating environment factor. The clinical data on coprecipitation with other hypercoagulable or hypofibrinolytic factor presents the impact of FVL as more pronounced, supporting the idea of the multifactorial etiology of PE.

The prevalence of prothrombin G20210A mutation in our patients was about 5.7% (heterozygous) comparing to 2.0% in controls OR=2.654 (95% CI; 0.755-11.789; p -value >0.05). There was no homozygous carrier of this mutation. The risk of recurrence was high: OR=2.556 (95% CI, 0.349-13.691) but not significant. The prevalence of prothrombin G20210A is reported to be about 7% [32].

PTM G20210A increases the risk by 2- to 3-fold. The risk of recurrence is rather modest. The results reported are controversial: of the 4 high-quality studies, only one found a significant increase in risk of recurrence associated with heterozygous carriage of the G20210A polymorphism (OR=2.4; 95% CI, 1.3-4.7) [33].

PIA2 IIb/IIIa carriage was extended in 27.3 % of patients as compared to 19.9% of the control group with OR =2,038, and non significant, p -

value >0.05 , in the range of the data from other authors: 14.5% to 31.6% In spite of the comparatively high prevalence of this polymorphism, only one patient was homozygous. The impact of this mutation to the development of VTE and PE in particular is under discussion. The recurrent incidents were the same level as in the patients who were non carriers of thrombophilic mutation (OR=1.108; 95% CI, 0.322-3.90). In more than 75% of cases the patients had an additional precipitating environment factor. To understand the importance of this polymorphism we have to keep in mind that it affects platelet membrane glycoprotein IIb/IIIa, which is a member of the integrin family of adhesive molecules, by binding fibrinogen and von Willebrand factor, promotes platelet aggregation and as a consequence contributes to thrombogenesis.

The prevalence of plasminogen PAI-1 4G/4G allele was about 27.8% (heterozygous), as compared to 22.6 % in the controls (OR= 1.934; $p>0.05$). The contribution to recurrence was also insignificant (OR=1.365; 95% CI 0.371-4.743). PAI-1 was investigated as a fibrinolytic factor playing a critical regulatory role in a variety of pathologic processes, including inflammatory responses [34], heart and lung fibrosis [35], coronary artery disease, myocardial infarction [36], ischemic stroke [37], sepsis, and endometriosis. Although the results are disputable, these conditions are often associated with elevated gene expression both in patients with cardiovascular and metabolic disorders and for endometriosis, but not in healthy individuals. There are investigations confirming that carriage of 4G allele appears to increase the risk of venous thrombosis, particularly in subjects with other genetic thrombophilic defects [38]. However, investigations on the relationship of carriage of 4G and VTE are controversial, as far as the impact of allele 4G is concerned.

As for variant allele C677T MTHFR, there was an adverse relationship between polymorphism carriage and VTE events. The prevalence of this mutation in the patients was lower than in the controls, 6.7% and 13.4%, respectively (OR=0.461, p -value 0.5). There were no carriers of this mutation among the patients with recurrent events. That is why when considering the contribution of genetic disorders to the development of VTE events, the C677T MTHFR polymorphism could not be considered as

rigorous. Extracting this mutation from pooled thrombophilic factors slightly reduces the impact of thrombophilic mutation on VTE development.

Recently, hyperhomocysteinemia has been widely discussed as a factor contributing to the development of both arterial and venous thrombosis. A C677T point mutation within the MTHFR gene is the most common genetic defect, which causes hyperhomocysteinemia. In turn, elevated homocysteine levels exert numerous vasotoxic effects on the endothelium, leading to endothelial cell dysfunction, platelet activation, thrombus formation and increased risk of thrombotic events. However, the contribution of MTHFR polymorphism to the development of episodes of PE was not established in this investigation. What is more, there was a paradox of inverse relationship of mutation carriage and VTE events.

Incidence of recurrent thromboembolic events in patients with inherited and modifiable risk factors

The prevalence of recurrent thrombotic events (two or more incidents) was 15.6% among the VTE patients with available medical records. Eleven of these patients (61.0%) had at least one mutation, FVL and PAI-1 being the most frequently presented in 5 carriers. The mean age of the patients with recurrent events who carried at least one mutation was 48.2 years, as compared to those without mutation, which was significantly higher—55.2 years.

Although there is a great deal of controversy regarding the significance of factor V Leiden in increasing the risk of recurrent venous thrombosis [39,40], our investigation indicated that the prevalence of carriage of FVL and prothrombin G20210A was more frequent in patients with recurrent VTE (27.7% and 11.2%, respectively) than among those who had had no recurrences (16.5% and 5.6%, respectively). The relative risk of occurrence was calculated in patients who had a thrombophilic mutation and then compared to that in the patients without mutation, that is in accordance with a relative risks of recurrence ranging about 2 to 4 discussed by Simioni et al. [41].

The most frequent modifiable factors associated with a significant risk of pulmonary event were surgery and/or trauma (37.39%), obesity (26.69%) and smoking (26.69%). The contribution of obesity and smoking was the highest, when the group of surgery was divided from those with trauma.

Obese and smoking patients had the same risk of recurrence as the total group of the patients. The patients with surgery, trauma, immobilization were at a lower risk of recurrent events and underwent a standard therapy.

The results showed that the greater the triggering factor was, the lower the risk of recurrence after anticoagulant withdrawal. These results were similar to those reported by other authors [42]. The low risk of recurrence after postoperative VTE was presented in a study comparing various durations of anticoagulant therapy. Prandoni et al. [43] have shown that surgery and recent trauma or fracture are associated with a lower risk of recurrent VTE, as compared to unprovoked VTE (hazard ratio 0.36; 95% CI; 0.21 to 0.62). Subsequent studies have confirmed that the risk of recurrent VTE is much lower among patients whose initial VTE is provoked by modifiable/reversible risk factors than among patients with thrombophilia.

Conclusion

- The early onset of PE was confirmed for thrombophilic mutation carriers
- Both FVL and prothrombin G2010A mutation carriage was related to a higher risk of recurrence
- PAI-1 4G and PLA2Ib/IIIa polymorphisms play a certain role in the development of VTE
- For variant allele C677T MTHFR there was an adverse relationship between polymorphism carriage and VTE event.
- Obese and smoking patients had the highest risk of VTE development among the patients with modifiable risk factors
- The association between thrombophilic genetic factors FVL, prothrombin G2010A, PLA2Ib/IIIa and hypofibrinolytic PAI-1 4G and PE confirms the significance of applying genetic testing in patients with PE.

Physicians should be aware of risk factors and risk stratification for VTE development, particularly in certain medical situation, so as to avoid VTE development and recurrent events. Genetic testing in patients with thrombosis and VTE would allow prevention of recurrent thrombotic incidents in individuals with inherited predisposition. Genetic testing may benefit patients with inherited tendency to hypercoagulability and hypofibrinolysis and PE through various approaches: longer duration of anticoagulation therapy; preventive anticoagulation for high risk medical conditions and live

situations; avoidance of predisposing risk factors with synergistic action, family testing [44], counselling etc. All these could contribute to lower incidence of VTE, and PE in particular.

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