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Genetic polymorphisms associated with acute pulmonary embolism and deep venous thrombosis

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ABSTRACT: Frequently an inherited predisposition to thrombosis remains clinically silent until an additional environmental factor intervenes. The present study aimed to assess distribution of inherited risk factors of venous thrombosis in patients with venous thromboembolism (VTE).

The prevalences of factor V Leiden (FV Leiden), prothrombin factor II G20210A (FII G20210A), C677T and A1298C of methylenetetrahydrofolate reductase (MTHFR) mutations were studied in 149 VTE patients and 100 controls.

The following key risks were established: previous deep venous thrombosis or pulmonary embolism (23.5%), bed rest (34.2%), immobilisation of lower limb (10.1%), hospitalisation (30.9%) and obesity (28.9%). In 29 (19%) patients and in three (3%) controls FV Leiden was found. A significant association between VTE and FV Leiden was established. There were six (4%) carriers of the FII G20210A among VTE patients and one in the controls. No associations between VTE and MTHFR polymorphisms (C677T, A1298C) were found. In three of 149 patients both FV Leiden and FII G20210A polymorphisms were observed. The mean protein C activity was slightly, though nonsignificantly, smaller in VTE patients.

In conclusion, there was a positive association between venous thromboembolism and factor V Leiden. Only a weak trend favouring a relationship between prothrombin factor II G20210A and venous thrombolism was present. No associations between common polymorphisms of methylenetetrahydrofolate reductase and venous thromboembolism were found.

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Venous thromboembolism (VTE) is a common vascular disease with multifactorial pathogenesis that results in two major clinical manifestations: deep venous thrombosis (DVT) and pulmonary embolism (PE). Their annual incidence ranges 1–5 per 1,000 individuals [1]. In spite of its high incidence, it is often difficult to diagnose and hence frequently missed. On many occasions an inherited predisposition remains clinically silent until an additional environmental factor intervenes. The key acquired risk factors for VTE comprise trauma, surgery, prolonged immobilisation, advanced age, pregnancy and malignancy [1, 2]. In the recently published French Multicenter Registry it was revealed that nearly 50% of PE and DVT cases occurred in the absence of a classical predisposing risk factor [3]. Within the last decade many investigators have focused on identifying congenital predisposition to thrombosis. Inherited thrombophilia should therefore be considered whenever a patient has a documented history of unexplained thrombotic events sustained below the age of 45–50 yrs, recurrent DVT and PE, a positive family history or has no apparent acquired risk factors, or in the case of female patients, a history of multiple abortions or stillbirth [1, 2]. Advances in the understanding of blood clotting allow the determination of an underlying inborn cause of thrombosis in ~30% of patients with recurrent thromboembolic disease [1]. The most common identified mutations are: factor V Leiden (FV Leiden), prothrombin factor II G20210A (FII G20210A) and deficiencies of protein C (PC), protein S (PS) or antithrombin III (AT-III). FV Leiden is present in ~5% of the general white population. Heterozygous carriers of this mutation have a risk of VTE almost 7-fold higher than in a general population, whereas for homozygous carriers it is as much as 80-times higher [4]. The relationships between both C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene and VTE have been analysed [5, 6]. Some studies have suggested that hyperhomocysteinaemia is an independent risk factor for VTE [6, 7].

Admittedly, manifestation of thrombophilia varies substantially among individuals carrying genotypically identical deficiencies of AT-III, PC and PS. Only 50% of them develop venous thrombosis below 45 yrs of age [1]. It follows that other genetic abnormalities could well modify the expression of these mutations.

The present paper aims to analyse genetic predisposition to venous thrombosis in patients hospitalised for VTE disease.

Material and methods

Study population

A total of 149 consecutive eligible patients (91 males, 58 females), aged 19–86 yrs (mean 47.9±14.3), with episodes of DVT and/or PE were studied at the Dept of Medicine, Jagiellonian School of Medicine, Cracow, Poland, throughout 1998–2000. The study patients were divided up into three subgroups (table 1): 1) DVT group with 33 subjects (22.2%); 2) DVT complicated by PE group with 68 subjects (45.6%); and 3) isolated PE group with 48 subjects (32.2%). Additionally, the analyses were also performed on patients $\leqslant$50 yrs of age and on patients >50 yrs.

Healthy subjects (60 males, 40 females), aged 19–61 yrs (mean 45.4±7.4), who denied both personal and family history of VTE, were selected as controls from amongst the medical staff of the University Hospital. They all tested normal in routine lab tests. Both the VTE patients and controls came from the same geographical area, *i.e.* Malopolska, the southern region of Poland, as evidenced by the respective personal histories.

The study was approved by the local ethics committee and informed consent was obtained from all study subjects.

Diagnostic techniques

DVT was diagnosed in compliance with the recommendations of the American Heart Association using ultrasonography (Acuson 128XP/10; Mountain View, CA, USA) and contrast venography, when indicated [8]. Clinical assessment of the likelihood of acute PE was based upon all available noninvasive data (history, physical examination, chest radiography, electrocardiogram, arterial blood gases, echocardiography, level of D-dimers) [9, 10]. The findings were confirmed by objective imaging methods: spiral computed tomography (Helicatflash, Eliscint, Israel) and pulmonary angiography, when indicated (Integris V 3000, Philips, Eindhoven, the Netherlands) [11].

Table 1. – Characteristics of patients with venous thromboembolism

Characteristics	DVT	DVT and PE	PE
Subjects n	33 (22.2)	68 (45.6)	48 (32.2)
Age yrs	43.4±11.8 (19–66)	48.8±15.1 (18–76)	49.6±14.2 (18–86)
Sex			
Female	18 (12)	18 (12)	22 (15)
Male	15 (10)	50 (34)	26 (17)

Data are expressed as n (%) or mean±SD (range). DVT: deep venous thrombosis; PE: pulmonary embolism.

Laboratory tests and genetic analysis

Genomic deoxyribonucleic acid was isolated from peripheral blood leukocytes and genotyped for FV Leiden, FII G20210A, C677T and A1298C polymorphisms of MTHFR, as previously described [12–15].

The global test ProC Global (Dade-Behring, Marburg, Germany) was used for the identification of defects in the PC pathway. AT-III [16] and PC [17] activity were assessed by the chromogenic method using the Behring Coagulation Timer Instrument (Dade-Behring). Free PS levels were assessed by the enzyme-linked immunosorbent assay (ELISA) method (Diagnostica Stago, Asnieres, France) [18]. Plasma total homocysteine levels were determined by high-performance liquid chromotography with fluorometric detector according to MANSOOR *et al.* [19]. Antiphospholipid antibodies (anticardiolipin antibodies, lupus anticoagulant) were assessed by the ELISA method, in compliance with the recommendations of the International Society on Thrombosis and Haemostasis [20].

Statistical analysis

The statistical significance of any differences was evaluated by the nonparametric Mann-Whitney U-test, whereas the differences in the genotype distributions were tested using the Chi-squared test. For all mutations the odds ratio (OR) and their 95% confidence intervals (CI) were calculated according to SHEEHE [21] to estimate the risk for VTE. Statistical significance was set as $p<0.05$.

Results

In the studied group of 149 patients the males suffered significantly more frequently from PE and DVT complicated by PE than the females (p=0.03, p=0.004, respectively). The difference in prevalence of DVT, DVT complicated by PE and isolated PE in relation to age was not statistically significant. However, when the overall number of patients with PE (DVT complicated by PE in conjunction with isolated PE) was analysed, the older patients (>50 yrs) were found to fall sick more frequently than the younger patients ($\leqslant$50 yrs) (p=0.03). In the PE group of patients, DVT was diagnosed in 68 subjects (58%).

After stratification of the subgroups in terms of the acquired risk factors, statistically significant immobilisation of lower limb was found more frequently in patients with DVT complicated by PE (n=13, 8.7%, p=0.005). Personal history of venous thrombosis was observed significantly more frequently in patients with DVT (n=13, 8.7%, p=0.002). In the isolated PE subgroup, bed rest and hospitalisation were most common (n=17, 11.4%, p=0.006 and n=15, 10%, p=0.019, respectively). The estimated combined number of acquired risk factors for VTE was comparable in all

studied subgroups. No relationship between age and the acquired risk factors was found.

In 29 (19%) patients FV Leiden was found; 28 individuals (99.4%) were heterozygous for FV Leiden, whereas one subject (0.6%) was homozygous. Conversely, three (3%) heterozygotes were found in the controls. A significant association between VTE and FV Leiden was established (OR=6.9, 95% CI 2.35–20.46, p=0.00013). The allele frequency for FV Leiden was notably lower in the subgroup of patients with isolated PE (p=0.05) than in patients with DVT combined with patients with DVT complicated by PE. Although a tendency for lower allele frequency for FV Leiden was observed between patients with isolated PE and the other subgroups, no significant differences were found (table 2).

There were six (4%) carriers of the FII G20210A among VTE patients and one in the controls. Nobody was found to be homozygous for this mutation. In the calculated OR for VTE related to FII G20210A (OR=3.28, 95% CI 0.7–16.1, p=0.08) there was a perceptible trend in favour of FII G20210A, a risk factor of VTE (fig. 1). It should also be noted that no differences in the allele and the carrier frequencies of the G20210A mutation in the prothrombin gene were found between the studied subgroups (DVT, DVT and PE, PE).

The allele, carrier and homozygote frequencies of FV Leiden and FII G20210A for patients with VTE and controls are presented in table 3. In three (3%) patients both FV Leiden and the G20210A mutation in the prothrombin gene were found.

The percentage of patients with FV Leiden was 19–22% with respect to the decade of age. In the case of FII G20210A it ranged 4–5%.

Among subjects with VTE there were 35 individuals (23.5%) with recurrent thrombotic events. When 114 subjects with the first episode of venous thrombosis were analysed, amongst the individuals <50 yrs, FV Leiden was found in 11 patients (19%). In older patients with VTE there were seven individuals with FV Leiden (14%). No differences in the frequency of FV Leiden were found between the younger and older patients with VTE.

Table 2. – The comparison of allele and carrier frequencies of factor V Leiden in patients with deep venous thrombosis (DVT), DVT complicated by pulmonary embolism (PE) and isolated PE

	Carrier frequency p-value	Allele frequency p-value
PE *versus* (DVT and PE)+DVT	0.06	0.05
PE *versus* (DVT and PE)	0.09	0.07
PE *versus* DVT	0.09	0.10
DVT *versus* (DVT and PE)+PE	0.23	0.28
DVT *versus* (DVT and PE)	0.48	0.54
(DVT and PE) *versus* (PE)+(DVT)	0.26	0.21

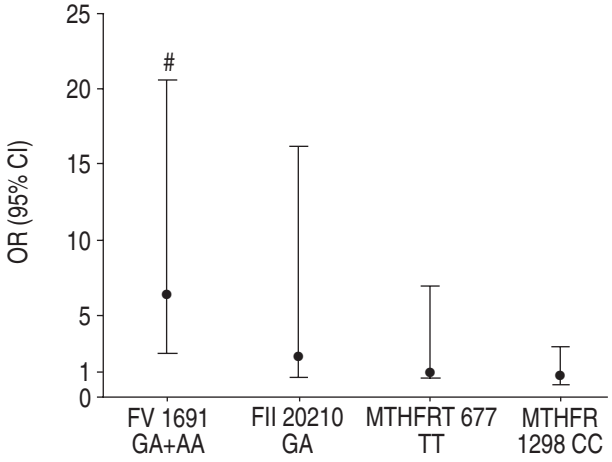

Fig. 1. – The odds ratios (OR) and 95% confidence intervals (CI) associated with carriership of analysed allele variants. FV: Factor V Leiden; FII: prothrombin factor II; MTHFR: methylenetetrahydrofolate reductase. #: p=0.00013 comparing patients with venous thromboembolism and healthy subjects.

No associations between VTE and MTHFR polymorphisms (C677T, A1298C) were found. The OR for VTE related to the mutated homozygotes of MTHFR was 0.86 (95% CI 0.3–2.6) for 677TT, whereas for 1298CC it was 0.66 (95% CI 0.8–6.7). No difference in the frequencies of 677TT and 1298CC was found between patients with DVT, DVT complicated by PE and with isolated PE.

The mean plasma homocysteine levels in the VTE patients and the controls was similar (12.82±4.95 $\mu mol \cdot L^{-1}$ *versus* 12.83±2.91 $\mu mol \cdot L^{-1}$, p=0.90). The individual homocysteine concentrations in both VTE patients and controls are presented in fig. 2. There were 55 (36.9%) subjects with hyperhomocysteinaemia among the VTE patients and 33 (33%) among the controls. In line with the suggested consensus terminology [22], a plasma total homocysteine level of 16.0 $\mu mol \cdot L^{-1}$ was assumed as the upper limit of normal range in the fasting state.

Decreased activity of PC was found in eight (5%) patients with VTE and in one (1%) control (OR=5.6, 95% CI 0.7–45.6). A decreased level of free PS antigen was found in seven patients with VTE (4.7%) and in one (1%) control (OR=4.9, 95% CI

Table 3. – The allele, carrier and homozygote frequencies of factor V Leiden (FV G1691A) and prothrombin factor II G20210A (FII G20210A) for patients with venous thromboembolism (VTE) and controls

	FV G1691A			FII G20210A		
	Patients	Controls	p-value	Patients	Controls	p-value
Allele frequency	0.102	0.015	0.00013	0.022	0.005	0.08
Carrier frequency	0.197	0.03	0.04	0.044	0.01	0.09
Homozygote frequency	0.007	0		0	0	

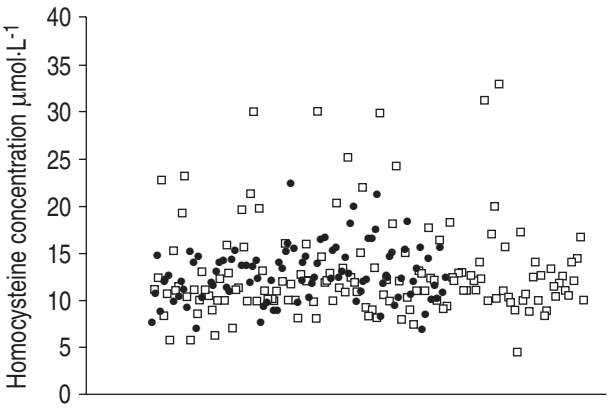

Fig. 2. – Plasma homocysteine levels in venous thromboembolism (VTE) patients (□; n=149) and controls (●; n=100). The x-axis represents the number of consecutive patients with VTE or healthy subjects enrolled in the study.

0.6–40.3). Decreased AT-III activity was observed in three (2%) subjects with venous thrombosis, whereas in the controls it was not encountered.

The mean PC activity was not significantly higher in the controls than in patients with VTE (121.1 *versus* 110.2%, p=0.05). There was a similar mean level of free PS antigen in the VTE subjects and the controls (89.4 *versus* 91.9%, p=NS). The mean AT-III activity was comparable in both studied groups (104.9 *versus* 109.3%, p=NS). When the subgroups (DVT, DVT complicated by PE and isolated PE) were compared, no differences in mean PC activity, mean level of free PS antigen and mean AT-III activity were encountered.

Increased titres of immunoglobulin (Ig)M anticardiolipin antibodies (MPL) (normal range <20) were found in 29 (20%) subjects with VTE and in nine (9%) of the controls (OR=2.4, 95% CI 1.1–5.4), whereas IgG antibodies (GPL) (normal range <16) were found in 21 (14%) and six (6%) patients and controls (OR=2.6, 95% CI 0.9–6.6), respectively. Calculated mean titres of anticardiolipin antibodies (IgM, IgG) were higher in the subjects with venous thrombosis than in the controls (14.3 MPL *versus* 9.18 MPL, p=0.004) and (6.15 GPL *versus* 3.2 GPL, p=0.016), respectively. A higher titre of anticardiolipin IgM antibody was found in subjects with DVT alone, in comparison with the other subgroups (DVT complicated PE, isolated PE) (p=0.016). In eight (27.5%) thrombotic subjects with FV Leiden an increased titre of IgM and IgG anticardiolipin antibodies was found. The distributions of FV Leiden in all subjects with thrombosis showed no significant differences when compared to subjects with a higher titre of anticardiolipin antibodies. Primary antiphospholipid syndrome was diagnosed in three (2%) patients of the 149 subjects studied. The presence of lupus anticoagulant was encountered in five (3.4%) patients.

Discussion

The present study aimed to assess the distribution of inherited risk factors of venous thrombosis in patients with DVT and/or PE. The subjects with FV Leiden were at significantly higher thrombotic risk than the ones without this genetic factor. The prevalence of FII G20210A was slightly higher in VTE patients than in controls while the 677TT and 1298CC of MTHFR was the same in subjects with VTE and in controls. The impact of the acquired and other genetic factors on the risk of venous thrombosis was also assessed.

Before establishing the diagnosis of inherited thrombophilia it is essential to rule out any acquired conditions that might account for similar clinical manifestations. The set of environmental risk factors and coexisting factors was based on the studies by Celi *et al.* [9] and Wells *et al.* [10]. In the recently published 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 DVT or PE (24.9%) [23], whereas in 19% of their study subjects no environmental risk factors were reported. In the present study, the following key risk factors were established: previous DVT or PE (23.5%), bed rest (34.2%), immobilisation of lower limb (10.1%), hospitalisation (30.9%) and obesity (28.9%), whereas 24% of patients had no apparent predisposing factors.

Most cases of VTE tend to occur at an older age, 60–70 yrs [1] and the incidence rate of venous thrombosis increases with age [2]. This tendency might be due to a relationship between age and other concomitant disorders. The same was observed in the current group of patients with PE (isolated PE and DVT complicated by PE). However, in contrast to the findings of others [2], the present authors did not manage to find any association between age and the environmental risk factors. The authors based their analysis on the 21 commonly addressed risk factors. Information about the "power of assessed risk factors" might enhance the analysis of these parameters. Regretfully, some of the factors were encountered in too few subjects, hence the analysis in the study may well be inadmissibly biased.

The association between sex and VTE is ambiguous. The data based on autopsies are also inconclusive [24]. In some studies no differences were reported in the prevalence of DVT [25] and PE [14] between males and females. In the Silverstein study VTE was more common in males [26]. The ICOPER study revealed that 55% of patients with acute PE were females [23]. In contrast, the study by Kasper *et al.* [27] reported that PE was more frequently encountered in males.

The relationship between FV Leiden and VTE is commonly recognised [4]. In the present study the carriers of FV Leiden were at an approximately sevenfold increased risk of venous thrombosis. The current observations are consistent with other reports [4, 28]. The prevalence of FV Leiden is not regularly encountered in white Caucasian populations and the prevalence in the current study is similar to that recently reported by others [29].

Frequency of FV Leiden (19%) and FII G20210A (4%) for various decades of age in subjects with VTE

was similar, which might be due to the specific inclusion criteria, *i.e.* incidence of any thrombotic episodes. Whilst analysing subjects who had been diagnosed with the first thrombotic event (n=115), statistically insignificant higher number of younger subjects (<50 yrs) with FV Leiden was found. Presence of FV Leiden, however, does not seem to have an impact on the longevity of the carriers.

The lower frequency of FV Leiden in patients with PE alone seems to be related to the "factor Leiden paradox". In these circumstances the thrombus is believed to be more stable and well bound to the vessel wall, hence the migration of emboli is less likely to happen. The FV Leiden may enhance the local thrombin generation process or impair the profibrinolytic response to the activated protein C. Furthermore, it was established that thrombi were smaller among the carriers of FV Leiden and seldom located at the level of the femoral and iliac veins, where emboli most commonly originate. It was also hypothesised that a val34leu mutation in factor XIII might protect against PE but not DVT [30].

In contrast to other studies [14, 28], only a weak trend in favour of the relationship between FII G20210A and VTE was observed, which might be due to a relatively small sized study population. The G20210A mutation in the prothrombin gene is associated with an increased level of plasma prothrombin [31]. It has been suggested that the carriers of this mutation are characterised by higher effectiveness of translation or more stable messenger ribonucleic acid, which might result in the increase of thrombin generation [14, 31]. In addition, the frequency of the FII 620210A mutation was not reduced in patients with PE alone, which is consistent with other reports [28].

It has been shown previously that common polymorphisms of MTHFR (C677T, A1298C) appear to be related to early coronary artery disease [32], however studies on their relationship with VTE have been conflicting [5, 6, 33]. In the present study, there was no association between MTHFR mutations and venous thrombo-embolic disease.

Hyperhomocysteinaemia seems to be only a weak risk factor for VTE [33]. The mean value of total plasma homocysteine levels in the patients with venous thrombosis and in the controls in the current study did not differ. In the group of older patients (>50 yrs) a significantly higher number of individuals with total plasma homocysteine levels >16 $\mu mol \cdot L^{-1}$ was found than in the younger patients. This might be related to age, sex, vitamin status and other lifestyle factors commonly known to influence plasma homocysteine levels.

The analysis of natural anticoagulant deficiencies is hard to accomplish due to their extremely rare incidence in population. The mean PC activity was slightly, albeit not significantly, smaller in patients with VTE than in the controls, which might be related to an impairment in the PC pathway in persons experiencing VTE in the previous 6 months.

Anticardiolipin antibodies are heterogenic and various reports yield conflicting data regarding their prevalence [1, 17]. Data from the literature shows that in patients with VTE they ranged 8.5–14%, whereas in the present study anticardiolipin antibodies were encountered in 14–20% of subjects. Since the tests were actually carried out during the active phase of the thrombotic process, they should be repeated after 3 months, *i.e.* outside the acute episode. In spite of the unequivocal relationship between thrombosis and anticardiolipin antibodies, the mechanism of procoagulant effect is far from clear and further studies are required.

All patients with multiple genetic disorders are prone to recurrent thrombosis. Recurrence is more common in patients with deficiency of AT-III, PC, PS, in those with more than one inherited factor of thrombophilia and in those who are homozygous for FV Leiden [1]. In the present study a double mutation in three subjects (FV Leiden, FII G20210A) and in one individual homozygous for FV Leiden was found. The recently published data pool [25] originating from 2,310 patients and 3,204 healthy subjects revealed an unadjusted OR of 20.0 (95% CI 11.1–36.1) of VTE for both FV Leiden and FII G20210A.

In conclusion, the overall body of evidence yielded by the present study prompted the authors to conclude that there was a positive association between venous thromboembolism and factor V Leiden, only a weak trend in favour of the relationship between prothrombin factor II G20210A and venous thromboembolism, and an absence of any association whatsoever between common polymorphisms of methylenetetrahydrofolate reductase (C677T, A1298C) and venous thromboembolism. It may be reasonably assumed that had the size of the study population been substantially larger, prothrombin factor II G20210A may well have reached statistical significance. In view of the results yielded by this study, the authors suggest that all young patients with venous thromboembolism and older patients with recurrent venous thromboembolism should be screened for factor V Leiden and prothrombin factor II G20210A mutations, however, screening venous thromboembolism patients for methylenetetrahydrofolate reductase polymorphisms appears to be futile.

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