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ORIGINAL ARTICLE

Impact of environmental and hereditary risk factors on the clinical manifestation of thrombophilia in homozygous carriers of factor V:G1691A

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Summary. *Background*: Limited data exist on the clinical manifestations of homozygous factor (F)V:G1691A mutation (FV Leiden) and the impact of environmental and genetic risk factors. *Objectives*: To assess the contribution of these factors on the thrombophilic phenotype. *Patients and methods*: In a retrospective multicenter cohort study 165 individuals with homozygous FV:G1691A mutation, of whom 129 had previous venous thromboembolism (VTE), were included. To study the role of environmental risk factors, patients were compared by the use of a standardized questionnaire to 165 sex- and age-matched individuals (reference group A); of these, two had previous VTE. To assess the role of genetic risk factors, factor (F)II:G20210A and MTHFR:C677T were determined in individuals homozygous for FV:G1691A and in 177 healthy individuals without previous VTE (reference group B). *Results*: The first VTE occurred significantly earlier in women (median age 25 years) than men (35.5 years). In 81% of women and 29% of men an environmental risk factor was present before first VTE. Oral contraceptives increased the risk of thrombosis 4-fold [odds ratio (OR) 4.0, 95% confidence interval (CI) 1.7, 10.4] in women with homozygous FV:G1691A. Postoperative and post-traumatic VTE as first manifestation occurred in 13% and 15% of surgical/traumatic events in patients and in 0.7% and 1.8% in reference group A, respectively (OR 19.7, 95% CI 2.5, 154 and OR 9.2, 95% CI 1.1, 79.4). Heterozygous FII:G20210A was more prevalent in symptomatic patients (11.7%) compared with reference group B (2.8%, OR 4.6, 95% CI 1.6, 13.2). The prevalence of homozygous MTHFR:C677T genotype was similar in patients and reference group B. *Conclusions*: Our study supports the concept of thrombophilia as a multifactorial disorder. The knowledge of coexisting factors predisposing to VTE is useful for medical advice for primary and secondary prophylaxis in these patients.

Keywords: factor V Leiden, homozygous factor V:G1691A, oral contraceptives, prothrombin G20210A variation, thrombophilia, venous thrombosis.

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Introduction

In the last decades genetic defects in proteins regulating blood coagulation have been established as risk factors predisposing to venous thromboembolism (VTE). The most important are antithrombin, protein C and protein S deficiency, resistance to activated protein C due to the factor (F)V G1691A gene mutation (FV:R506Q, FV Leiden) and the factor (F)II gene G20210A variant [1–5]. Homozygosity of the C to T substitution at nucleotide 677 within the methylene tetrahydrofolate reductase gene has been shown to be associated with mild to moderate hyperhomocysteinemia, which is recognized as a risk factor for vascular disease [6–17]. However, the contributing role of homozygous MTHFR:C677T to venous thrombosis remains unclear [8].

Among the risk factors mentioned, the FV:G1691A mutation has been identified as the most common with an average prevalence of 20% in unselected patients with first VTE, and approximately 3–7% in the general Caucasian population [1,2,9–12]. The prevalence of individuals homozygous for FV Leiden in the general population is around 1 in 2500 [13]. In a

previous study the prevalence of homozygous FV:G1691A carriers was 4.1% among 1200 consecutive patients with a history of an objectively confirmed juvenile VTE compared with 0% among 450 healthy controls [14]. Whereas the relative risk of VTE is moderately increased in heterozygous FV:G1691A carriers (3–8-fold), it is 30–140-fold increased in homozygous individuals [4,5,13,14]. Coinheritance of the FV:G1691A mutation with other genetic defects in the hemostatic system potentiates the thrombotic risk, as does the presence of acquired prothrombotic risk factors [15–17].

Although both the thrombotic tendency and importance of coexistent prothrombotic risk factors have been investigated in carriers of FV:G1691A, most studies included mainly heterozygotes. This prompted us to analyze the clinical feature in a large cohort of FV:G1691A homozygotes and to assess how the concomitant presence of genetic coagulation disorders or environmental risk factors contributes to the thrombophilic phenotype observed in these subjects.

Subjects and methods

Five European centers from Austria (Vienna), Germany (Frankfurt, Cologne, Kaiserslautern) and Hungary (Budapest) with laboratory and clinical experience in diagnosing and managing patients with thrombophilia participated in this study.

Patients

A total of 172 homozygous FV:G1691A outpatients were registered at the participating centers at the time of the study and 165 (102 women, 63 men) with a median age of 38.5 years (range 11–84 years) were enrolled (Table 1). Only three patients from Frankfurt and four from Vienna could not be enrolled because of unwillingness to participate. Some of the patients were also enrolled in the European Prospective Cohort on Thrombophilia Study (EPCOT). Information on the thromboembolic disease was obtained from patient documentation files and personal interview. Follow-up covered the life time of each study individual.

A questionnaire was completed for each patient during a visit to the participating center, which emphasized history and characteristics of thrombotic manifestations, including date of occurrence, site of every thrombotic episode, and the presence of precipitating factors (e.g. surgery, trauma, prolonged immobilization, pregnancy or puerperium, and oral contraceptive intake). Of 165 homozygous carriers, 129 (78 women, 51 men) had a history of at least one VTE prior to their referral to the thrombosis centers. The vast majority of the symptomatic patients was referred for thrombophilia screening. We used the term VTE for deep vein thrombosis (DVT) and pulmonary embolism (PE) and also for major thromboembolic events at unusual sites, but not for superficial thrombophlebitis (STP). Diagnosis of VTE was made by objective methods, including ultrasonography, duplex scanning, contrast venography, computed tomography (CT) or magnetic resonance imaging. Diagnosis of PE had been confirmed by the presence of ventilation-perfusion mismatch on a lung scan and/or CT and/or the presence of thrombus in the pulmonary vasculature confirmed by pulmonary angiography or other appropriate objective methods for thrombosis at unusual sites. Events that were not objectively confirmed were included, if the clinical feature was typical and had led to hospitalization and/or anticoagulant treatment. Thirty-six subjects (24 women, 12 men) were diagnosed during family studies ($n = 16$) or screening before prescription of oral contraceptives (OC, $n = 20$) and had no history of DVT or PE, while seven had a history of only STP. The diagnosis of STP was based on typical clinical symptoms and was confirmed by ultrasonography in some cases. None of the homozygous carriers had antiphospholipid antibody syndrome. The concomitant presence of a natural inhibitor deficiency was identified in six symptomatic patients (heterozygous type I deficiency of antithrombin, protein C, and protein S in two cases each).

Reference population

To study the effect of environmental factors on the clinical manifestation of thrombosis, a reference group (reference group A) consisting of 165 subjects (102 women, 63 men) from the normal population of the same geographic region and the same ethnic background as the patients, who were matched for sex and age (± 5 years) with the FV Leiden carriers (median age 39 years, range 12–86 years), was included. Individuals in this reference group had to be unrelated to the patients, and were either acquaintances of the patient, or from hospital staff or friends or relatives of hospital staff. They were evaluated in the same way as the patients by a questionnaire either by telephone or direct personal interview. There were no further exclusion criteria for recruitment of these reference subjects and

Table 1 Characteristics of patients with homozygous factor V:G1691A

	Women	Men	Total
Subjects, *n*	102	63	165
Median age, years (range)	34.5 (11–75)	48 (15–84)	38.5 (11–84)
Patients with VTE, *n* (%)	78 (76)	51 (81)	129 (78)
Median age, years (range)	35 (18–75)	48 (18–84)	39 (18–84)
Patients with recurrent VTE, *n* (%)*	29 (38)	26 (51)	55 (43)
Patients without VTE, *n* (%)†	24 (24)	12 (19)	36 (22)
Median age, years (range)	32.5 (11–65)	41.5 (15–78)	34 (11–78)

*Percent of patients with venous thromboembolism (VTE). †Seven patients had a history of superficial thrombophlebitis.

no laboratory investigations were performed in this group to exclude thrombophilic defects. Two individuals from this reference group (both women) had a history of VTE, one a DVT and the other a PE. A further 13 persons (10 women and three men) of this group had a history of STP.

To study the effect of OCs among women homozygous for FV:G1691A, we compared the prevalence of OC use at the time of thrombosis in the symptomatic women with FV:G1691A with the prevalence of OC use in the population reference group A, with the assumption that this prevalence would estimate fairly the prevalence in asymptomatic and undiagnosed FV:G1691A carriers. This approach was chosen because it was not possible to study OC use in a large asymptomatic group of FV:G1691A carrier women.

To study the impact of major surgical procedures and leg trauma as precipitating conditions for VTE, we assessed such risk factors in homozygous carriers, and the frequency of subsequent thrombosis. We compared the frequency and distribution of these risk factors in the patients and in reference group A and included all individuals from reference group A in this analysis. Only surgical procedures and leg injuries that had occurred before the first VTE were evaluated and therefore a comparable prophylactic anticoagulation scheme in patients and controls can be assumed. Only thrombotic events that occurred in close temporal connection to surgery or trauma (≤ 6 weeks) were regarded as triggered events.

To evaluate the prevalence of the FII:G20210A and the homozygous MTHFR:C677T variants in the normal population a second reference group of 177 individuals (median age 36 years, range 19–84, 57% women) from the same geographic region and the same ethnic background as the patients, who had no history of arterial or venous thromboembolic events, and who were previously diagnosed as carrying the wild-type FV:G1691A genotype, were investigated (reference group B). Here we used the same approach as in studying the additional risk of OCs among homozygous individuals, i.e. we used the prevalence of prothrombin and MTHFR variant among healthy controls as a proxy for the prevalence among homozygous patients without thrombosis. The odds ratio (OR) estimates the relative risk for these variants among individuals with homozygous FV:G1691A carriers.

Blood sampling and assays for gene analysis

For genetic analysis EDTA or citrated blood samples were collected by peripheral venepuncture. DNA was extracted by standard procedures. Determination of the FII:G20210A, FV:G1691A, and MTHFR:C677T genotypes by polymerase chain reaction was done according to published protocols [4,6,10].

Statistical analysis

Statistical analysis was performed using the Stat View 5.0 program (SAS Institute Inc., Cary, NY, USA). Statistical tests were based on contingency tables (χ^2 test and Fischer's exact test). In addition, relative risks of VTE were calculated based on the incidence of events in the various groups, with 95% confidence intervals (95% CI) based on the assumption of a Poisson distribution for the number of events. The method of Kaplan–Meier was used to show graphically the age at occurrence of VTE. For comparison of men and women the log rank test was applied.

Ethics

The present multicenter study was performed in accordance with the ethical standards laid down in the relevant version of the Declaration of Helsinki. All study subjects gave their informed consent to perform genetic analysis to test for mutations associated with an increased risk of thrombosis.

Results

Thromboembolic episodes

The main characteristics of the study group are listed in Table 1. Of the 165 homozygous FV:G1691A carriers, 129 (78.2%, 78/102 women and 51/63 men) had suffered at least one DVT or PE, 55 of these (29 women/26 men) had recurrent VTE (42.6%). Thirty-six homozygous carriers were either completely asymptomatic ($n = 29$) or had a history of STP only ($n = 7$).

In Table 2 the characteristics of the first VTE are listed. In most of the 129 symptomatic homozygous FV:G1691A carriers the first thrombotic event was DVT of the leg, associated with PE in 23%. Unusual manifestations of VTE were documented in five patients, while primary PE occurred in one case only. At the time of the first VTE episode, none of the 129 symptomatic FV:G1691A homozygotes had overt evidence of an underlying disorder known to be associated with increased risk of VTE, such as autoimmune, neoplastic or severe cardiac disease. Only 19.2% of women developed the first VTE without any known environmental risk factor, whereas this was the case in 73% of men ($P < 0.0001$). Forty-three women developed VTE during intake of OC, in four of these an additional triggering condition was present. In Fig. 1 the age at occurrence of VTE in women and men is shown by using the method of Kaplan–Meier. Women developed VTE significantly earlier compared with men (log rank test $P = 0.0001$).

Vascular events that occurred in the symptomatic patients during 5322 patient observation years are listed in Table 3. Isolated DVT was the most common event. PE was diagnosed in 46 patients: in seven patients PE occurred as an apparently isolated event, while in the remaining 39 patients PE was associated with lower extremity DVT. Superficial thrombophlebitis was reported 293 times in 64 patients, and thus was more frequent than any other vascular complication.

OC intake as triggering condition for the first VTE in women

To evaluate the influence of OC intake for risk of VTE in women homozygous for FV:G1691A, the frequency of OC

Table 2 Clinical characteristics of first venous thromboembolism (VTE) in 129 symptomatic patients with homozygous factor V:G1691A

	Women $n = 78$	Men $n = 51$	Total $n = 129$
Site of VTE			
DVT without symptomatic PE, *n* (%)*	53 (68)	41 (80)	94 (73)
DVT + PE, *n* (%)†	20 (26)	9 (18)	29 (23)
Isolated PE, *n* (%)	1 (1.3)	0	1 (0.8)
Unusual site, *n* (%)‡	4 (5.1)	1 (2)	5 (3.9)
Age at first manifestation			
Age (years), median (range)	25 (0.1–65)§	35.5 (14–82)§	29 (0.1–82)
Prothrombotic conditions associated with first VTE episode¶			
None, *n* (%)	15 (19.2)	37 (73)	51 (39.5)
Major surgery, *n* (%)	5 (6.4)	8 (15.7)	14 (10.9)
Trauma, *n* (%)	4 (5.1)	5 (9.8)	9 (7.0)
Immobilization, *n* (%)	2 (2.6)	3 (5.9)	5 (3.9)
Pregnancy, *n* (%)	8 (10.3)		
Puerperium, *n* (%)	6 (7.7)		
Intake of oral contraceptives, *n* (%)	42 (54)		

DVT, Deep vein thrombosis; PE, pulmonary embolism. *One woman extending into the caval vein. †Two men extending into the caval vein. ‡One man with retinal, one woman with cerebral, renal, jugular and arm vein thrombosis, respectively. §$P < 0.0001$ difference women vs. men. ¶Two men and four women had more than one single risk factor.

intake in women with FV:G1691A at the time of thrombosis was compared with the frequency of OC intake in control women of reference group A of the same age. Fifty women with homozygous FV:G1691A fulfilled the criteria of having had a thrombosis and having a matched control woman in whom OC intake was exactly known. Of the 50 women with homozygous FV:G1691A and thrombosis 38 took OC (76%), whereas 12 (24%) did not. Of the 50 control women 22 (44%) had OC, whereas 28 (56%) had not. This indicates that among women homozygous for FV:G1691A, OCs increase the risk of thrombosis 4-fold (OR 4.0, 95% CI 1.6, 10.4).

Surgical procedures and leg injury as triggering events for the first VTE (Table 4)

Thirteen percent of operations (12/95) in patients were complicated by a first symptomatic VTE, whereas this was the case in only 0.7% (1/136) of persons of reference group A (OR 19.7, 95% CI 2.5, 154). Abdominal surgery was followed by VTE in only 7.3% of procedures, whereas orthopedic and urological procedures had the highest risk of VTE (20% or higher).

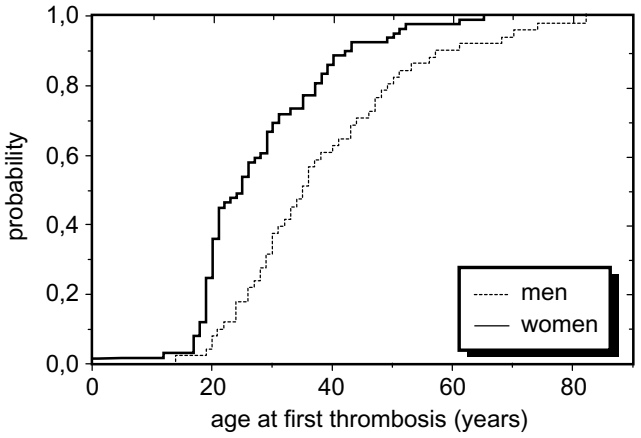

Fig. 1. Probability of development of thrombosis in symptomatic women ($n = 78$) and men ($n = 51$), log rank test, $P = 0.0001$.

Table 3 Vascular events in 129 symptomatic patients with homozygous factor V:G1691A during 5322 patient years

	Vascular events $n = 531$	Patients* $n = 129$
Venous thromboembolic episodes		
Deep leg vein thrombosis†	165	101
Deep leg vein thrombosis + PE‡	44	39
Isolated PE	10	7
Deep arm vein thrombosis	9	8
Retinal vein thrombosis	5	3
Renal vein thrombosis	1	1
Cerebral vein thrombosis	3	3
Isolated caval vein thrombosis	1	1
Superficial thrombophlebitis, *n*	293	64

PE, Pulmonary embolism. *Some of the patients had events at different sites. †One female extending into the caval vein. ‡Four patients extending into the caval vein.

Table 4 Thrombotic complications (deep vein thrombosis and/or pulmonary embolism) in association with major surgical procedures and trauma of the leg in homozygous FV:G1691A carriers

	Number of procedures/venous thromboembolism episodes (%) $n = 165$
Type of surgery	
Abdominal	55/4 (7.3)
Gynecological	10/1 (10)
Orthopedic	17/4 (24)
Urological	7/3 (43)
Thoracic	1/0
Various	5/0
All surgical procedures	95/12 (13)
Trauma of the leg	40/6 (15)

Table 5 Coinheritance of factor (F)II:G20210A and MTHFR:C677T in symptomatic homozygous factor (F)V:G1691A carriers

	Homozygous FV:G1691A	Ref. group B	OR (95% CI)
Subjects, *n*	111	177	
Median age, years (range)	40 (18–84)	36 (19–84)	
FII:G20210A	13 (11.7%)	5 (2.8%)	4.6 (1.6, 13.2)
Homozygous MTHFR:C677T	19 (17.1%)	25 (14.1%)	1.3 (0.7, 2.4)

None of the subjects had homozygous MTHFR:C677T combined with FII:G20210A, none was homozygous for the FII:G20210A variation.

Leg trauma (fractures or soft-tissue trauma leading to immobilization) were followed by a first VTE in 15% (6/40) of patients and in 1.8% (1/55) of reference group A (OR 9.2, 95% CI 1.2, 79.4).

Coinheritance of FII:G20210A and MTHFR:C677T genotypes

To asses the role of FII:G20210A and MTHFR:C677T as additional risk factors predisposing to thrombosis in FV:G1691A homozygotes, 111 symptomatic homozygous FV:G1691A carriers were investigated for these mutations. To focus on FII:G20210A and MTHFR:C677T as potential factors contributing to the thrombophilic phenotype in homozygous FV:G1691A carriers, patients with coexisting deficiencies of antithrombin, protein C, and protein S ($n = 6$) were excluded from this analysis. Data on the prevalence of the FII:G20210A and MTHFR:C677T mutations are shown in Table 5.

Among the 111 homozygous FV:G1691A carriers a coexistence with the FII:G20210A was detected in 8.9% of the patients compared with 2.8% in reference group B. This difference was highly significant. No increase in the frequency of the homozygous MTHFR:C677T genotype was observed in symptomatic homozygous FV:G1691A carriers compared with reference group B.

Discussion

Only limited data on the clinical features of thrombophilia and the contribution of circumstantial and/or genetic risk factors are available for homozygotes [17–22]. Therefore, we present the results of a multicenter study on the thrombophilic phenotype and the concurrent effects of additional prothrombotic risk factors in a large cohort of homozygous FV:G1691A carriers.

In women with the homozygous FV:G1691A mutation the first VTE occurred approximately 10 years earlier compared with men. This difference is most probably due to the increased risk of thrombosis in homozygous women associated with OC [23] and pregnancy [24]. A higher prevalence of environmental prothrombotic risk factors at first thrombotic manifestation was present in women compared with men. OC and pregnancy were the most frequent additional risk factors in women. For thrombosis during OC use an OR of 4.0 was found. This is similar to the VTE risk of OC known from case–control studies [25] and this increased risk is superimposed on the increased basic risk of VTE in homozygous FV Leiden patients. These data confirm the previous assumption that subjects with homozygous FV:G1691A are particularly predisposed to VTE while using OC [21–23]. Due to this high risk, OCs containing ethinylestradiol should not be prescribed to women known to be homozygous for FV:G1691A mutation.

DVT without and with PE was the most prevalent major thrombotic manifestation. STP was more frequently reported than any other event. STP is a less severe manifestation within the phenotypic spectrum of venous thrombophilia, and is therefore often disregarded by physicians. In patients with idiopathic STP Martinelli *et al*. reported an OR of 6.1 for the FV:G1691A mutation, 4.3 for the FII:G20210A genotype and 12.9 for deficiencies of a natural coagulation inhibitor [26]. The early recognition and treatment of STP is important, because progression of thrombosis into the deep venous system may occur in a high proportion of cases [27–29].

To investigate further the interactions between genetic and environmental factors we evaluated the prevalence of a first VTE after surgery and trauma in patients with homozygous FV:G1691A and controls from reference group A. The overall prevalence was considerably higher in patients than in controls. Furthermore, 15% of leg injuries documented in FV:G1691A carriers were followed by VTE episodes, which was almost 10-fold higher than in the control group. Thus, both major surgery and leg trauma were associated with a significantly increased risk of VTE in homozygous FV:G1691A patients. In patients with known homozygous FV:G1691A, antithrombotic prophylaxis should be recommended at the time of both conditions. However, due to the low prevalence of the homozygous FV:G1691A genotype in the normal population, general preoperative screening seems not to be reasonable.

As one of the most marked findings of our study we observed that the prevalence of the FII:G20210A variation was higher among homozygous FV:G1691A carriers with a history of VTE compared with the group of healthy individuals. This indicates that the additional presence of FII:G20210A increases the penetrance of thrombotic disease in patients carrying FV:G1691A. The findings confirm and extend those reported in several previous reports, which also described an enhanced risk of VTE in heterozygotes for FV:G1691A when associated with FII:G20210A [17,30–33]. The data from our study and the literature support the importance of the FII:G20210A variant as a common manifesting risk factor for VT in carriers of the FV:G1691A mutant.

Between 5 and 20% of unselected Caucasians are homozygous for a C → T substitution at nucleotide 677 within the MTHFR gene [34,35]. In the present study, a similar prevalence

of the homozygous MTHFR:C677T genotype in the patients who had suffered VTE and in healthy controls was found. We therefore conclude that homozygosity of the MTHFR:C677T allele itself does not contribute to the development of VTE in FV:G1691A homozygotes, a conclusion similar to those of several studies of mainly FV:G1691A heterozygotes [35–37], and that routine investigation for this mutation is not justified. These results are in contradiction to some other studies, in which homozygosity for MTHFR:C677T was identified as a significant risk factor for VT when coinherited with the heterozygous FV:G1691A mutation [34,38]. The contradictory observations may reflect different influences of various other predisposing factors.

Since data were collected retrospectively and were mainly based on information given by the patient, a recall bias cannot be excluded. This may have affected both the rate of reporting as its accuracy. Moreover, fatal cases were not included. Since life expectancy does not seem much reduced by thrombophilia [39], this will not have introduced major bias. Obviously, the proportion of symptomatic individuals in this cohort does not represent the true distribution, since individuals were mostly selected because of thrombosis. This implies that the risks and age-at-onset presented here are likely to be overestimates. Therefore, we have not presented direct risk estimates for FV Leiden carriership, either as absolute rates or in a comparison with population controls. However, with regard to collection of data on peri- and postoperative thrombosis, as well as OC use, the evaluation of patients and controls was similar, and data pertaining to this period preceded the diagnosis or the thrombotic event. Therefore, it seems reasonable to expect only limited bias in the evaluation of these factors. Similarly, the preferential inclusion of thrombosis patients does not affect the analysis of effect modification by other genetic factors (FII:G20210A, MTHFR:C677T), since their prevalence in asymptomatic carriers can be estimated via the population controls.

In conclusion, our study of a large cohort of homozygous FV:G1691A carriers supports the concept of thrombophilia as a multifactorial disorder. In the majority of symptomatic patients an additional genetic or environmental risk factor was present at first thrombotic onset. The identification of homozygous individuals may contribute to the prevention of VTE episodes. By investigation of siblings of homozygous patients individuals at high risk of thrombosis can be identified. The knowledge of coexisting factors predisposing to VTE is useful for medical advice on primary and secondary prophylaxis in individuals homozygous for the FV:G1691A mutation.

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