Haplotypes of the *EPCR* gene, prothrombin levels, and the risk of venous thrombosis in carriers of the prothrombin G20210A mutation

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ABSTRACT

Background

Haplotypes A1 and A3 in the endothelial protein C receptor (*EPCR*) gene are tagged by 4678G/C and 4600A/G respectively. We assessed whether these haplotypes modify the risk of venous thromboembolism in carriers of the prothrombin 20210A allele.

Design and Methods

We genotyped 4678G/C and 4600A/G in 246 20210A carriers: 84 venous thromboembolism propositi and 162 relatives (13 symptomatic), and in 140 relatives not carrying the 20210A variant. Prothrombin and soluble EPCR (sEPCR) levels were also measured.

Results

Among propositi, the mean age at first onset was lower in carriers (35±8 years) than non-carriers of the 4600G allele (44±14 years) (p=0.004). The probability of being free of thrombosis at age 40 was lower in 20210A carriers with the *EPCR* 4600G allele (p=0.015). The frequency of the 4600G allele (p=0.002) and the levels of prothrombin antigen (p=0.002) and sEPCR (p<0.001) were higher in the propositi than in their asymptomatic relatives. Multivariate analyses showed that the presence of the 4600G allele (OR=2.5, 95% confidence interval 1.3-5.0), sEPCR >147 ng/mL (2.8, 1.5-5.2) and prothrombin >129% (3.8, 1.8-8.3) all increased the thrombotic risk. In bivariate analysis, including the 4600G allele and sEPCR>147 ng/mL, only the latter remained associated with risk.

Conclusions

These results show that in 20210A carriers the venous thromboembolism risk is influenced both by the actual prothrombin levels and by the *EPCR* A3 haplotype, via its effect on sEPCR levels.

Key words: endothelial protein C receptor, prothrombin G20210A mutation, venous thromboembolism, prothrombin level, activated protein C.

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Introduction

The prothrombin G20210A mutation is associated with increased plasma prothrombin levels and risk of venous thromboembolism (VTE).[1-3] The prevalence of the 20210A allele in the Caucasian population is about 2% but this is higher in Southern Europe.[4] Carriers of the mutant allele present with higher prothrombin levels in plasma, probably due to a change in polyadenylation efficiency,[5] and it has been suggested that this increase is responsible for the higher risk of thrombosis in carriers of the 20210A variant.[1] Carriership of the 20210A allele is associated with a slight increase in the risk of recurrent VTE,[6] and the presence of other VTE risk factors increases the risk of VTE in 20210A carriers.[7-11]

Polymorphisms in the anticoagulant pathways may also modify the VTE risk in 20210A carriers. The endothelial protein C receptor (EPCR) enhances the rate of protein C activation, thus contributing to the regulation of thrombin formation by the protein C anticoagulant pathway.[12] Four *EPCR* haplotypes have been reported. Haplotype 3 (A3), tagged by the rare allele of 4600A/G, is associated with increased plasma levels of soluble EPCR (sEPCR) which might be due to increased sensitivity to ADAM17.[13] However, its association with risk of VTE is controversial.[14-16] sEPCR retains its ability to bind both protein C and activated protein C (APC), and blocks APC anticoagulant activity.[17,18] Haplotype 1 (A1), tagged by the rare allele of 4678G/C, was reported to be associated with increased levels of APC and a reduced risk of VTE.[15,19] Finally, haplotype 4 (A4), tagged by the rare allele of 3811 G/A, was reported to be associated with a slight increase in the risk of VTE.[16]

The aim of this study was to investigate whether the A1 and A3 haplotypes of *EPCR* modify the risk of VTE in carriers of the 20210A variant. In addition, we measured the effect of prothrombin and sEPCR levels on VTE risk, since these parameters have been associated with the 20210A and 4600G alleles respectively.

Design and Methods

Study population

Our institute became a reference hospital for thrombophilia screening in 2004. Unrelated carriers of the 20210A allele with at least one episode of objectively diagnosed VTE were identified from the records of all patients referred to our institute for thrombophilia examination over an 11-year period (from 1996 to 2006). Patients with VTE were considered for thrombophilia screening if the thrombotic event occurred when they were under 50 years of age, when they presented with recurrent thrombosis, when the event occurred without any circumstantial risk factor (idiopathic), when the thrombosis had an unusual location, or when they had a positive family history. Of the 125 Caucasian patients identified, 7 died before the study, 13 were not localized, 12 did not agree to participate in the study, and in 9 family studies were not possible. Therefore, 84 propositi participated in the study (47 women and 37 men). The mean age of the first thrombotic event was 40 years (women, 37 years; men, 44 years). Idiopathic VTE was defined as VTE without known precipitating risk factors (use of oral contraceptives, surgery, pregnancy or delivery, trauma, major immobilization).

Altogether, the 84 propositi had 405 first-degree relatives who were still alive, and 302 of these participated in the study. Relatives under 16 years of age were not included. A total of 162 family members were identified as carriers of the 20210A variant (93 women and 69 men). Of them, 13 had experienced a venous thrombotic event (6 women and 7 men). Among the remaining 140 relatives (64 women, mean age 42 years; 76 men, mean age 44 years) who did not carry the 20210A variant, 2 had experienced a thrombotic event (one was protein C deficient and the other was a carrier of factor V Leiden).

All subjects gave their informed consent. The study was approved by the institutional Ethics Committee.

Blood collection

Blood samples were collected at least six months after the acute event. Atraumatic venipunctures were performed after 12 h fasting, into 0.1 vol of 0.129 M trisodium citrate and centrifuged at 1,500 g for 30 min. at 4°C. Plasma was snap frozen in small aliquots, stored at –80°C and used within six months. For APC determination, blood was drawn into two tubes containing 0.5 mL of 0.129 M trisodium citrate. Immediately (within 10 sec), 46mL of 1,000 U/mL heparin was added to one tube and the mixture was incubated at 37°C for 30 min to force all circulating APC to form complexes with its major plasma inhibitor, protein C inhibitor (PCI). The second blood tube was immediately mixed (within 10 sec) with 46 mL of a mixture of 0.58 M benzamidine. HCl and 0.5 mM PPACK to prevent formation of complexes of APC with its inhibitors.

Activated protein C assay

Levels of circulating APC were measured as previously reported[20] with slight modifications.[15]

Prothrombin assays

Prothrombin antigen levels were determined by a specific enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 0.01 mg/mL sheep anti-human prothrombin polyclonal antibody (Enzyme Research Laboratories, South Bend, IN, USA). Samples were diluted 1:10,000 and 1:20,000, added to the wells and incubated for 1 h at room temperature. Calibration curves were constructed with serial dilutions of normal plasma pooled from 40 healthy subjects. Plates were washed with phosphate buffered saline buffer containing 0.05% Tween 20 (Sigma Chem. Co., St. Luis, MO, USA) pH 7.4, and sheep anti-human prothrombin polyclonal antibody conjugated with horseradish peroxidase (HRP) (Enzyme Research Laboratories) diluted 1/300 was added. After 1 h at room temperature, plates were washed and HRP activity was detected with OPD substrate. Coefficients of variation were 4.8 to 6.5%

for intra-series and 6.7 to 8.9% for inter-series determinations. Values are given as percentage of the prothrombin level in the pooled normal plasma (100%).

Prothrombin time was determined with an automated method using a very high sensitivity calcium thromboplastin (Instrumentation Laboratory Company, Lexington, MA, USA). Prothrombin activity was measured with an automated, modified prothrombin time test that included human plasma immunodepleted of factor II (Instrumentation Laboratory Company).

sEPCR assay

Levels of sEPCR were measured by ELISA (Asserachrom sEPCR, Diagnostica Stago, Asnieres, France).

Genetic analysis

Prothrombin gene G20210A variant[1] and FV Leiden mutation[21] were detected by polymerase chain reaction amplification and restriction analysis as reported. The 4600A/G (rs 867186) and the 4678G/C (rs 9574) polymorphisms were assayed as previously described.[15,19]

Statistical analysis

Statistical analysis was performed using the SPSS for Windows release 11.5 statistical software (SPSS Inc., Chicago). Continuous variables are reported as means and standard deviations or as medians and 10th-90th percentile. Allele frequencies were calculated by gene counting. The χ^2 test was used to compare percentages. Parameter levels were compared between genotypes using the Kruskal-Wallis test. The Kaplan-Meier method was used to construct thrombosis-free survival curves. Time to first thrombosis was used as the failure event. Thrombosis-free survival times were compared for 4600A/G and 4678G/C genotypes using the log-rank test. Differences in the mean age at onset were analyzed with the One-Way ANOVA test. Logistical regression analysis was used to identify associations between genotypes and VTE. For plasma levels of prothrombin, we used a cut-off of 129% (the 90th percentile of the distribution amongst asymptomatic 20210A carriers) and for sEPCR we used a cut-off of 147 ng/mL (the 80th percentile of the distribution amongst asymptomatic 20210A carriers). For prothrombin and sEPCR levels we also performed analyses by quartiles, according to the distribution among asymptomatic 20210A carriers. For sEPCR levels in carriers of the 4600G allele, the analysis by quartiles was performed according to the distribution among asymptomatic 20210A carriers with the 4600G allele. In all these analyses, the group with the lowest level (i.e. ≤90th percentile, ≤80th percentile, ≤25th percentile) served as the reference category to which risk was expressed. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from the logistical model. A two-tailed p value <0.05 was considered statistically significant.

Results

Main characteristics of the study subjects are shown in Table 1. There were no significant differences in any of the parameters studied between symptomatic and asymptomatic relatives carrying the 20210A allele. Three female propositi (20, 32 and 33 years of age) and one asymptomatic female relative (28 years of age) were homozygous 20210AA carriers.

EPCR polymorphisms and VTE risk

All subjects were successfully genotyped for the *EPCR* 4600A/G and 4678G/C polymorphisms. Among the 84 propositi, the frequency of the 4600G allele (A3 haplotype) was 0.167 (Table 2), which is clearly higher than that observed in previous studies of unselected VTE patients (0.094),[15] VTE patients with FV Leiden[19] (0.100) and healthy subjects (0.092).[15] The frequency of the 4678C allele (A1 haplotype) was 0.369, slightly lower than that observed in unselected VTE patients[15] (0.409) and VTE patients with FV Leiden[19] (0.395), and significantly lower than the 0.470 observed in healthy subjects.[15]

The frequency of the 4600G allele was higher in the 84 propositi (0.167) than in their 149 asymptomatic relatives (0.070) (p<0.001), whereas that of the 4678C allele tended to be lower in the propositi (0.369) than in their asymptomatic relatives (0.456) (p=0.078) (Table 2). In addition, the frequency of the 4600G allele was higher in the 13 symptomatic than in the 149 asymptomatic relatives with the 20210A allele (OR 3.0, 1.0-8.9).

We performed logistical regression analyses (Table 2) to identify associations between genotypes and VTE among the propositi and their asymptomatic 20210A carrier relatives. In univariate analysis, the presence of the 4600G allele significantly increased the risk of VTE (OR=2.6, 1.4-4.8). In multivariate analysis, adjustment for sex, age, the presence of antiphospholipid antibodies, the presence of factor V Leiden and the presence of the other *EPCR* polymorphis did not significantly modify the OR (2.5, 1.3-5.0). With respect to the 4678G/C polymorphism, a decrease in the OR (protective effect) was observed when the number of 4678C alleles increased (Table 2), although it did not reach statistical significance (Adjusted OR for CC *vs.* GG genotype=0.6; 0.2-1.3).

Among propositi, idiopathic VTE events, i.e. venous events without known precipitating risk factors, occurred more frequently in 4600G allele carriers (73%) than in non-carriers (57%), although the difference was not statistically significant (p=0.224), and the mean age at the first VTE episode was lower in carriers of the 4600G allele than in non-carriers (p=0.001) (Table 3). Kaplan-Meyer analysis showed that the probability of being free of thrombosis at 40 years of age was lower in the 26 carriers (21%) than in the 58 non-carriers (53%) of the 4600G allele (p=0.010). By contrast, the age at which 50% of the propositi with the 4678GG or CC genotype had experienced VTE was similar (p=0.329), as was the probability of being free of thrombosis at 40 years of age (p=0.217). When the analysis was restricted to relatives carrying the 20210A allelle (13 symptomatic and 149 asymptomatic), the mean age at the first VTE episode was 28±10 years for 4600G allele carriers and 43±10 years for non-carriers (p=0.019), and the probability of being free of thrombo-

sis at 40 years of age was lower in the 26 carriers of the 4600G allele (20%) than in the 136 non-carriers (60%) (p=0.038).

Among the 140 relatives not carrying the 20210A variant, the frequency of the 4600G allele was 0.093: 115 individuals carried the 4600AA genotype, 24 the AG and 1 the GG genotype. The frequency of the 4678C allele was 0.471: 39 individuals carried the 4678GG genotype, 70 the GC and 31 the CC genotype.

sEPCR levels and VTE risk

We compared the levels of sEPCR in the 84 propositi and in their 149 asymptomatic relatives studied to investigate whether sEPCR levels were associated with the risk of VTE. After excluding the 16 propositi under coumarin therapy, sEPCR was significantly higher in the 68 propositi (134, 87-417 ng/mL; median and 10th-90th percentile) than in the 149 asymptomatic (110, 74-258 ng/mL) 20210A carriers from the families studied (p<0.001). As the sEPCR plasma level is strongly influenced by the presence of the *EPCR* 4600G allele, we also calculated these levels separately for carriers and non-carriers of the 4600G allele. Propositi non-carriers of the 4600G allele showed higher sEPCR levels (126, 87-157 ng/mL) than asymptomatic relatives (105, 72-140 ng/mL) (p<0.001). Symptomatic carriers of the 4600G allele also tended to have higher sEPCR levels (344, 180-610 ng/mL) than the asymptomatic ones (287, 174-411 ng/mL) (p=0.124).

Using a cut-off of 147 ng/mL for sEPCR (the 80th percentile of sEPCR in the asymptomatic 20210A carriers), out of the 68 propositi without coumarin therapy, 27 (40%) exceeded this cut-off compared with 29 (19%) in the 149 asymptomatic relatives (OR adjusted for sex, age, the presence of factor V Leiden and the presence of antiphospholipid antibodies 2.8, 1.5-5.2). A dose-response relationship was observed between sEPCR levels and the risk of VTE when the subjects were stratified in quartiles according to the sEPCR levels in asymptomatic subjects. Taking the first quartile as the reference group, the ORs for the 2nd, 3rd and 4th quartile were 1.6 (0.4-5.1), 3.2 (1.3-9.6) and 4.6 (1.6-10.5) respectively, after adjusting for sex, age, the presence of factor V Leiden, the presence of antiphospholipid antibodies and the two *EPCR* polymorphisms (p for trend <0.001).

Since high sEPCR levels are strongly associated with the *EPCR* 4600G allele, we performed a bivariate analysis entering in the model both sEPCR >147 ng/mL and the presence of the 4600G allele. The OR for the presence of the 4600G allele decreased from 2.8 (1.4-5.5) to 1.5 (0.5-4.3), whereas the OR for sEPCR did not significantly decrease (from 2.8, 1.5-5.2 to 2.3, 1.1-5.4). These results suggest that the increased VTE risk observed in carriers of the 20210A variant with the 4600G allele (A3 haplotype) is due to the higher sEPCR levels. To further study whether the effect of the 4600G allele is due to its effect on sEPCR levels, we calculated the adjusted ORs of subjects carrying the 4600G allele according to quartiles of sEPCR. Compared with the first quartile, the ORs for the 2nd, 3rd and 4th quartile were 1.8 (0.2-15.4), 3.0 (0.4-22.7) and 6.0 (0.9-41.2) respectively.

Table 1. Clinical characteristics of prothrombin G20210A carriers.

Characteristics	*Propositi (n=84)*	*Symptomatic relatives (n=13)*	*Asymptomatic relatives (n=149)*
Age at sampling, yrs.	44±14 (20-74)	48±13 (31-78)	42±16 (16-84)
Age at onset, yrs.	40±13 (18-70)	37±12 (20-57)	N.A.
Male, n (%)	37 (44)	7 (54)	62 (42)
20210AA carriers	3 (4)	0 (0)	1 (1)
Factor V Leiden, n (%)	6 (7)	2 (15)	5 (3)
Protein C deficiency, n (%)	2(2)	1 (8)	2 (1)

Age is given as mean±SD (range); N.A., not applicable. In all cases, the statistical difference between groups was p>0.15.

Table 2. Influence of the 4600A/G and 4678G/C polymorphisms in EPCR on the thrombosis risk in the 233 prothrombin G20210A mutation carriers from the 84 families studied.

Polymorphism	*Propositi (n=84)*		*Asymptomatic relatives (n=149)*		*OR (95% CI)*
	n	*Frequency*	*n*	*Frequency*	
4600A/G					
A allele	140	0.833	277	0.930	1*
G allele	28	0.167	21	0.070	2.6 (1.4-4.8)[§] 2.5 (1.3-5.0)[†]
AA genotype	58	0.690	128	0.859	1*
AG genotype	24	0.286	21	0.141	2.7 (1.4-5.2)[§&] 2.6 (1.3-4.8)[†&]
GG genotype	2	0.024	0	0.000	-
4678G/C					
G allele	106	0.631	162	0.544	1*
C allele	62	0.369	136	0.456	0.7 (0.5-1.0)[§] 0.7 (0.6-1.1)†
GG genotype	33	0.393	44	0.295	1*
GC genotype	40	0.476	74	0.497	0.7 (0.4-1.3)[§] 0.8 (0.5-1.4)[†]
CC genotype	11	0.131	31	0.208	0.5 (0.2-1.1)[§] 0.6 (0.2-1.3)[†]

**Reference group; [§]Univariate analysis. [†]Multivariate analysis for sex, age, the presence of antiphospholipid antibodies, the presence of factor V Leiden and the 4600A/G or 4678G/C polymorphism. [&]Includes the 2 symptomatic carriers with the GG genotype.*

Table 3. Age (mean±SD) and risk factors (challenge) at onset of thrombosis in the 84 propositi according to the EPCR 4600A/G polymorphism.

	Genotype		*Statistical significance (p)*
	AA (n=58)	*AG+GG (n=26)*	
Age, years	42±14	33±9	0.001
Idiopathic	33 (57)	19 (73)	0.224
Oral contraceptives	4 (7)	1 (4)	0.889
Surgery	10 (17)	2 (8)	0.326
Pregnancy or delivery	4 (6)	2 (8)	0.968
Trauma	7 (11)	2 (8)	0.714
Immobilization	1 (1)	0 (0)	–

Data are expressed as n (%), except for age (mean±SD).

Prothrombin levels and risk of VTE

Given that at least one of the mechanisms by which carriers of the 20210A variant have an increased risk of VTE is the presence of increased plasma levels of prothrombin, we also determined the plasma levels of prothrombin antigen and activity in all 20210A carriers of the 84 families studied, excluding those subjects under coumarin therapy. The mean prothrombin antigen and activity levels were higher in the 68 propositi without coumarin therapy (121±10% and 120±9%) than in their asymptomatic relatives (117±10% and 116±10%) (p=0.002 and <0.001 respectively), whereas the prothrombin time was lower in the propositi (11.0±0.5 sec) than in their asymptomatic relatives (11.3±0.6 sec) (p=0.012). Using a cut-off of 129% (the 90th percentile of the prothrombin antigen levels in the asymptomatic 20210A carriers), out of the 68 propositi 20210A carriers not taking coumarin, 19 (28%) exceeded this cut-off compared with 14 (9%) in the asymptomatic 20210A carriers (n=149) (adjusted OR 3.8, 95% CI 1.8-8.3). A dose-response relationship was observed between prothrombin antigen levels and the risk of VTE when the subjects were stratified in quartiles according to the prothrombin levels. Compared with the first quartile, the ORs for the 2nd, 3rd, and 4th quartile were 1.5 (0.5-4.3), 2.1 (0.8-6.0 and 3.4 (1.4-8.1) respectively, after adjusting for sex, age, the presence of factor V Leiden, the presence of antiphospholipid antibodies and the two EPCR polymorphisms (p for trend=0.003). Similar results were found for prothrombin activity levels.

Activated protein C

After excluding those subjects under coumarin therapy, APC levels were lower in the 68 propositi (1.12±0.24 ng/mL) than in their 149 asymptomatic relatives (1.19±0.25 ng/mL) (p=0.046), whereas protein C antigen was similar in both groups (106±25% and 104±19% respectively, p=0.519) (Table 4). APC levels were also lower in all (n=42) 20210A carriers with the 4600G allele (1.05±0.24 ng/mL) than in the 175 with the 4600A allele (1.19±0.25 ng/mL) (p=0.003).

Discussion

In this study, we found that the 4600G allele (A3 haplotype) of *EPCR* was more frequently present in unselected unrelated symptomatic 20210A carriers (16.7%) than previously observed in symptomatic (10.0%) and asymptomatic (10.2%) carriers of the factor V Leiden mutation,[19] in healthy subjects (9.2%) and in unselected patients with VTE (9.4%).[15] A study of the families of these symptomatic 20210A carriers revealed that the 4600G allele (A3 haplotype) of *EPCR* was associated with an increased risk of VTE in carriers of the 20210A allele. Furthermore, carriers of the 4600G allele experienced the first VTE episode at a younger age and were free of thrombosis at 40 years of age in a lower proportion than those carrying the 4600A allele. By contrast, the 4678C allele (A1 haplotype) did not modify the VTE risk in carriers of the 20210A allele. However the OR tended to decrease when the number of 4678C alleles increased, suggesting a protective effect similar to that observed in factor V Leiden carriers (Table 2).[19] The lack of significance may be due to the low number of subjects carrying the 4678CC genotype.

The mechanism by which A3 haplotype increases the risk of VTE in carriers of the 20210A allele is not clear. There is no consensus as to whether the presence of the A3 haplotype *per se* increases the risk of VTE. Three reports indicated that the A3 haplotype is not associated with an increased VTE risk[15,16] and had no effect on the risk of VTE in factor V Leiden carriers,[19] whereas another report showed that the A3 haplotype was associated with a 2.5-fold increased risk of VTE in men but not in women.[14] The A3 haplotype has been associated with increased levels of plasma sEPCR.[14-16] Given that sEPCR binds both protein C and APC, it has been hypothesized that elevated sEPCR levels may result in decreased APC generation and in APC inhibition,[14] inducing decreased plasma APC levels and increased risk of VTE.[22] Our finding that the presence of the 4600G allele is associated with higher sEPCR levels and lower APC levels, and that increased sEPCR levels are associated with VTE in carriers of the 20210A allele, independently of the 4600A/G polymorphism, would support this hypothesis. So far, the effect of the 4600G allele on VTE risk is most convincingly present in prothrombin 20210A carriers, which might indicate a modest prothrombotic effect of the A3 haplotype (via increased sEPCR levels) that is only evidenced in the presence of another risk factor. The question then is why the prothrombotic effect of the A3 haplotype is seen in 20210A carriers (this study) and not in Factor V Leiden carriers.[19] More information is needed on the mechanisms underlying the prothrombotic effect(s) of these genetic risk factors before this can be answered. However, as both sEPCR and prothrombin are APC inhibitors,[23] it could be hypothesized that the *EPCR* A3 haplotype specifically synergizes with the prothrombin mutation. Alternatively, it is possible that the families under study are enriched in prothrombotic risk factors (the prothrombin mutation alone being a mild risk factor) and that this background favours the detection of the EPCR polymorphism as a contributing risk factor.

The protective effect of the A1 haplotype in carriers of the prothrombin 20210A mutation observed in the present study was less effective than that seen in subjects carrying factor V Leiden.[19] The presence of this haplotype is associated with moderately higher circulating APC levels, which may counteract the APC-resistance associated with the presence of factor V Leiden.

The mechanism by which prothrombin 20210A increases VTE risk via elevated prothrombin levels is not fully understood. There is some evidence that prothrombin levels affect the APC-resistance phenotype, but the precise mechanism is not known. As to the EPCR 4600G allele, this allele has been associated *in vivo* and *in vitro* with increased sEPCR levels,[14-16] which might be due in part to increased sensitivity to ADAM17.[13] However, a recent report also attributes this to increased production of a soluble alternative splice product of *EPCR*.[24] Both soluble forms of EPCR

Table 4. Plasma levels of prothrombin and other hemostatic parameters in the 217 prothrombin G20210A mutation carriers of the 68 families studied without coumarin therapy.

Parameter	*Propositi (n=68)*	*Asymptomatic relatives (n=149)*	*Statistical significance (p)*
Prothrombin ag, (%)	121±10	117±10	0.002
Prothrombin act, (%)	120±9	116±10	<0.001
Prothrombin time, secs.	11.0±0.5	11.3±0.6	0.012
Activated protein C, ng/mL	1.12±0.24	1.19±0.25	0.046
Protein C, (%)	106±25	104±19	0.519
sEPCR, ng/mL			
All individuals	134 (87-417)	110 (74-258)	<0.001
4600 AA carriers	126 (87-157)	105 (72-140)	<0.001
4600 AG+GG carriers	344 (180-610)	287 (174-411)	0.124

Values are expressed as mean±SD or as median (10^{th}-90^{th} percentile).

have procoagulant effects by binding APC and protein C. The overall effect of the 4600G allele on the concentration of EPCR in the endothelial membrane is still unknown.

Our results confirm and extend previous data showing that the presence of the 20210A variant is associated with increased VTE risk because it is associated with increased plasma prothrombin levels[1,2] and increased plasma prothrombin levels are associated with increased VTE risk.[1-3] Castoldi *et al.*[3] reported higher prothrombin activity levels (122±14%) in 25 symptomatic 20210A carriers than in 128 asymptomatic carriers (112±14%). In the present study, we found that in 20210A carriers, prothrombin levels above 129% were associated with increased risk of VTE, with an OR of 4.1. Stratification of the prothrombin levels in quartiles demonstrated a dose response relationship between prothrombin level and VTE risk.

In conclusion, our results show that, among VTE patients carrying the 20210A variant, the presence of the *EPCR* A3 haplotype significantly reduced the age at the first onset and the probability of being free of thrombosis at 40 years of age. They also show that the presence of the EPCR A3 haplotype results in an approximately two-fold increase in the risk of VTE in family members carrying the prothrombin 20210A allele due to its association with increased sEPCR levels. Our data also show that symptomatic family members carrying the 20210A allele have significantly higher prothrombin levels than asymptomatic members, confirming that the mechanism by which the G20210A mutation increases VTE risk is because it induces an increase in plasma prothrombin levels. In family members carrying the 20210A allele, both an increase in prothrombin levels and an increase in sEPCR are associated with VTE risk.

Authorship and Disclosures

FE designed research; SN, PM, AE, PV and FE performed research; AE, RMB and FE analyzed data; YM, FF and AV took care of the clinical aspects of the study. FE, AE and RMB wrote the paper. All authors revised and approved the final version of the manuscript. The authors reported no potential conflicts of interest.

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