

# Polymorphisms of clotting factors modify the risk for primary intracranial hemorrhage

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Intracranial hemorrhage is the third most frequent cause of cerebrovascular disease, but few genetic risk factors have been associated with its development. Recently, it has been reported that some polymorphisms that affect clotting factors increase the risk for thrombosis. However, reports have analyzed the effect of polymorphisms influencing the hemostatic state in bleeding disorders insufficiently. A case-control study was conducted of 201 patients with spontaneous intracranial hemorrhage and 201 control subjects matched for age, race, sex, and selected risk factors (hypertension, smoking, and alcohol consumption). Genomic

polymerase chain reaction was used to analyze the prevalence of 4 polymorphisms: factor V Leiden, prothrombin 20210A, factor VII–323 Del/Ins of a decanucleotide, and factor XIII V34L. Subjects with factor V Leiden had decreased risk for spontaneous intracranial hemorrhage (odds ratio, 0.19; 95% confidence interval, 0.03-0.95). The frequency of the prothrombin 20210A/G genotype was also lower among patients than controls (1.5% vs 3%, respectively). Moreover, carriers of the –323 Ins allele of factor VII had a 1.54-fold risk for intracranial hemorrhage (95% CI, 1.03-2.72). Finally, no significant differences were observed in the preva-

lence of factor XIII V34L polymorphism between patients and controls. Therefore, new genetic factors affecting the risk for spontaneous intracranial hemorrhage were identified. These data, together with the relevance of these polymorphisms in thrombotic diseases, support the idea that a polymorphism may play opposite roles in thrombosis and hemorrhage, suggesting an explanation for the high frequency of these polymorphisms in the general population. (Blood. 2001;97:2979-2982)

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## Introduction

Exquisitely regulated hemostatic mechanisms have evolved within the animal kingdom to protect against the ever-present danger of fatal hemorrhage. Platelets and coagulation factors interact to generate the protective hemostatic plug that prevents blood loss at sites of vascular injury. However, structural anomalies or changes in the levels of different elements of the hemostatic system can result in disturbances that may cause bleeding or thrombotic disorders.

Because thromboembolic disease is the most common cause of death in developed countries,<sup>1</sup> attention during the last 30 years has been focused primarily on identifying molecular changes that play important roles in the predisposition to persistent hypercoagulable states. Initially, it was shown that an increased risk for venous thrombosis is associated with a rare deficiency of anticoagulant proteins (antithrombin, protein C, or protein S).<sup>2,3</sup> Recently, 2 common genetic risk factors for venous thrombosis have been identified—the resistance to activated protein C, caused mainly by the R/Q 506 mutation in the factor V gene (FV Leiden), and the G/A transition at position 20210 in the 3' untranslated region of the prothrombin gene.<sup>4,5</sup> Finally, the thrombotic role of factor VII (FVII) polymorphisms associated with the levels of FVII in plasma is controversial. Well-designed studies have failed to find any association, but 2 recent reports showed the significance of these polymorphisms in myocardial infarction.<sup>6,7</sup>

By contrast, most studies analyzing the role of hemostatic alterations in bleeding disorders have been focused on rare deficiencies of different elements of the hemostatic system: platelet glycoproteins or coagulation factors. Surprisingly, few studies have analyzed prothrombotic mutations in bleeding disorders, especially common polymorphisms, with functional consequences in the coagulation response.

Intracranial hemorrhage is the third most frequent cause of cerebrovascular disorder; it accounts for 12% of all stroke events.<sup>8</sup> However, it is remarkable that only occasional brief reports, mainly in newborns, analyzed the role of coagulation factor disorders in the pathogenesis of cerebral hemorrhage. Of note is the reported relevance of the factor XIII (FXIII) V34L polymorphism found in a small series of 62 patients with primary intracranial hemorrhage.<sup>9</sup>

The aim of the present study is to characterize genetic alterations of the hemostatic system that might promote or protect against spontaneous bleeding in the brain. We analyzed the prevalence of 4 polymorphisms that affect clotting factors with relevant effect in the levels or function of the encoded proteins that could therefore determine procoagulant or prohemorrhagic states: FV Leiden, prothrombin 20210A/G, deletion/insertion (Del/Ins) of 10 nucleotides in the promoter (–323) of FVII, and FXIII V34L.

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## Materials and methods

### Selection of patients and control subjects

Between April 1998 and April 2000, we studied 236 consecutive, unrelated, white adult patients with a first episode of nontraumatic spontaneous intracranial hemorrhage who were admitted to University General Hospital and Arrixaca Hospital in Murcia, Spain. Patients with hemorrhagic transformation of a previous infarct (4), with acquired or congenital bleeding disorders (2), or with primary or metastatic brain tumors (8) and patients in anticoagulant or antiaggregant treatment (21) were not included in the study. Therefore, our analyses were restricted to 201 patients who fulfilled these criteria. Diagnosis of spontaneous intracranial hemorrhage was verified by computed tomography for all patients on admission; it was performed within 24 hours of the onset of symptoms in each patient. Cerebral angiography or magnetic resonance angiography was performed in patients with subarachnoid hemorrhage to identify aneurysmal or vascular malformation origin. All patients underwent standard hematologic screening tests (platelet count, activated partial thromboplastin time, and prothrombin time). The presence of selected risk factors for nontraumatic intracranial hemorrhage (hypertension, smoking history, and alcohol consumption)<sup>8</sup> was also recorded in all study participants. Confounding risk factors for patients unable to provide a meaningful clinical history were obtained from family members. Finally, the frequency of deaths that occurred within 30 days of the first intracranial hemorrhagic episode was also registered (Table 1).

During the same period of time, we selected 201 control subjects by reviewing the charts of a population of patients admitted to the hospital who had no history of vascular, thromboembolic, or hemorrhagic disease and who were not undergoing antithrombotic therapy. These control subjects were chosen based on age, race, sex, and selected risk factors for primary intracranial hemorrhage (smoking history, blood pressure, and alcohol consumption) to match those of the respective patient. Finally, we analyzed the frequency of the studied polymorphisms in the general population from our region in 490 additional healthy white subjects, mainly blood donors (Table 1).

Patients, controls, and family members of those patients with low levels of consciousness at presentation were fully informed of the aim of this study. All subjects investigated gave their informed consent to enter the study, which had been approved by the local ethics committee and was performed in accordance with the tenets of the Declaration of Helsinki, as amended in Venice in 1983.

### Blood collection and DNA isolation

Blood samples were obtained by atraumatic venipuncture collection into 1:10 volume of trisodium citrate (Vacutainer; Becton Dickinson, Meylon, France). Total genomic DNA was obtained from peripheral blood after lysis

with sodium dodecyl sulfate and proteinase K treatment of buffy coat. DNA was purified using phenol–chloroform and ethanol precipitation.

### DNA studies

Genotyping of FV Leiden, prothrombin 20210A/G, the –323 decanucleotide Del/Ins polymorphism of the *FVII* gene, and FXIII V34L was performed by genomic polymerase chain reaction amplification as described.<sup>4,5,7,10</sup>

### Statistical analysis

Data for continuous variables were expressed as mean  $\pm$  SD. Two-tailed Student *t* test was used to compare continuous variables. Discrete variables were analyzed by the  $\chi^2$  test. *P* < .05 was considered to indicate statistical significance. The strength of the association of the polymorphisms with the occurrence of hemorrhage was estimated by calculation of the odds ratio (OR) with the EpiInfo software and the Cornfield method for the calculation of 95% confidence intervals (CI).

## Results

### Characteristics of the study population

We assumed that a single genetic polymorphism by itself was unlikely to be responsible for the development of thrombus or hemorrhage but that it could influence disease risk in association with other factors. Therefore, the selection of patients and controls presented 2 special features to avoid the interference of other factors and to determine the role of polymorphisms that predisposed to or protected against disease. First, patients at high risk for intracranial hemorrhage, such as those with a personal history of acquired or congenital bleeding disorders or primary or secondary brain tumors, as well as patients undergoing antithrombotic therapy, were excluded. Second, to avoid the overrepresentation of classic hemorrhagic risk factors among patients, their respective controls were selected to match for age, race, sex, and selected risk factors for spontaneous intracranial hemorrhage (smoking history, blood pressure, and alcohol consumption). The general characteristics of patients and control subjects are shown in Table 1. According to this inclusion design, no significant differences were found in age, sex, and risk factor prevalence among patients and controls. Remarkably, we did not detect significant differences in coagulation tests or in number of platelets between patients and controls (Table 1).

Our study did not include patients who died during transport to the hospital or at home; therefore, we cannot completely exclude an effect of survival bias. Table 1 shows the survival percentage 30

**Table 1. Characteristics, clinical features, prevalence of selected risk factors for intracranial hemorrhage, coagulation parameters, and platelet count among patients, controls, and general population**

	Patients with hemorrhage			Controls	<i>P</i>	General population N = 490
	PIH N = 141	SAH N = 60	Total N = 201	N = 201		
Age (y), mean $\pm$ SD	68.7 $\pm$ 13.1	60.9 $\pm$ 12.3	66.4 $\pm$ 13.5	67.8 $\pm$ 12.8	.282	49.5 $\pm$ 19.5
Male sex (%)	63.8	38.3	56.2	56.2	1.000	48.6
Hypertension (%)	65.3	36.7	56.7	59.2	.572	ND
Current/former smoker (%)	27.7	30.0	28.4	26.4	.655	ND
Alcohol consumption (%)	7.8	8.3	8.0	6.0	.441	ND
APTT(s): mean $\pm$ SD	26.4 $\pm$ 2.2	26.6 $\pm$ 2.6	26.5 $\pm$ 2.3	26.7 $\pm$ 2.2	.452	ND
Platelets $\times 10^6/L$ : mean $\pm$ SD	204.0 $\pm$ 35.3	221.6 $\pm$ 51.4	209.3 $\pm$ 41.5	202.7 $\pm$ 18.9	.078	ND
30-day survival (%)	80.1	76.7	79.1	—	—	—
Aneurysm (%)	—	70.0	—	—	—	—

PIH, primary intracerebral hemorrhage; SAH, subarachnoid hemorrhage; APTT, activated partial thromboplastin time; ND, not determined. Hypertension was defined as blood pressure exceeding 140 mm Hg systolic or 90 mm Hg diastolic on repeated observations over 3 months or if no blood pressure values were available when the subject was under treatment with chronic antihypertensive therapy. Current/former smoker, subject smoked more than 10 cigarettes per day. Alcohol consumption, subject consumed more than 300 g alcohol per week.

days after admission to the hospital; it does not differ from that of previous reports.<sup>8</sup>

Based on the type of intracranial hemorrhage and the risk factors associated with it, our data fit properly with those of previous reports of large numbers of patients.<sup>8</sup> Sixty patients had subarachnoid hemorrhage (SAH), and most (70%) of them also had aneurysms. Primary intracerebral hemorrhage (PIH) was diagnosed in 141 of 201 patients. As expected, hypertension was the most prevalent risk factor in patients with PIH (Table 1).

**Prevalence of the FV Leiden, prothrombin 20210A/G, FVII-323 Del/Ins, and FXIII V34L polymorphisms in the case-control study**

Genotypic and allelic frequencies for the analyzed polymorphisms in the case-control study are shown in Table 2. The frequencies of these polymorphisms in the control group were similar to those identified in the general population of our region and did not differ from those previously reported in other Mediterranean countries.<sup>7,10-12</sup> However, the frequency of FV Leiden, prothrombin 20210A/G, and FVII -323 Del/Ins in patients with intracranial hemorrhage differed in patients and controls. The mutated 20210A allele of the prothrombin gene was present in 1.5% of patients, whereas its frequency in controls was 2-fold (3%), a difference that did not achieve statistical significance ( $P = .312$ ). The frequency of FV Leiden was also lower in patients than in controls (1% vs 4.9%;  $P = .019$ ). According to these results, carriers of FV Leiden show almost a 5-fold decreased risk for intracranial hemorrhage than those lacking the genetic variant under the same environmental risk factors.

To our knowledge, the FXIII Leu 34 variant was the first polymorphism to increase the risk for PIH in the white population, according to recent data obtained in 62 patients with this disease.<sup>9</sup> However, our results in a larger number of patients did not support such a suggestion because the FXIII V34L polymorphism showed a similar distribution among patients and controls. By contrast, in our case-control study, we found statistically significant differences in the prevalence of FVII -323 polymorphism between patients and controls (Table 2). Carriers of the -323 Ins allele, associated with significant lower levels of FVII in plasma,<sup>7</sup> displayed an increased risk for intracranial hemorrhage (OR, 1.5; 95% CI, 1.03-2.72); therefore, this genetic change could be involved in the etiology of this disease.

We then analyzed the involvement of the studied polymorphisms in subtypes of intracranial hemorrhage. The prevalence of FVII polymorphism was shown to be similar in PIH or SAH, suggesting that low levels of FVII could increase the risk for both types of intracranial hemorrhage (Table 2). However, the frequency

of FV Leiden was lower in patients with PIH than in those with SAH, indicating that this polymorphism could play a more relevant role in PIH. On the contrary, the prothrombin 20210A/G was not found in any of 60 patients with SAH (Table 2). Finally, the prevalence of the FXIII Leu34 allele was higher in SAH than PIH, though differences did not reach statistical significance (Table 2).

We did not detect differences in genotype or allele frequency associated with age, sex, or any of the studied risk factors (hypertension, smoking, or alcohol consumption) (data not shown). Finally, no relation was found between the presence of a polymorphism and the 30-day posthemorrhagic episode survival (data not shown).

**Discussion**

During the past 20 years, substantial progress has been made in understanding the multifactorial and multigene nature of hemostatic diseases. It is well established that interactions between environmental and genetic factors determine the risk for a thrombotic episode.<sup>13</sup> In bleeding disorders, the frequently observed variability in the phenotypic expression even among persons carrying identical genetic mutations suggests the influence of multiple factors in the pathogenesis of these diseases. Thus, co-inheritance of genetic abnormalities has previously been reported for deficiencies of several coagulation factors, including FVIII and FXI,<sup>14</sup> as well as FXI and von Willebrand factor,<sup>15</sup> though reports of combined coagulation factor deficiencies are scant. The remarkably high prevalence of FV Leiden in the general population (2%-12%)<sup>11</sup> and the procoagulant state associated with this genetic change make FV Leiden a tantalizing possibility as a key factor in bleeding disorders. Certainly, 2 pieces of evidence support a protective role of FV Leiden in hemophilia. "In vitro" studies recreating the condition of moderate to severe hemophilia A and the distinct clinical phenotype of hemophilia with identical mutations in factor VIII but distinct genotypes in FV Leiden suggest that FV Leiden might have a beneficial effect on hemophilia.<sup>16,17</sup> Our report is the first one showing a protective role of FV Leiden polymorphism in acquired bleeding disorders, thus providing further evidence of such a potentially protective role of FV Leiden in hemorrhagic diseases. Thus, we found that the presence of FV Leiden reduces by 5-fold the risk for spontaneous intracranial hemorrhage. This result is also in accordance with the suggested protective role of this prothrombotic polymorphism in other bleeding disorders such as the partum.<sup>18</sup> Accordingly, the high prevalence of this potentially harmful mutation in the white population could be explained as a consequence of an evolutionary

**Table 2. Genetic and allelic frequencies of FV Leiden, prothrombin 20210A/G, FVII-323 Del/Ins, and FXIII V34L polymorphisms in patients, controls, and general population**

	FV Leiden			Prothrombin 20210			FVII-323			FXIII V34L		
	± N (%)	-/- N	+ Freq	A/G N (%)	G/G N	A Freq	D/I + I/I N (%)	D/D N	I Freq	V/L + L/L N (%)	V/V N	L Freq
PIH (N = 141)	1 (0.7)	140	0.004	3 (2.1)	138	0.011	40 + 2 (29.8)	99	0.156	41 + 8 (34.8)	92	0.202
SAH (N = 60)	1 (1.7)	59	0.008	0	60	0	14 + 3 (28.3)	43	0.167	17 + 7 (40.0)	36	0.258
Total (N = 201)	2 (1.0)	199	0.005	3 (1.5)	198	0.007	54 + 5 (29.4)	142	0.159	58 + 15 (36.3)	128	0.219
Control (N = 201)	10 (4.9)	191	0.025	6 (3.0)	195	0.015	36 + 4 (19.9)	161	0.109	64 + 6 (34.8)	131	0.189
<i>P</i>	.019		.020	.312		.315	.028		.039	.755		.294
OR	0.19		0.20	0.49		0.50	1.54		1.54	1.07		1.20
95% CI	0.03-0.95		0.03-0.96	0.10-2.25		0.10-2.24	1.03-2.72		1.0-2.37	0.69-1.64		0.84-1.72
GP (N = 490)	19 (3.9)	471	0.019	11 (2.3)	479	0.011	99 + 6 (21.4)	385	0.112	159 + 15 (35.6)	316	0.193

PIH, primary intracerebral hemorrhage; SAH, subarachnoid hemorrhage; OR, odds ratio; CI, confidence interval; Freq, frequency; GP, general population.

selection mechanism conferring survival advantages in bleeding situations to the carriers of this prothrombotic mutation, as has been previously proposed.<sup>11,19-21</sup> This attractive hypothesis could also be applied to the 20210A polymorphism of the prothrombin gene, the second prothrombotic polymorphism identified so far. We observed a lower prevalence of this polymorphism in patients with intracranial hemorrhage (1.5%) than in controls (3.0%). However, the low prevalence of this polymorphism in the white population and the lower prevalence of this genetic change in the prothrombotic state of carriership<sup>12</sup> suggest that further studies with larger numbers of participants should be performed.

Another example of the dichotomy of the hemostatic system is the prevalence of FVII in thrombosis and hemorrhage. During the last decade evidence has accumulated indicating that raised plasma FVII levels increase the risk for ischemic heart disease, though in other studies such an association was not evidenced.<sup>22</sup> Multiple studies also investigated the role of common polymorphisms influencing the FVII plasma levels in thrombotic disorders with conflicting results. Several well-designed studies have failed to find any association of FVII polymorphisms and arterial or venous thrombosis.<sup>23</sup> However, recent reports suggest that those alleles associated with low levels of FVII could play a protective role against myocardial infarction.<sup>6,7</sup> By contrast, FVII plays a key role in bleeding disorders. Thus, congenital deficiencies of this protein predispose to spontaneous bleeding and bleeding after surgery.<sup>24</sup> FVII deficiency displays considerable phenotypic and molecular heterogeneity. Clinically, patients range from those without symptoms to those with severe hemorrhagic tendencies associated with zygotosity and FVII level.<sup>24</sup> Interestingly, 16% of FVII-deficient homozygotes have episodes of cerebral hemorrhage.<sup>25</sup> Moreover, 70% of the FVII knockout mice had fatal intra-abdominal bleeding within the first 24 hours, whereas most of the remaining neonates died of intracranial hemorrhage before the age of 24 days.<sup>26</sup> Recombinant activated factor VII has become an interesting and

effective treatment for multiple bleeding disorders.<sup>27</sup> We herein show the first evidence for the association of the promoter decamer Del/Ins -323 polymorphism with intracranial hemorrhage, supporting the idea that the FVII Del -323 allele, associated with lower levels of FVII, could play opposite roles in thrombosis and hemorrhage.

Genetic polymorphisms underlie the diversity of any specie. Most of such inherited changes in DNA structure are neutral, but others could affect the function of proteins and, with more or less severity, the efficiency of a whole physiological system, thus modifying susceptibility to a particular disease. These genetic changes could have particular strength in very sensitive systems such as the hemostatic system. Because of the dichotomy of this system, polymorphic changes affecting hemostatic factors could have mild but opposite effects in the pathogenesis of thrombotic and hemorrhagic disorders. Our results in patients with intracranial hemorrhage support this assessment for 3 polymorphisms affecting the level or function of 3 relevant clotting factors: II, V, and VII. Thus, one polymorphism conferring a specific procoagulant state could have a distinct pathologic effect, increasing the risk for thrombosis or reducing that of hemorrhage, depending of the presence of specific conditions and other risk factors for thrombosis or bleeding. Similar assessments can be made for a polymorphism with prohemorrhagic consequences, changing the effect for each disease. This new concept opens new perspectives in the investigation of bleeding disorders and in the relevance of polymorphisms affecting hemostatic factors.

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