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# The prothrombin 20210A allele influences clinical manifestations of hemophilia A in patients with intron 22 inversion and without inhibitors

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*Background and Objectives*. The modulation of disease severity in hemophilia A (HA) patients may be related to the co-inheritance of mutations in genes with a known thrombotic effect such as factor V Leiden (FVL) and prothrombin. In the Spanish population, the prothrombin 20210A (PT20210A) allele is the most prevalent genetic risk factor for venous thromboembolism.

*Design and Methods*. We investigated the presence of both mutations in a cohort of 265 hemophiliac patients divided into two groups: I) 140 unrelated patients with moderate and mild HA and II) 125 unrelated patients with severe HA (83 carrying an inversion of intron 22).

*Results*. In group I, 4 patients had the FVL (2.8% vs. 2.98% controls) and 5 had the PT20210A (3.6% vs. 6.46% controls). In group II, two patients with inversion had the FVL (1.6%) and PT20210A was found in 10 patients (8%), five of them with inversion of intron 22 without inhibitors. One of these patients had the FVL and PT20210A mutations concomitantly. In the subgroup of patients with inversion who were carriers of the PT20210A, three parameters i.e. spontaneous bleeding ($p$=0.008), factor VIII utilization ($p$=0.016) and number of hemophilic arthropathies ($p$<0.0005) were significantly lower than in a subgroup of 11 age-matched non-PT20210A severe HA patients with inversion and without inhibitors.

*Interpretation and Conclusions*. These results indicate that the inheritance of PT20210A could be a protective factor that mitigates the clinical severity of HA.


Key words: hemophilia A, bleeding, FVIII utilization, hemophilic arthropathy, inversion, FVL, PT20210A.

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Hemophilia A (HA) is an X-linked disorder characterized by a deficiency of coagulation factor VIII (FcVIII) activity. Mild hemophilia results as the consequence of FcVIII activity ranging between 5-30% whereas in moderate cases this activity varies from 2-5%. These cases are usually not diagnosed during infancy and the clinical course is generally benign. On the other hand, patients with severe hemophilia A (factor activity less than 1%) experience frequent bleeding episodes in joints or soft tissues.[1,2]

However, there is considerable variation in the extent and frequency of bleeding in such patients and even in those sharing the same level of deficient factor or the same molecular abnormality. Apart from the molecular pathology of the FcVIII gene, the final phenotype and evolution of the disease could also be influenced by genetic risk factors for thrombosis which could modulate the severity of HA by compensating for a defective FVIII. In fact, the role of prothrombotic risk factors, especially the factor V Leiden (FVL) mutation, in hemophilia has been the subject of a number of studies.[3-6]

The FVL mutation is a G to A substitution at nucleotide 1691 in factor V replacing Arg by Gln at position 509. Cleavage by activated protein C at this Arg site is required for the efficient inactivation of activated factor V. FVL is the major cause of activated protein C resistance and has been identified as one of the most frequent inherited risk factors for venous thrombosis.[7-9]

The 20210 G to A mutation in the prothrombin gene (PT20210A), occurs in the 3´untranslated region of the prothrombin gene. This variant, which was first described in 1996,[10] was found to be linked to elevated plasma prothrombin levels and with a moderately increased risk for first venous

thrombotic events.[11] Moreover, in the Spanish population, the PT20210A allele is the most prevalent genetic risk factor for venous thromboembolism.[12]

We investigated the prevalence of both mutations in a cohort of 265 Spanish HA patients in an attempt to determine whether both mutations were associated with decreased bleeding, less FcVIII utilization and a reduced number of hemophilic arthropathies. Based on these three parameters, we found significant evidence that the PT20210A mutation could mitigates the severity of the phenotype in HA patients with inversion of intron.[22]

## Design and Methods

### *Patients*

Study subjects consisted of consecutive regular patients with HA attending clinics of different Spanish Hemophilia Units. We studied 265 Spanish HA patients who were divided into two groups. Group I: 140 patients with moderate and mild HA (defined by FcVIII activity). The mutation in the Fc VIII gene was identified in 26 of these patients. Group II: 125 patients with severe HA (defined by FcVIII coagulation activity less than 1%). Eighty-three of these patients had intron 22 inversion; 25 had other mutations and in 17 patients the identification of the mutation was in progress. FVL and PT20210A variant frequencies from 201 unrelated individuals of the Spanish population were available.[12]

### *Assessment of clinical severity of bleeding*

Markers of bleeding severity were defined *a priori* and retrospectively ascertained for each study subject in a blind fashion. We determined three parameters for comparison between carriers and non-carriers of FVL and PT20210A:

1) average of total factor concentrate utilization for the three-year period from January 1998 to December 2000 expressed as Units/year. This information was available for all patients with the exception of cases 1-3, 6 and 7 (Table 1) who died before or during these dates. In these cases the information recorded was from the last three years of life. Information on the use of prophylactic treatment with FcVIII concentrate and on the presence or absence of inhibitors was also available for each patient. The criteria for administering FcVIII replacement were similar for all patients;

**Table 1. Clinical information on patients with severe HA with FVL and /or PT20210A (PT). 1-5 and 6-11 correspond to cases without and with inversion, respectively. C1-C11 correspond to the patients with severe HA with inversion without the FVL or PT20210A allele.**

| *Patient I.D.* | *Age (years) HIV* | *FcVIII activity* | *Mutation in FcVIII gene* | *Inhibitors* | *MBF* | *FcVIII Utilization** | *Hemophilic Arthropathy* | *FVL/PT* |
|---|---|---|---|---|---|---|---|---|
| 1 | † 19 | <1% | us | Yes | >6 | 80,000 | 4 | PT |
| 2 | † 14+ | <1% | del exon 23 | No | na | na | na | PT |
| 3 | † 26 | <1% | us | No | <5 | 45,000 | 3 | PT |
| 4 | 2 | <1% | K1040X | Yes | >6 | rFVIIa | none | PT |
| 5 | 9 | <1% | W1567X | No | Prophylaxis | Prophylaxis | none | PT |
| 6 | † 32+ | <1% | Inversion (D) | No | <5 | 20,000 | 2 | PT |
| 7 | † 26+ | <1% | Inversion (D) | No | <5 | 6,000 | 2 | PT |
| 8 | 28 | <1% | Inversion (D) | No | 5 | 45,000 | 3 | PT |
| 9 | 27 | <1% | Inversion (D) | No | none | none | 1 | PT |
| 10 | 38 | <1% | Inversion (D) | No | <5 | 6,000 | 2 | FVL/PT |
| 11 | 22 | <1% | Inversion (D) | No | >6 | 64,200 | 1 | FVL |
| C1 | 34 | <1% | Inversion (D) | No | > 6 | 110,200 | >4 | No |
| C2 | 30 | <1% | Inversion (D) | No | > 6 | 78,400 | >4 | No |
| C3 | 28 | <1% | Inversion (D) | No | > 6 | 196,000 | >4 | No |
| C4 | 39 | <1% | Inversion (D) | No | 6 | 40,000 | >4 | No |
| C5 | 35 | <1% | Inversion (D) | No | <5 | 13,200 | 4 | No |
| C6 | 40 | <1% | Inversion (D) | No | > 6 | 72,600 | >4 | No |
| C7 | 39 | <1% | Inversion (D) | No | > 6 | 95,800 | >4 | No |
| C8 | 39 | <1% | Inversion (D) | No | >6 | 104,800 | >4 | No |
| C9 | 23 | <1% | Inversion (D) | No | >6 | 145,000 | 4 | No |
| C10 | 18 | <1% | Inversion (P) | No | 5 | 72,000 | 4 | No |
| C11 | 21 | <1% | Inversion (P) | No | 5 | 69,000 | 4 | No |

*Abbreviations: * = units/year; MBF = maximum bleeding frequency per year; us = under study; D = distal; P = proximal; †death from major trauma, except case #6 who died of renal failure; †+ death from HIV complications; na=data not available.*

2) the number of bleeding episodes per year experienced by the patient over the last three years. Bleeding episodes were defined as spontaneous or traumatic bleeding events requiring treatment of FcVIII concentrate. The precise number of bleeding episodes was difficult to ascertain retrospectively in some cases. Therefore, we determined the maximum bleeding frequency to categorize the patients as those with either a low tendency (up to 5 episodes) or a high tendency (more than 6 episodes) in their worst year;

3) data from severely affected subjects under study included the presence and number of hemophilic arthropathies caused by hemophilic bleeding, which interfered with the daily activities because of pain or deformity.

### *Genotyping*

DNA was extracted using a standard protocol.[13] The presence or absence of inversion of intron 22 was ascertained as previously described.[14] All other mutations were detected by SSCP analysis according to David *et al.*[15] with minor modifications in the reaction conditions. Fragments with mobility shift were then purified with a QIAquick column polymerase chain reaction (PCR) purification kit (QIAGEN), and analyzed by direct forward and reverse sequencing using a DNA sequencing kit (Perkin Elmer-Applied Biosystems) on an ABI PRISM 310 DNA automatic sequencer. We genotyped the FVL and the PT20210A variants using four previously described primers[16] in a multiplex-PCR. Control DNA samples of wild type, heterozygous and homozygous FVL and PT were included with each PCR batch. Briefly, 50 μL of mixture containing 20 mM TRIS HCl pH 8.2, 2 mM MgCl, 0.2 mM of each of the four dNTPs, 0.5 μM of each primer, 250 ng of DNA and 1.5 U Taq-gold (Perkin-Elmer) was subjected to 40 cycles of 10 min at 95°C, 1 min at 55°C (for FVL-PT20210A) and 1 min at 72°C, with a final extension step of 10 min at 72°C. In the FVL-PT20210 multiplex PCR, the 175 bp and 118 bp PCR products were digested with *TaqI* (Biolabs) and electrophoresed on a 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland, ME, USA).

### *Statistical analysis*

We performed a $\chi^2$ test for group comparison of frequencies and a t-test to compare the mean values of FcVIII utilization in the group with and without FVL/PT20210A. A non-parametric approach (Mann-Whitney test) was used to compare the variables of maximum bleeding frequencies and the number of hemophilic arthropathies after replacing *less than* or *greater than* by concrete values (i.e. <5 was replaced by 4; >6 was replaced by 7). In all the tests, $p$ values <0.05 were considered statistically significant.

## Results

### *Prevalence of FVL and PT20210A in Spanish HA patients*

In group I, including 140 patients with moderate and mild HA, we found 4 unrelated FVL carriers (2.8% vs. 2.9% controls) (Fisher´s exact test $p$=1) and 5 unrelated PT20210A carriers (3.6% vs. 6.5% controls) ($p$=0.32). In group II, including 125 patients with severe HA, two patients had the FVL (1.6% vs. 2.9% controls) (Fisher´s exact test $p$=0.65) whereas the PT20210A was found in 10 patients (8% vs. 6.5%) ($p$=0.76). Nine of them had a known molecular defect in the FcVIII gene: two patients with different stop mutations: K1040X (novel) and W1567X, one patient with a deletion of exon 23 and six patients with inversion of intron 22. The characteristics of patients with severe HA with FVL or PT20210A variants are shown in Table 1. Patient #10 carried both FVL and PT20210A alleles.

### *Bleeding episodes and FcVIII utilization*

In group I no bleeding episodes requiring utilization of FcVIII were recorded in patients with FVL or PT20210A mutations. This situation was very similar to that in the moderate and mild cases without FVL or PT20210A. In fact, these cases are generally treated with desmopressin instead of FcVIII.

Patients with FVL or PT20210A mutations from group II were classified into two subgroups: those without inversion and those with inversion. The subgroup without inversion included 5 patients, three of whom had died before the start of this study (Table 1, patients #1-5). Patient #1 had a history of inhibitors and died at the age of 19 in a road accident. Patient #2 was infected with HIV and died at 14 years old due to HIV complications. No reliable data on bleeding episodes and factor VIII utilization were available for this patient. Patient #3, despite a history of few bleeding episodes, died at the age of 26 in a road accident. Patient #4, who is two years old, developed high response inhibitors and is currently receiving rFVIIa in bleeding episodes. Finally, patient #5, who is 9 years old, is receiving prophylaxis with FcVIII. The heterogeneity of the patients in this group in terms of age, presence of inhibitors, prophylaxis, rVIIa factor therapy and different genotype prevented an adequate comparison of bleeding and factor VIII utilization in patients #1, 4 and 5. Given that clinical data were unavailable for patient #2, only case #3

could be included in the group of PT20210A for comparison (Table 1). The subgroup with inversion and FVL or PT20210 mutations was more homogeneous in age (all were adults) although two of them had died before the study. None of them had developed inhibitors prior to or during the period of the study, despite the fact that inhibitors were present in a proportion of the remaining inversion cases (0/6 vs. 31/77) (Fisher´s exact test $p$=0.08). We compared the bleeding episodes, FVIII utilization and the number of hemophilic arthropathies in the subgroup of patients with inversion of intron 22 and PT20210A and /or FVL with the same parameters in a subgroup of patients with severe HA and inversion but without PT20210A or FVL. Out of 77 patients with inversion without PT20210A/FVL, 31 had inhibitors, 30 were infants and adolescents receiving prophylaxis and data were not available for 5 patients. Information on the remaining 11 patients was used for comparison with the PT20210A group and the cases with FVL (Table 1, C1-C11). The average age in the subgroup with PT20210 (patients #6-10) was 30 (ranging from 26 to 38) and that in the subgroup without PT20210A was 32 (ranging from 20 to 40). The number of bleeding episodes in the subgroup with PT20210A was always less than or equal to 5 episodes per year (median=4) and case #9 had not had bleeding episodes during the previous three years. The average FcVIII utilization in this subgroup was 15,400 Units per year (±18105). The range was from no required factor in recent years in patient #9 to 45,000 units of FcVIII utilization per year in patient #8. The average bleeding tendency in the subgroup without PT20210A was more than 6 episodes per year (with the exception of case C5) (median=7) and the average FcVIII utilization was 90,636 Units per year (± 49,515). The difference between both subgroups with respect to maximum bleeding frequency ($p$=0.008) and FcVIII utilization ($p$=0.016) reached statistical significance. The 95% confidence interval for differences between groups in FcVIII utilization was 10,212 to 131,959. Considering the subgroup with inversion and PT20210A (6-10) together with the patient #3 (PT2010A without inversion) and #11 (FVL with inversion) (Table 1), the average FcVIII utilization was 26600 Units per year (± 24,802) ($p$=0.002). The difference with the subgroup without prothrombotic risk factors was still significant when considering maximum bleeding frequency ($p$=0.009).

*Hemophilic arthropathy*

Data on hemophilic arthropathy indicated that patients with severe HA with FVL and/or PT20210A tended to have less than 4 arthopathies (median=2) interfering with daily activities whereas all the cases without PT20210A had 4 or more arthropathies (median=5) ($p$<0.0005)(Table 1). There were insufficient data to determine orthopedic joint scores.

## Discussion

To the best of our knowledge, this is the first report indicating the association of a thrombotic risk factor, PT20210A, with a significant difference in clinical outcome in patients with severe HA with an identical defect in the FcVIII gene. Considering all patients with HA and prothrombotic risk factors (PT20210A and FVL), there was a significant difference in FcVIII utilization, maximum bleeding frequencies and the number of arthropathies. This result was even more marked when considering the subgroup with PT20210A and inversion. We found that the co-inheritance of PT20210A in patients with severe HA and inversion of intron 22 was independently associated with fewer bleeding episodes, with a reduced FcVIII concentrate utilization and with a tendency to have a reduced number of hemophilic arthropathies when compared with a similar subgroup of HA patients without the PT20210A variant. These results evidence a possible mitigating effect of the PT20210 mutation in severe HA. The fact that only two patients with severe HA were detected to have FVL (one of these concomitantly with PT20210) precluded a separate adequate comparison.

In hemophilia, bleeding tendency, FcVIII utilization and joint damage are influenced by a number of known factors such as the type of mutation in FcVIII and the development of inhibitors. Although pediatric patients with severe HA experience frequent bleeding episodes, this tendency declines with age.[17] There is also considerable variation in the extent and frequency of bleeding in such patients. A possible explanation for this variable expression of hemophilia A could be the influence of gene mutations at other loci. Given that the procoagulant system is in equilibrium with anticoagulant and fibrinolytic systems in normal homeostasis, genetic factors such as FVL or PT20210A mutations which increase the risk of thrombosis are potential modifier loci for the clinical expression of HA. Nichols *et al.*, studying two HA patients, suggested that co-inheritance of the FVL mutation could influence the phenotype of patients with severe HA sharing an identical FcVIII mutation.[4] However, this observation has not been confirmed by other reports. Arbini *et al.*, studying 21 patients

with severe HA who had milder bleeding, found only one carrying the FVL mutation.[3] Arruda *et al.*, in a cohort of 113 patients with mild to severe HA, reported 3 patients with severe HA and co-inheritance of FVL with no difference in the frequency of bleeding episodes or FcVIII utilization.[5] Lee *et al.*, studying 137 patients with severe HA, found 6 carriers of FVL mutation who utilized less FcVIII concentrates and tended to have fewer bleeding episodes, suggesting a mitigating effect of the FVL on HA. On the other hand, FVL has not been associated with the absence of arthropathy.[6] These authors acknowledged that their results require a cautious interpretation given the small number of patients with FVL, their different characteristics and that the effect of other variables such as the FcVIII molecular defect could not be ruled out in this study. Finally in a recent report, Escuriola Ettinghausen *et al.*[18] observed that the onset of symptomatic bleeding in children with severe HA carrying prothrombotic factors occurred later in life than in non-carriers. In a cohort of 92 patients with severe HA, they found 6 with FVL, 3 with the PT20210A and one with protein C type I deficiency. Considering these ten cases as a group, they found a significantly earlier onset of symptoms (0.9 vs. 1.6 years). However, the underlying molecular defect in the Fc VIII gene in these patients was not considered.

The frequencies of FVL and PT20210A in the Spanish hemophiliac population were fairly similar to those in the general Spanish population.[12] The prevalence in mild, moderate and severe hemophilia was roughly the same. The population of HA patients in whom FVL and PT20210A mutations were detected varied considerably in age, HIV infection, molecular defect in FcVIII, inhibitor development and type of treatment. In our group of patients with moderate and mild HA it was not possible to obtain conclusive data about a protective effect of these mutations since, in practice, such patients do not need FcVIII replacement.

The results in the group with severe HA without inversion were not conclusive because of the heterogeneity of the group. On the other hand, the patients with inversion, severe HA and allele variants were all adults without a history of inhibitors, immunotolerance or prophylactic FcVIII administration. Interestingly, none of the patients with PT20210A and inversion developed high response inhibitors although inhibitor development was present in 37.5% of our Spanish patients with inversion.[19] Bleeding episodes, FcVIII utilization and the number of arthropathies were significantly lower in the inversion subgroup with PT20210A. Given that all these patients have the same molecular defect in FcVIII, a similar age and received a similar protocol of therapy, it may be concluded that the presence or absence of PT20210A is the only known demonstrable factor that could account for their different clinical outcome. Moreover, neither bleeding episodes nor FcVIII utilization were recorded for case #9 during the period of the study even though this patient had a normal daily activity. One case from the subgroup of patients with inversion without PT20210A had an average FcVIII utilization within the range of the subgroup with PT20210A. This patient, suffering from mental impairment, had a very limited activity that may explain the few bleeding episodes and the low FcVIII utilization recorded. However, we cannot exclude the possibility that case #9 in the first subgroup and patient C5 in the second subgroup were carriers of additional unknown genetic risk factors for thrombosis, which could influence their hemophilic phenotype. Indeed, reduced activated protein C independently of FVL mutation,[20] high factor XI levels[21] and high factor IX levels[22] have been associated with a risk of thrombosis. Furthermore, the importance of genetic factors in determining variations in hemostasis-related phenotypes has recently been highlighted.[23,24]

Patient #10 of our series was a carrier of both defects, FVL and PT20210A. Double-heterozygosity for these mutations is the most common combined condition associated with thrombophilia.[25,26] Although carriers of both genetic defects have a higher risk of thrombosis than those with single gene defects, we were unable to obtain evidence of a synergistic effect of both prothrombotic factors. This patient has not hitherto shown evidence of thrombotic episodes. Thrombotic episodes constitute a very rare complication in the treatment of severe HA.[27] Most of the cases have been reported in patients with inhibitors receiving continuous activated prothrombin complex or rFVIIa administration during severe bleeding episodes.[28-31] One patient with severe HA who was a carrier of the FVL, developed a portal vein thrombosis after continuous FcVIII infusion to treat a post-traumatic intramural jejunal hematoma.[32] Further investigations are necessary to determine the extent of the *in vivo* or *in vitro* effect of both mutations-FVL and PT20210A- in hemophilic patients.

In conclusion, our results provide evidence that the presence of PT20210A could modify different biological parameters in patients with severe HA, especially those with intron 22 inversion. There was only one patient with severe HA and FVL alone, pre-

venting a specific comparison. The study of other additional genetic and acquired factors that influence the delicate balance between blood fluidity and blood coagulation is warranted to understand the phenotypic differences of severe hemophilia patients and to yield new insights into the prognosis and treatment of FVIII deficiency.

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*ET was primarily responsible for this work, from conception to submitted manuscript and should be considered as the principal author. JMS, JF and MB made substantial contributions to conception and design, interpretation of data and critical revision of the manuscript for important intellectual content. The remaining authors qualified for authorship according to the World Association of Medical Editors (WAME) criteria, and have taken specific responsibility for the following parts of the content: CA and MM, collection of clinical data and interpretation of the results; IC, BG, MC, MD, and ER carried out essential and indispensable laboratory experiments to achieve this work. Order of authorship: the authors are listed according to a criterion of decreasing individual contribution to the work, with the exception of the last two authors who had a major role as senior authors.*

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*Disclosures*

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## Peer Review Outcomes

### *Manuscript processing*

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received December 4, 2001; accepted January 23, 2002.

### *What is already known on this topic*

It has been suggested that the clinical manifestations in hemophilic patients may be related to the co-inheritance of prothrombotic mutations.

### *What this study adds*

This study investigates the clinical effect of the presence of factor V Leiden and prothrombin 20210A allele in a cohort of 265 hemophilic patients.

### *Potential implications for clinical practice*

The data suggest that co-inheritance of prothrombin 20210A allele could be a protective factor for bleeding manifestations in severe hemophilic patients.

*Vicente Vicente, Deputy Editor*