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# Analysis of biological phenotypes from 42 patients with inherited factor VII deficiency: can biological tests predict the bleeding risk?

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A B S T R A C T

**Background and Objectives.** Inherited factor VII (FVII) deficiency is a rare bleeding disorder characterized by a poor relationship between reported FVII clotting activity (FVII:C) and bleeding tendency. Our study was aimed at defining biological parameters that are possibly predictive for bleeding risk in this condition.

**Design and Methods.** Forty-two FVII-deficient patients (FVII:C <30%) were classified into two opposite clinical groups defined as severe and non-or-mild bleeders. For each patient, plasma samples were collected and then investigated for FVII:C (using a sensitive method and human recombinant thromboplastin as the reagent), FVII antigen, activated FVII coagulant activity (FVIIa:C) and the free-form of tissue factor pathway inhibitor.

**Results.** None of these tests could be used as highly accurate predictors of bleeding. Nevertheless, both FVII:C and FVIIa:C differed significantly between the two clinical groups. Using ROC-curve analysis, two critical values of 8% and 3mIU/mL for FVII:C and FVIIa:C, respectively, could be proposed to discriminate between severe bleeders and non-or-mild bleeders.

**Interpretation and Conclusions.** A highly accurate diagnostic test for predicting bleeding tendency in inherited FVII deficiency still eludes definition, highlighting the fact that factors other than FVII itself interfere with the expression of bleeding phenotypes in this condition. Nevertheless, potential critical values using sensitive FVII:C and FVIIa:C methods may be useful in clinical laboratories for FVII-deficient patients. Those patients with FVII:C levels higher than 8% FVII:C or FVIIa:C higher than 3 mIU/mL, with no other hemostatic defect, seem to have a minimal risk of severe bleeding. Extended clinical studies are needed to support these findings.

Key words: inherited FVII deficiency, activated FVII, human recombinant thromboplastin, ROC-curve.

Human factor VII (FVII) is a vitamin K-dependent plasma glycoprotein that plays a pivotal role in the initiation of coagulation. Upon contact with its receptor, the tissue factor (TF) on cell surfaces, FVII is converted to an active form (FVIIa) and triggers the hemostatic cascade.[1] The catalytic function of the FVIIa/TF complex is regulated by a specific trivalent Kunitz-type inhibitor, referred to as the tissue factor pathway inhibitor (TFPI).[2] Inherited FVII deficiency is a rare autosomal recessive disorder characterized by a wide phenotypic heterogeneity and a very poor correlation between reported procoagulant activity and severity of the bleeding diathesis.[3-4] Up to now, no conventional clotting assay has ever been found to be predictive of the bleeding tendency in FVII deficiency.[4] This is probably due to the presence of trace amounts of FVIIa that are required to initiate coagulation.[6] Therefore classical clotting assays may not be sensitive enough, failing to differentiate the real absence of FVII from the presence of trace amounts of FVII. Previous genotype investigations revealed that very severe clinical phenotypes were associated with genotypes including FVII gene mutations, on both alleles, incompatible with the generation of FVIIa.[7] Considering these data, we hypothesized that residual FVII or FVIIa levels might indicate the bleeding potential of an individual with inherited FVII deficiency. To test this hypothesis, we examined a cohort of 42 patients with inherited FVII deficiency (clotting FVII activity (FVII:C) <30%) using highly sensitive clotting assays for FVII (FVII:C) and FVIIa (FVIIa:C) coagulant activities. In addition, we investigated a potential failure in the regulation of the catalytic function of the FVIIa/TF complex.

To this end, we measured the circulating free-form of TFPI antigen.

## Design and Methods

### *Plasma collection*

The panel consisted of 42 plasma samples from 25 females and 17 males with inherited FVII deficiency (FVII:C level below 30% of normal). The series included some bothers and sisters.[7] Clinical features, including epistaxis, menorrhagia, hematomas, hemarthrosis, digestive tract or cerebral hemorrhages and bleeding outcomes of hemostatic challenges with or without replacement therapy, were recorded by the physician in charge of each patient in a standardized questionnaire focused on bleeding traits. Plasma samples were collected in standard 0.129mol/L trisodium citrate tubes and sent frozen in dry ice to the central laboratory where the phenotype analyses were performed.

### *Coagulation assays*

The levels of both FVII antigen (FVII:Ag) and the free-form of TFPI antigen (TFPI:Ag) were determined by enzyme-linked-immunosorbant-assays using the Asserachrom™ FVII:Ag and the Asserachrom™ Free TFPI kits, respectively (Diagnostica Stago, Asnières-sur-Seine, France). Activated FVII levels (FVIIa:C) were determined using the Staclot™ VIIa-rTF kit (Diagnostica Stago, Asnières-sur-Seine, France). FVII coagulant activity (FVII:C) was assayed by a one-stage method based on the prothrombin time using human recombinant thromboplastin (Recombiplastin™ Instrumentation-Laboratory, Lexington, USA), an FVII-immunodepleted plasma, STA-deficient VII™ (Diagnostica Stago, Asnières-sur-Seine, France) as the substrate and STA-Unicalibrator™ (Diagnostica Stago, Asnières-sur-Seine, France) as the calibration plasma. The last two techniques were performed automatically (STA™ Diagnostica Stago, Asnières-sur-Seine, France) according to the manufacturer's instructions. The calibration curves were generated using 1/8, 1/16, 1/32 and 1/80 dilutions of working standard solutions, and the patients' plasma was diluted 1/5 in FVII-deficient reagent plasma. The FVII genotypes were characterized as previously described.[7]

### *Clinical classification*

Based on the clinical questionnaire, the 42 patients investigated were retrospectively classified by two independent physicians into two opposite groups using the classification proposed by Mac Vey *et al.*[8] Group A included patients with no bleeding history or with a mild bleeding diathesis including menorrhagia and epistaxis, but without a history of surgical or traumatic hemorrhage that required blood or FVII concentrate infusions. Group B comprised patients who presented with spontaneous hematoma, hemarthrosis, digestive tract or intracerebral hemorrhages or bleeding after haemostatic challenges that had required blood or FVII concentrate infusions.

### *Statistical analysis*

As the distribution of the numerous variables was not normal, descriptive statistics are given, using the median and range. Clinical groups were compared using non-parametric methods (Mann-Whitney test). When this Mann-Whitney test gave significant results ($p<0.05$), the cut-off was determined using the method of receiver-operating-characteristic (ROC)-curves.[9] Analyses were carried out using the statistical package SAS 8.2. To assess the reproducibility of both sensitive FVII:C and FVIIa:C methods, coefficients of variation (i.e., the ratio between the standard deviation and the mean) were calculated for the cut-off and surrounding values.

## Results

### *Bleeding risk parameters*

The FVII genotypes, clinical data and laboratory parameters (plasma FVII:C, FVII:Ag, FVIIa:C and free TFPI:Ag levels) of the 42 patients with inherited FVII deficiency (FVII:C<30%) are shown in Table 1. Medians and Mann-Whitney test values are summarized in Table 2. Both FVII:C and FVIIa:C hemostatic parameters were significantly different between the two clinical groups (severe bleeders and non-or-mild bleeders). For these tests, intra and inter-assay precision were determined by replicate analysis of plasma samples. The intra-assay coefficient of variation ranged from 2.5% to 3.6% for the FVII:C assay (n=8) and from 1.4% to 3.1% for the FVIIa:C assay (n=8). The inter-assay coefficient of variation ranged from 2.4% to 3.2% for FVII:C (n=4) and from 4.2% to 5.2% for FVIIa:C (n=4). Normal individuals have an average circulating FVIIa corresponding to approximately 1% of their total FVIIAg levels[10] or 100 mIU/mL (64-138) determined on 20 healthy individuals (*personal unpublished data*). Among the 42 FVII-deficient patients of our series, no correlation could be found between either FVIIa:C and FVIIAg or FVIIa and FVII:C levels.

### *ROC curve analysis*

The performances of both FVII:C and FVIIa:C assays were evaluated as predictive criteria for bleeding risk in FVII deficiency using ROC analysis. The underlying assumption of ROC analysis is that the diagnostic variable is used to discriminate between two mutually exclusive states, which correspond to *severe bleeders*

**Table 1. FVII genotypes, clinical and laboratory data of 42 patients highlighting the molecular heterogeneity of inherited FVII deficiency.**

| *Patients* | *FVII :C %* | *FVII :Ag %* | *FVIIa :C mIU/mL* | *TFPI %* | *FVII genotypes* | | *R353Q alleles* | *Clinical group* |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.64 | 66 | 0.92 | 9.5 | Cys310Phe | Cys310Phe | M1 M1 | A |
| 2 (I) | 0.69 | 8 | 0.83 | 14 | Gln100Arg | Gly97Cys | M1 M2 | A |
| 3 | 0.73 | 0.9 | 1.23 | 10.7 | Thr359Met | Thr359Met | M1 M1 | A |
| 4 | 0.76 | 9.3 | 0.88 | 5.6 | 11125 delC and Ala294Val | Leu13Gln and Ala294Val | M2 M2 | A |
| 5 | 0.96 | 17 | 1.37 | 9.5 | 3933+1G→A | 3762G→A | M1 M1 | A |
| 6 (II) | 1.11 | 56 | 0.43 | 2.2 | 10543 del gcgagcacgacctca | Cys310Phe | M1 M1 | A |
| 7 | 1.13 | 6 | 1.11 | 12.5 | Arg379Gly | Arg379Gly | M2 M2 | A |
| 8 (III) | 1.21 | 47 | 0.4 | 5.21 | Met327Ile | Met327Ile | M1 M1 | A |
| 9 | 1.21 | 19 | 0.98 | 7 | Cys102Tyr | Wt | M1 M1 | A |
| 10 (III) | 1.28 | 65 | 0.4 | 6.91 | Met327Ile | Met327Ile | M1 M | A |
| 11 | 1.71 | 80 | 0.57 | 4.2 | Gly331Ser | Gly331Ser | M1 M | A |
| 12 | 2.17 | 14 | 1.26 | 4.05 | 3762G→A | 3762G→A | M1 M1 | A |
| 13 | 2.33 | 40 | 1.64 | 4 | Met298Ile | Trp364Stop | M1 M1 | A |
| 14 | 2.41 | 17 | 1.05 | 9.5 | Arg277Cys | Arg28Gly | M1 M2 | A |
| 15 | 2.77 | 37 | 2.97 | 18.64 | Met298Ile | Wt | M1 M1 | A |
| 16 | 3.29 | 7 | 1.59 | 7.5 | ND | ND | ND | A |
| 17 | 3.85 | 15 | 1.32 | 15.1 | ND | ND | ND | A |
| 18 | 3.89 | 7 | 1.15 | 9.29 | Ala244Val | Wt | M2 M2 | A |
| 19 | 4.65 | 10 | 2.86 | 10.5 | Ala244Val | Gly179Arg | M2 M2 | A |
| 20 | 6.48 | 25 | ND | 6.23 | Ala191Thr | Ala191Thr | M1 M1 | A |
| 21 | 6.55 | 34 | 1.4 | 11.39 | Arg28Gly | Arg28Gly | M1 M1 | A |
| 22 | 7.4 | 65 | 1.52 | 63 | Arg304Trp | Arg304Trp | M1 M1 | A |
| 23 | 8.16 | 17 | 5.43 | 17.16 | Gly162Arg | Gly162Arg | M1 M1 | A |
| 24 | 11.12 | 27 | 1.3 | 5.85 | Arg152Stop | Thr324Met | M1 M1 | A |
| 25 | 13.55 | 55 | 1.32 | 6.75 | Arg304Gln | Cys135Arg | M1 M1 | A |
| 26 | 19.7 | 72 | 1.78 | 7.4 | Arg304Gln | Arg304Gln | M1 M1 | A |
| 27 | 28.62 | 75 | 3.66 | 6.2 | Arg304Gln | Arg304Gln | M1 M1 | A |
| 28 | 29.27 | 70 | 3.35 | 6.8 | Arg304Gln | Arg304Gln | M1 M1 | A |
| 29 | 0.59 | 7 | 0.36 | 50 | 6070+1G>A | Cys135Arg | M1 M1 | B |
| 30 | 0.6 | 8 | 0.92 | 7.5 | Gln49Stop | Gln100Arg | M1 M1 | B |
| 31 | 0.64 | 63 | 0.83 | 10 | Cys310Phe | Cys310Phe | M1 M1 | B |
| 22 | 0.67 | 0.9 | 0.88 | 14.8 | Cys135Arg | Cys135Arg | M1 M1 | B |
| 33 (I) | 0.71 | 8 | 0.85 | 7.6 | Gln100Arg | Gly97Cys | M1 M2 | B |
| 34 | 0.71 | 2 | 1.37 | 7.6 | Asn57Ile | Asn57Ile | M1 M1 | B |
| 35 | 0.74 | 65 | 0.72 | 5.6 | Phe328Ser | Asp343Asn | M1 M1 | B |
| 36 (II) | 0.82 | 58 | 1.1 | 4.05 | 10543 del gcgagcacgacctca | Cys310Phe | M1 M1 | B |
| 37 (III) | 0.88 | 52 | 0.4 | 3.76 | Met327Ile | Met327Ile | M1 M1 | B |
| 38 | 0.94 | 0.9 | 0.97 | 4.06 | -61T→C | -55C→T | M1 M1 | B |
| 39 | 1.01 | 25 | 0.48 | 10.74 | 5886+5G→A | Glu16Lys | M1 M1 | B |
| 40 | 3.31 | 2 | 0.4 | 3.76 | His348Gln | His348Gln | M1 M1 | B |
| 41 | 5.74 | 48 | 2.01 | 30.89 | 11125 delC and Ala294Val | Ala294Val | M2 M2 | B |
| 42 | 7.44 | 76 | 2.59 | 6.47 | Met298Ile | Met298Ile | M1 M1 | B |

*Clinical groups A and B correspond to non-or-mild bleeders and severe bleeders, respectively. I, II, III numbers referred to the three kindred. FVII:C, FVII:Ag and TFPI:Ag levels are expressed as percentages of normal pooled plasma samples. M1: allele Arg353 CGG, M2: allele Gln353 CAG. ND: not determined. Wt: wild type sequence.*



and *non-or-mild bleeders* in our study. Comparison of the test results against the true status of the individuals allows estimation of the diagnostic sensitivity (Se) and specificity (Sp).[9] Both Se and Sp are a function of the selected test value or *cut-off value*. ROC-analysis allows the diagnostic performance of a test, in terms of its Se and its Sp, to be estimated for each possible cut-off. All possible combinations of Se and Sp that can be calculated by changing the test's cut-off value are summarized using the area under the ROC-curve (AUC).[11] In our series, the AUC were calculated to be 0.784 (CI 0.63-0.94) and 0.716 (CI 0.54-0.88) for the FVII:C and FVIIa:C tests, respectively. According to an arbitrary guideline,[12] these values correspond to moderately accurate tests. The calculated cut-off value corresponding to the combination of the minimal per-

**Table 2. Clinical relevance of the five parameters tested in this study.**

| *Phenotype* | *A (n=28)* | *B (n=14)* | *Mann-Whitney test p value* |
|---|---|---|---|
| FVII :C (%) | 2.59 (0.69-28.62) | 0.78 (0.59-7.44) | 0.0026 |
| FVII :Ag (%) | 26 (6-75) | 16.5 (0.9-76) | NS |
| FVIIa :C (mU/mL) | 1.3 (0.4-3.66) | 0.86 (0.36-2.59) | 0.0258 |
| TFPI (%) | 7.2 (4-17.16) | 7.5 (3.76-50) | NS |

*Presented values indicate medians ($5^{th}$-$95^{th}$). NS means p>0.05. Group A includes non-or-mild bleeders whereas group B consists of patients with a severe bleeding phenotype.*

centages of false positives and false negatives was 1.01% (Se: 78.6%; Sp: 82.1%) for the FVII:C parameter. This cut-off value would lead to 21.4% of false negative cases (severe bleeders displaying values of FVII:C > 1.01%) being missed. Thus, such a mathematically calculated threshold cannot be retained. It is well recognized that Se and Sp at a single cut-off value do not describe a test's performance at other potential cut-off values. Therefore, to maximize the sensitivity of FVII:C and FVIIa:C tests, we selected values toward the upper part of the curve: 7.44% (Se: 100%; Sp: 21%) for the FVII:C test and 2.59 mIU/mL (Se: 100%; Sp: 18.5%) for the FVIIa:C parameter (Figure 1). These critical values were corrected according to the inter-assay coefficient of variation of each test: 7.67% (7.44 + [0.032×7.44]) and 2.72 mIU/mL (2.59 + [0.052× 2.59]) for FVII:C and FVIIa:C, respectively.

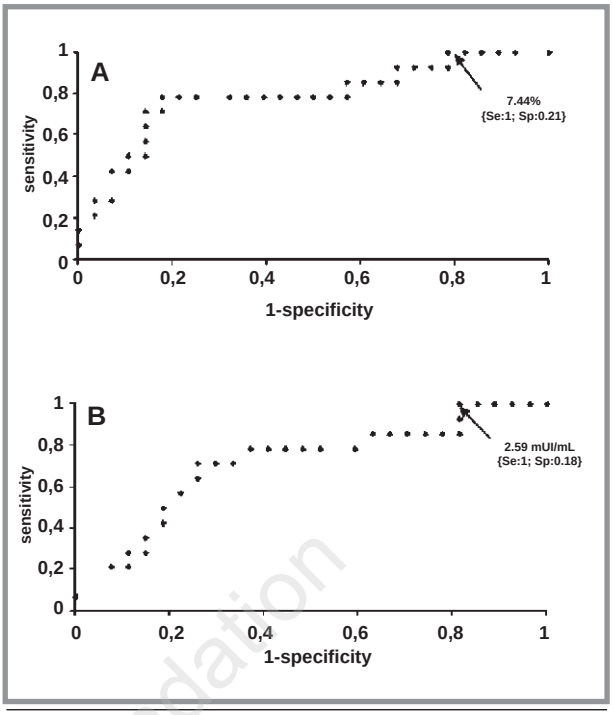


**Figure 1. ROC curves for FVII:C and FVIIa:C parameters. Critical values are indicated in labels. Se and Sp denote sensitivity and specificity, respectively. A: FVII:C ROC curve, B: FVIIa:C ROC curve.**

### *Kindred comparisons*

Brothers and sisters sharing the same FVII genotype displayed nearly the same FVII:C, FVII:Ag and FVIIa:C levels but had different clinical phenotypes (Table 3).

## Discussion

We analyzed the clinical relevance of four biological parameters involved in the first step of the clotting process in a large series of 42 patients with inherited



**Table 3. Laboratory phenotype of the 3 kindred.**

| *Kindred* | *Patient* | *Sex/Age* | *Clinical group* | *FVII :C %* | *FVII :Ag %* | *FVIIa :C mIU/mL* | *FVII genotypes* |
|---|---|---|---|---|---|---|---|
| I | 33 | F/46 | B | 0.71 | 8 | 0.85 | Gln100Arg / Gly97Cys |
| | 2 | M/51 | A | 0.69 | 8 | 0.83 | |
| II | 36 | F/23 | B | 0.82 | 58 | 1.1 | 10543del15bp / Cys310Phe |
| | 6 | F/20 | A | 1.11 | 56 | 0.43 | |
| III | 37 | M/14 | B | 0.88 | 52 | 0.4 | Met327Ile / Met327Ile |
| | 8 | M/11 | A | 1.21 | 47 | 0.4 | |
| | 10 | F/3 | A | 1.28 | 65 | 0.4 | |

*FVII :C and FVII:Ag values are expressed as percentage of normal plasmas. The genotypes were determined as previously described.*[7]

FVII deficiency (FVII:C<30%). In an effort to standardize the assays and in order to provide reproducible and sensitized clotting assays for FVII:C and FVIIa:C coagulant activities, we used recombinant human thromboplastin. Indeed, tissue factor extracts from other species can under or overestimate FVII levels for some FVII variants.[13] Statistical analysis of the four studied biological parameters showed that FVII:C and FVIIa:C were the only assays that differed significantly between severe and non-or-mild-bleeders. In agreement with literature data, there was no relationship between FVII:Ag levels and bleeding tendency.[5,14] In addition, no difference was found between the two clinical groups regarding the plasma levels of the free-form of TFPI:Ag, even though the plasma free-form of this antigen is supposed to have a stronger anticoagulant significance than the truncated lipoprotein-bound form.[15] In fact, plasma TFPI levels do not reflect the total body TFPI levels, since the endothelium-associated inhibitor accounts for 85% of total TFPI.[16] Further studies are expected to evaluate the whole inhibitory nature of the tissue factor induced pathway.

As FVII:C and FVIIa:C differed significantly between the two clinical groups of our series, we used ROC-curve analysis to determine whether these tests could be used to discriminate between severe bleeders and non-or-mild bleeders. Analyses of the results argued against these parameters having highly accurate discriminatory power. First, the area under the ROC-curve, which is a global summary statistic of the test's accuracy, had low values (0.5< AUC<0.9). A diagnostic test with a good ability to discriminate between severe and non-or-mild bleeders would have an AUC of nearly 1. Second, siblings had nearly the same laboratory values for FVII parameters but had different clinical phenotypes (Table 3), as previously reported by a study on thrombin generation.[17] This last point highlights the real puzzle about inherited FVII deficiency. These patients shared the same FVII genotype, including mutations and polymorphisms in the FVII gene, which have been shown to contribute to circulating FVII concentrations.[18] Indeed, twin studies in normal populations reported that genetic factors contribute 63% of the variations of FVII levels.[19] Likewise, as previously published, patients bearing the same FVII genotypes could display different clinical phenotypes. For instance, patients with a homozygous His348Gln mutation have been reported at least three times (FVII mutation database, *http://europium.csc.mrc.ac.uk*) and once in our study. Of these patients, two were classified as having a mild phenotype whereas the other two presented with a severe bleeding diathesis. By contrast, some FVII genotypes, such as a homozygous Arg304Gln mutation, have never been found to be associated with severe bleeding phenotypes. Thus, the lack of correlation between clinical and laboratory phenotypes probably reflects the fact that other factors are involved in the expression of bleeding tendency in FVII deficiency. These could be other hemostatic factors such as TF, von Willebrand factor, platelets or unknown regulators. TF expression may vary between people due to genetic polymorphisms or environmental factors. As classical clotting assays use an excess of TF extracts, private variations of endogenous TF were not evaluated. Even though patients with von Willebrand's disease were excluded from our series, borderline levels of Willebrand factor are common and could increase the severity of a bleeding tendency in patients with inherited FVII deficiency.[20] In addition, it has been suggested that activated platelets might provide a suitable physiologic surface for the action of FVIIa:C independently of TF. Moreover, it was observed that similar numbers of platelets taken from different individuals and assayed at the same time with the same TF-initiated model system, generated variable levels of thrombin.[21] Thus, it is tempting to speculate that this variable platelet *activity* might be relevant in limiting FVII levels and might interfere with the bleeding tendency of FVII-deficient patients.

Even though neither FVII:C nor FVIIa:C can be considered as perfect discriminators between clinical groups, these parameters differed significantly between severe and mild-or-non bleeders. Our main goal was to find tests with critical values that would not miss false negative cases (severe bleeders displaying values above the critical thresholds). Indeed, missing any patient with a severe bleeding tendency would have serious consequences. Therefore, these critical values should be selected towards the upper part of the ROC-curve to maximize the sensitivity. Thus, we considered, for both the FVII:C and FVIIa:C parameters, the first values associated with a 100% sensitivity in our series. These values were corrected according to the coefficient of variation of each test and then adjusted to 8% (for FVII:C) and 3 mUI/mL (for FVIIa:C) for routine use in clinical laboratories. Considering these data, we assumed that biological phenotypes displaying levels higher than 8% for FVII:C or 3mUI/mL for FVIIa:C are associated with an extremely low risk of severe bleeding (defined as hemorrhage requiring blood or FVII concentrate infusions) during surgery. This is true in our series, but the FVII:C threshold determined in the present study also concords with many literature reports of levels in inherited FVII deficiency patients who did not experience excessive bleeding after surgery without replacement therapy.[5,22-23]

In conclusion, an accurate diagnostic test for discriminating between bleeders and non-bleeders remains to be defined in inherited FVII deficiency.

Studying platelet function in these patients might open interesting new trails. On the other hand, this retrospective study of 42 patients with inherited FVII deficiency allows us to propose potential critical values of classical FVII:C and FVIIa:C parameters that may be useful in clinical laboratories for FVII-deficient patients. According to the results of these sensitive methods, those patients with FVII:C levels higher than 8% or FVIIa:C higher than 3 mIU/mL with no other hemostatic abnormality, seem to have a minimal risk of severe bleeding. Additional clinical studies of other FVII deficient patients are needed to validate these findings.

*MG-B performed most of the laboratory analyses, wrote the paper and was involved in the design of the study, selection of patients, collection of plasma samples, analysis and interpretation of the data. RV performed the statistical analysis, CB-A was involved in the design of the study, JFS was involved in analysis and interpretation of data and supervised the whole study. He also played a part in the selection of patients. All the authors revised the manuscript and contributed to its intellectual content. The members of the study group of FVII deficiency, listed at the beginning of the paper, provided the 42 plasma samples.*

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