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ORIGINAL ARTICLE

Prediction of factor VIII inhibitor development in the SIPPET cohort by mutational analysis and factor VIII antigen measurement

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Essentials

- A residual factor VIII synthesis is likely to be protective towards inhibitor (INH) development.
- Mutation type-inhibitor risk association was explored in 231 patients with severe hemophilia A.
- A 2-fold increase in INH development for *in silico* null vs. non-null mutations was found.
- A 3.5-fold increase in INH risk for antigen negative vs. antigen positive mutations was found.

Summary. *Background:* The type of *F8* mutation is the main predictor of inhibitor development in patients with severe hemophilia A. Mutations expected to allow residual synthesis of factor VIII are likely to play a protective role against alloantibody development by inducing immune tolerance. According to the expected full or partial impairment of FVIII synthesis, *F8* variants are commonly classified as null and non-null. *Objectives:* To explore the mutation type–inhibitor risk association in a cohort of 231 patients with severe hemophilia A enrolled in the Survey of Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) randomized trial. *Methods:* The genetic defects in these patients, consisting of inversions of intron 22 (n = 110) and intron 1 (n = 6), large deletions (n = 16), and nonsense (n = 38), frameshift (n = 28), missense (n = 19) and splicing (n = 14) variants, of which 34 have been previously unreported, were reclassified according to two additional criteria: the functional effects of missense and splicing alterations as predicted by multiple *in silico* analyses, and the levels of FVIII antigen in patient plasma. *Results:* A two-fold increase in inhibitor development for *in silico* null mutations as compared with *in silico* non-null mutations (hazard ratio [HR] 2.08, 95% confidence interval [CI] 0.84–5.17) and a 3.5-fold increase in inhibitor development for antigen-negative mutations as compared with antigen-positive mutations (HR 3.61, 95% CI 0.89–14.74] were found. *Conclusions:* Our findings confirm an association between the synthesis of minute amounts of FVIII and inhibitor protection, and underline the importance of investigating the residual FVIII antigen levels associated with causative variants in order to understand their clinical relevance.

Keywords: antigen; factor VIII; hemophilia A; *in silico*; neutralizing antibodies.

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Introduction

Hemophilia A (OMIM #306700), which is one of the most common congenital coagulopathies, is characterized by variations in the frequency and severity of bleeding episodes related to the residual amount of factor VIII coagulant (FVIII:C) activity in patient plasma [1]. The current therapeutic approach for hemophilia A is aimed at correcting the inherited deficiency of FVIII by the administration of plasma-derived or recombinant FVIII products. This replacement therapy improves the management of the hemorrhagic diathesis, reduces mortality, and improves the quality of life for patients [2].

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A serious complication of this therapy is the development of inhibitors (i.e. neutralizing alloantibodies against FVIII), which nullify treatment benefits. The degree and severity of FVIII inactivation depend on the level of inhibitor present (> 5 Bethesda units [BU]) [1]. In these cases, hemostasis can be achieved only by using FVIII-bypassing agents [3], with a high cost of treatment. Inhibitor eradication is successful in approximately two-thirds of cases by means of immune tolerance induction, but with exorbitant costs [4].

The inhibitor incidence is associated with the degree of FVIII deficiency, as it occurs in only 3–13% of patients with mild and moderate hemophilia A, but in > 30% (of whom 60% have high titers) of those with severe hemophilia A [5]. The residual amount of endogenous FVIII offers a likely explanation for this difference, through the induction of natural immune tolerance [6]. Furthermore, established determinants of inhibitor formation are the patient's genetic background, and environmental factors [7–10]. Among them, the type of mutation in *F8* is the strongest risk factor for inhibitor development [11,12].

A wide spectrum of defects in *F8* are associated with severe hemophilia A [12]. A recent meta-analysis showed a high inhibitor risk for patients with severe hemophilia A carrying large deletions and nonsense variants, a medium risk for those carrying splicing variants and intron 1 and intron 22 inversions, and a low risk for those carrying small deletions/insertions and missense variants [12]. According to the putative degree of gene disruption and the genetically predicted lack of protein synthesis, genetic variants causing hemophilia A have also been classified as null and non-null [13].

In this study, the role of null and non-null *F8* mutations in inhibitor risk was evaluated in a cohort of 231 patients with severe hemophilia A enrolled in the framework of the Survey of Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) study [14]. We also assessed whether or not additional criteria, such as *in silico* analysis and plasma levels of FVIII antigen (FVIII:Ag), could help to improve the evaluation of inhibitor risk based on the null/non-null classification.

Materials and methods

Patient cohort

All cases analyzed in the present study were previously untreated or minimally treated patients with severe hemophilia A enrolled in the SIPPET study [14]. The patients were randomized 1 : 1 to receive plasma-derived or recombinant FVIII, and followed up to inhibitor development, for 50 exposure days (EDs), or for 3 years, whichever occurred first, or censored when follow-up was shorter.

At screening and before any FVIII exposure, severe deficiency of FVIII:C (< 0.01 IU mL^{-1}) and the absence of FVIII inhibitors (≤ 0.4 BU) were verified in patient plasma at the local laboratories, and confirmed at the central laboratory of the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center by means of the one-stage functional assay [15] and the Bethesda assay with the Nijmegen modification [16]. FVIII:Ag levels were measured at screening by enzyme immunoassay with the Asserachrom VIII:Ag kit (Stago, Asnières sur Seine, France) in all analyzed patients. After the first infusion of an FVIII-containing product, inhibitors were evaluated regularly every 2 weeks in patients receiving prophylactic treatment, and every 3–4 EDs during the first 20 infusions, and then every 10 EDs up to 50 consecutive EDs or every 3 months, whichever came first, plus at the final visit in on-demand-treated patients [14]. Positivity for inhibitors (> 0.4 BU) was confirmed twice within 14 days both at local and at central laboratories, and this was followed by monthly tests for the subsequent 6 months.

Detection of mutations in F8

Genomic DNA was extracted from 5 mL of peripheral blood samples collected in EDTA with the standard salting-out method [17]. Mutational scanning of *F8* was accomplished with a multistep approach. Detection of intron 22 and intron 1 inversions was performed with long-range PCR, as previously described [18,19]. Point mutation detection was performed by bidirectional sequencing of amplicons encompassing coding regions, splicing junctions, and 5′ and 3′ untranslated regions, as previously reported [20]. Exonic deletions were identified in PCR-negative samples by multiplex ligation-dependent probe amplification (MLPA) with the MLPA P178-A1 kit (MRC-Holland, Amsterdam, the Netherlands), according to the manufacturer's instructions. To rule out duplications, MLPA was also performed in samples negative for inversions and point mutations.

In silico *analyses*

The deleterious effects of missense variants were assessed with the web-based tools POLYPHEN-2 (http://genetics.bwh.harvard.edu/pph2/), CADD (http://cadd.gs.washington.edu/home), and MUTPRED (http://mutpred.mutdb.org/). The effects of variants at splice junctions were evaluated with ALAMUT VISUAL v.2.8.1 (http://www.interactive-biosoftware.com/alamut-visual/), which allows a simultaneous analysis with the programs SPLICE SITE FINDER-LIKE, MAXENT SCAN, NEURAL NETWORK SPLICE SITE, GENESPLICER, and HUMAN SPLICING FINDER, and with the NETGENE2 prediction tool (http://www.cbs.dtu.dk/services/NetGene2/). The impact on exonic splice enhancer motifs was evaluated with ESE FINDER 3.0 (rulai.cshl.edu/). Transcript ID ENST00000360256.8 from the Ensembl genome browser (https://www.ensembl.org/) was used to predict the effects of large deletions on FVIII protein.

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Statistical analysis

Kaplan–Meier survival analyses were performed to assess the cumulative incidence of inhibitors according to mutation groups. Incidence rates were compared by the use of Cox regression survival analyses, and CIs were obtained from this model. Statistical analyses were performed with SPSS, version 23.0 (IBM Corp., Armonk, NY, USA).

Results

Mutational spectrum

Of 251 randomized patients with severe hemophilia A involved in the SIPPET study [14], 235 provided blood and genomic DNA samples suitable for performance of *F8* mutational scanning. They came from 13 countries: India (82), Egypt (70), Iran (30), the USA (16), Italy (eight), Mexico and Spain (five each), Chile, Austria and Brazil (four each), Turkey (three), and Argentina and South Africa (two each). Genetic analysis of *F8* enabled the identification of a putative causative variant in 231 of them: 110 intron 22 and six intron 1 inversions, 16 single and multi-exon deletions, and 99 point mutations (mutation nomenclature according to the guidelines of the Human Genome Variation Society [http://varnomen.hgvs.org/] and to the *in silico* prediction described below) (Table 1).

Comparison of the mutational spectrum in the SIPPET cohort with that reported in a meta-analysis involving 5383 severe hemophilia A patients [12] showed a similar distribution, with nearly half of the patients carrying the intron 22 inversion, and a somewhat higher frequency of nonsense variants and lower frequency of missense variants (Fig. 1A). Similar results were found when non-redundant alterations listed in the FVIII Variant Database (http://www.factorviii-db.org/) were used (Fig. 1B). In Indian and Iranian patients, representing 35% and 13% of our cases, the mutation distribution was similar to those in previous reports from those countries [21–23].

Nonsense and frameshift variants

Nonsense and frameshift variants were scattered across the entire FVIII protein, and were mainly localized in the large B domain (Fig. 2A,B). Fourteen nonsense and eight frameshift variants were previously unreported (Fig. 2A,B). Nine recurrent variants were found in two or three patients (Fig. 2A,B). As it is known that *F8* transcripts harbouring premature stop codons escape degradation by nonsense-mediated decay [24], these nonsense and frameshift variants are predicted to cause the synthesis of FVIII proteins with C-terminal truncations ranging from 1.1% (p.Arg2326*) to 99.5% (p.Cys12*), and from 3.3% (p.Met2274Asnfs*79+32) to 97.6% (p.Phe57Serfs*25), respectively (Fig. 2A,B). The addition of variable stretches of aberrant amino acids (ranging from two to 111) at the C-terminus can also be predicted for frameshift variants (Fig. 2B). Taken together, these genetic defects feature a wide spectrum of possible effects on plasma FVIII levels related to the loss of specific FVIII domains.

Traces of FVIII:Ag were detected in two patients carrying the p.Tyr124* and p.Ser1026* nonsense variants (FVIII:Ag 1.4% and 3.2%, respectively) and the p.Ser946Ilefs*5 frameshift variant (FVIII:Ag 2%). Plasma FVIII:Ag was undetectable (FVIII:Ag < 1%) in patients carrying other nonsense and frameshift variants; no plasma samples of patients with p.Arg52*, p.Asn609* and p.Arg1985* were available.

Whereas the disease association of inversions in hemophilia A is well established and that of large deletions and truncating variants is self-explanatory, this is not the case for single or multiple missense and splicing variants. To distinguish deleterious from benign variants according to their predicted pathogenetic role, we

Table 1 Mutation distribution according to country

Country	Mutations, *n* (%)								
	Intron 22 inversion	Intron 1 inversion	Nonsense	Frameshift	Missense	Large deletion	Splicing	Unknown	Total
India	39 (47.6)	3 (3.7)	12 (14.6)	11 (13.4)	6 (7.3)	5 (6.1)	3 (3.7)	3 (3.7)	82 (34.9)
Egypt	34 (48.6)	1 (1.4)	10 (14.3)	8 (11.4)	6 (8.6)	4 (5.7)	7 (10.0)	0 (0.0)	70 (29.8)
Iran	12 (40.0)	1 (3.3)	5 (16.7)	3 (10.0)	5 (16.7)	1 (3.3)	3 (10.0)	0 (0.0)	30 (12.8)
USA	5 (31.3)	0 (0.0)	4 (25.0)	2 (12.5)	1 (6.3)	3 (18.8)	1 (6.3)	0 (0.0)	16 (6.8)
Italy	4 (50.0)	0 (0.0)	3 (37.5)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	8 (3.4)
Mexico	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	5 (2.1)
Spain	4 (80.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (2.1)
Chile	0 (0.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.7)
Austria	3 (75.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.7)
Brazil	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	1 (25.0)	1 (25.0)	0 (0.0)	1 (25.0)	4 (1.7)
Turkey	2 (66.7)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.3)
Argentina	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.85)
South Africa	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.85)
Total	110 (46.8)	6 (2.5)	38 (16.2)	28 (11.9)	19 (8.1)	16 (6.8)	14 (6.0)	4 (1.7)	235 (100.0)

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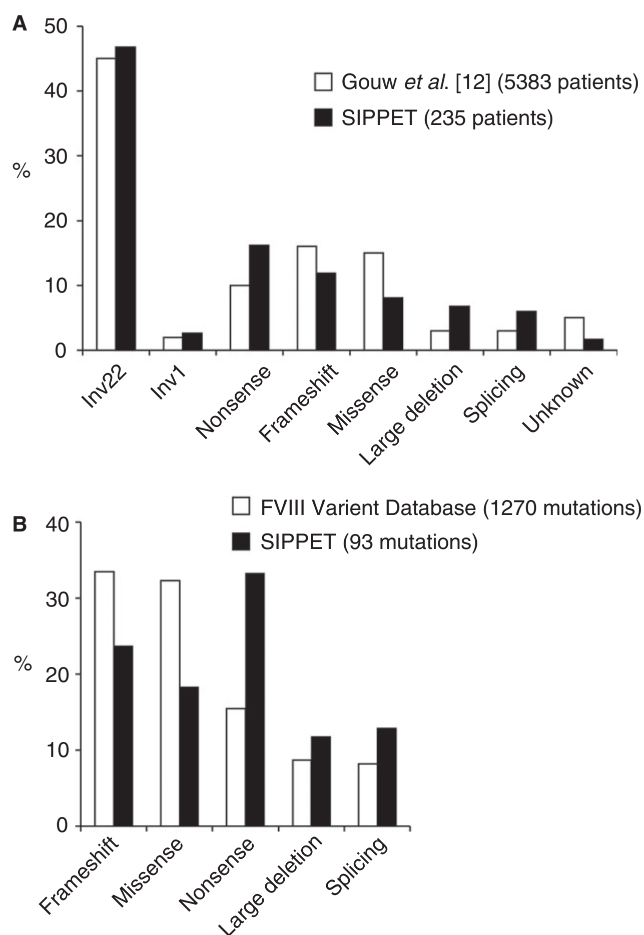

Fig. 1. Spectrum of *F8* mutations in different cohorts of patients with severe hemophilia A. (A) Percentages of *F8* mutation types. (B) Percentages of different types of unique mutation. Inv1, intron 1 inversion; Inv22, intron 22 inversion; SIPPET, Survey of Inhibitors in Plasma-Product Exposed Toddlers.

performed multiple *in silico* analyses on all the synonymous and non-synonymous substitutions (i.e. missense variants) (Table 2), and on the nucleotide substitutions localized in exon–intron boundaries that may affect donor and acceptor splice sites (i.e. splicing variants) (Table 3).

In silico *analysis of missense variants*

All of the 28 variants listed in Table 2 were analyzed by means of two of the most widely accessed prediction software packages, i.e. POLYPHEN-2 and MUTPRED, which base their prediction on information derived from multiple sequence alignment and the combination of structural (POLYPHEN-2) and functional (MUTPRED) parameters [25]. We also used the recently developed CADD software [26], which combines a wide range of annotations, including protein level scores (like POLYPHEN-2), functional genomic data, and transcript information.

All algorithms indicated disease-causative associations for 17 missense variants, four of them (p.Asn109Asp, p.Pro165Thr, p.Asp561Val, and p.Gly2107Val) not previously reported (Table 2; Fig. 2C). These missense variants were localized in the A1, A2, A3 and C1 FVIII domains, and two of them were found in two patients (Fig. 2C). POLYPHEN-2 predicted probable deleterious effects for these variants, with the highest score of 1 (Table 2). Concerning the MUTPRED output, a high probability of deleterious variants, indicated by a general score ranging from 0.669 to 0.987, was provided along with information on the potential pathogenetic molecular mechanisms and the likelihood of disease association (Table 2). CADD prediction allocated these variants to the range of the 0.1–1% most harmful substitutions, with scores ranging from 23.5 to 35 (Table 2). Taken together, these analyses suggest that all of the 17 missense variants are likely to be causative of hemophilia A. Conversely, no pathogenicity was predicted for the p.Arg1740Lys substitution, which is a new and unique genetic variant found in one patient only (Table 2).

In addition, no pathogenic effects were predicted for five previously reported polymorphisms and three new variants (p.Ile3Val, p.His1207=, and p.Ser1572=) (Table 2). Possible deleterious effects were predicted for two variants: the new p.Leu882Arg and p.Glu2023Lys,

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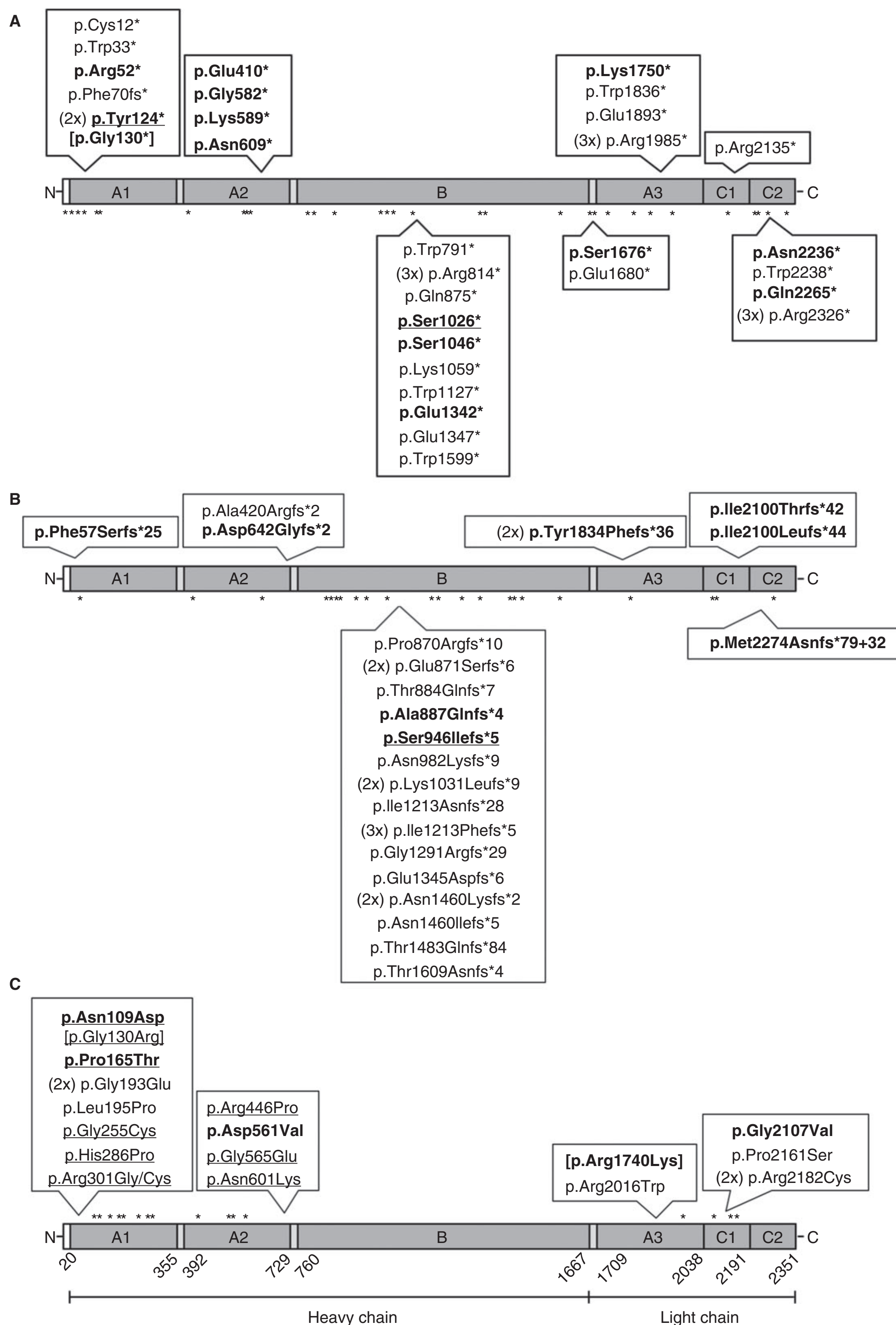

Fig. 2. Localization on the FVIII protein of nonsense, frameshift and missense mutations. (A–C) List and distribution of nonsense (A), frameshift (B) and missense (C) mutations. A schematic representation of FVIII domains A1, A2, B, A3, C1 and C2 drawn to scale is shown in each panel. Numbers below the FVIII protein in (C) refer to the amino acid boundaries of the different domains. Mutations are grouped according to their domain localization, and their distribution along the FVIII molecule is indicated by asterisks. Mutations not reported in the CHAMP Mutation database, EAHAD Coagulation Factor Variant Database or Human Gene Mutation Database are in bold. Mutations that recur in two or three patients are indicated in parentheses as 2× and 3×. Mutations in square brackets are predicted to have multiple effects at the protein level (see *in silico* analyses), and those with measurable FVIII antigen levels are underlined.

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Table 2 *In silico* analyses of the identified single amino acid substitutions

Variant*	Exon	cDNA†	POLYPHEN-2 Score prediction‡	CADD PHRED§	General score (g)	MUTPRED¶ Molecular mechanism disrupted (p)	Hypotheses	FVIII: Ag (%)
Missense								
p.Asn109Asp	3	c.325A>G	Probably damaging: 1	26.1	0.886	—	—	1.9
p.Gly130Arg	3	c.388G>A	Probably damaging: 1	27.2	0.773	Gain of solvent accessibility (0.0037)	Actionable	12.2
p.Pro165Thr	4	c.493C>A	Probably damaging: 1	25.4	0.669	—	—	1.2
p.Gly193Glu	4	c.578G>A	Probably damaging: 1	25.4	0.954	—	—	< 1
p.Leu195Pro	4	c.584T>C	Probably damaging: 1	24.5	0.925	Loss of stability (0.0239)	Confident	< 1
p.Gly255Cys	6	c.763G>T	Probably damaging: 1	32.0	0.987	—	—	1.5
p.His286Pro	7	c.857A>C	Probably damaging: 1	26.1	0.823	—	—	16.5
p.Arg301Gly	7	c.901C>G	Probably damaging: 1	32.0	0.763	—	—	1.2
p.Arg301Cys	7	c.901C>T	Probably damaging: 1	34.0	0.895	Loss of disorder (0.0429)	Confident	4.3
p.Arg446Pro	9	c.1337G>C	Probably damaging: 1	25.4	0.916	Loss of solvent accessibility (0.0299) Loss of loop (0.0374) Gain of helix (0.0425) Loss of sheet (0.0457) Gain of methylation at Lys444 (0.0481) Loss of MoRF binding (0.0029)	Confident Very confident	1
p.Asp561Val	11	c.1682A>T	Probably damaging: 1	28.9	0.891	—	—	< 1
p.Gly565Glu	11	c.1694G>A	Probably damaging: 1	30.0	0.845	Loss of catalytic residue at Gly565 (0.003)	Very confident	1.3
p.Asn601Lys	12	c.1803C>G	Probably damaging: 1	23.5	0.859	Gain of relative solvent accessibility (0.0166) Gain of ubiquitination (0.0185) Gain of solvent accessibility (0.0058) Gain of methylation at Asn601 (0.0087)	Confident Very confident	1.6
p.Arg1740Lys	14	c.5219G>A	Benign: 0.003	14.44	0.360	—	—	< 1
p.Arg2016Trp	19	c.6046C>T	Probably damaging: 1	34.0	0.933	Loss of sheet (0.0228)	Confident	< 1
p.Gly2107Val	22	c.6320G>T	Probably damaging: 1	34.0	0.926	Loss of disorder (0.0194)	Confident	< 1
p.Pro2161Ser	23	c.6481C>T	Probably damaging: 1	29.3	0.773	Loss of catalytic residue at Pro2161 (0.0083)	Actionable	< 1
p.Arg2182Cys	23	c.6544C>T	Probably damaging: 1	35.0	0.935	Gain of sheet (0.0344) Loss of MoRF binding (0.0474)	Confident	< 1
Polymorphism								
p.Ile3Val	1	c.7A>G	Benign: 0.003	0.002	0.185	—	—	/
p.Ala362=	8	c.1086G>A	NA	12.85	NA	NA	NA	/
p.Leu882Arg	14	c.2645T>G	Possibly damaging: 0.933	1.511	0.521	Loss of sheet (0.0037) Gain of loop (0.0079) Loss of stability (0.0126) Gain of methylation at Leu882 (0.018) Gain of disorder (0.0433)	Actionable	/
p.Arg1126Trp	14	c.3376A>T	Benign: 0.139	16.01	0.337	—	—	/
p.His1207=	14	c.3621C>T	NA	0.053	NA	NA	NA	/
p.Asp1260Glu	14	c.3780C>G	Benign: 0.003	0.002	0.096	—	—	/
p.Ser1288=	14	c.3864A>C	NA	0.105	NA	NA	NA	/
p.Ser1572=	14	c.4716C>T	NA	0.114	NA	NA	NA	/
p.Glu2023Lys	19	c.6067G>A	Possibly damaging: 0.741	28.9	0.840	Gain of ubiquitination at Glu2023 (0.023) Loss of stability (0.028) Gain of glycosylation at Glu2023 (0.0395)	Confident	/
p.Met2257Val	25	c.6769A>G	Benign: 0.152	7.091	0.600	Gain of methylation at Lys2258 (0.0387)	Actionable	/

FVIII:Ag, factor VIII antigen level expressed as a percentage (%) of normal plasma level and reported for the missense variants and not (/) for polymorphisms; g, general score; MoRF, Molecular Recognition Features; NA, not available; p, property score; —, no hypotheses regarding the molecular mechanism ($g > 0.5$ and $p > 0.05$). *Numbering refers to RefSeq NP_000123.1. Variants previously unreported are in bold. †Numbering refers to RefSeq NM_000132.3. ‡POLYPHEN-2 scores ranging from 0 (most probably benign) to 1 (most probably damaging). §CADD scaled score ranking a variant relative to all possible substitutions of the human genome in order of magnitude terms. Values of ≥ 20 indicate that these are predicted to be the 1% most deleterious substitutions in the human genome. ¶MUTPRED indicates the probability of a deleterious mutation according to the general score (g), information about the possible pathogenetic molecular mechanisms according to the property score (p), and the hypotheses of disease association on the basis of the combination of g and p scores: actionable ($g > 0.5$ and $p < 0.05$); confident ($g > 0.75$ and $p < 0.05$); and very confident ($g > 0.75$ and $p < 0.01$).

listed in the CHAMP *F8* Mutation Database, and associated with mild FVIII deficiency (FVIII:C > 0.05 IU mL^{-1}) (Table 2). Concerning p.Leu882Arg, all of the POLYPHEN, MUTPRED and CADD scores were below the lowest score for causative alterations; only the POLYPHEN score was below the lowest score for p.Glu2023Lys. The amino acid leucine 882 is localized in the FVIII B domain, where five amino acid substitutions at surrounding positions 806, 873, 963, 998 and 1225 have been experimentally demonstrated to be non-deleterious [27]. The potential impacts of p.His1207= (c.3621C>T) and p.Ser1572= (c.4716C>T) on exonic splice enhancer motifs were assessed with ESE FINDER. No disruptions of ESE motifs were predicted in either case. Taken together, these observations suggest that all of these 10 amino acid substitutions are likely to be neutral polymorphisms, but that p.Glu2023Lys may affect protein function to some degree. As p.Leu882Arg and p.Glu2023Lys were identified with

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p.Thr884Glnfs*7 and p.Ser1676* truncating variants, they are not expected to be the cause of disease in the corresponding patients. Among patients carrying missense variants, 10 had measurable FVIII:Ag levels up to 16.5% (Table 2).

In silico analysis of splicing variants

All of the 16 nucleotide variations identified in introns and at the exon–intron junctions were analyzed *in silico* with the prediction programs SPLICE SITE FINDER-LIKE, MAXENT SCAN, NEURAL NETWORK SPLICE SITE, GENESPLICER, and HUMAN SPLICING FINDER. Twelve variations that affect the conserved dinucleotides of donor and acceptor splice sites and the fourth and fifth nucleotides of donor splice sites were predicted to cause the disappearance of the corresponding physiological splice sites (Table 3). They include the unreported c.389-1G>A (found in two patients) and the c.601+2insC, c.670+1G>A, c.766_787+1del, c.1537+1G>T, c.5374-2A>G and c.6115+4delA variants. In only two cases (c.601+2insC and c.1903+5G>A) did some software programs predict weakening of the wild-type splice site instead of its complete abolishment. An additional *in silico* analysis of these variations, performed with the NETGENE2 prediction tool, confirmed the disappearance of the physiological splice sites (data not shown). In some cases, the activation of cryptic splice sites around the mutated site was predicted (Table 3). In addition to these splicing variants, four intronic variants (including the unreported c.388+76C>T and c.5586+7A>G transitions) were predicted not to affect the wild-type splice sites, and were thus considered to be polymorphisms (Table 3).

Moreover, effects on transcript processing of nucleotide substitutions affecting the last base of exon 3 (c.388G>T, p.Gly130*; c.388G>A, and p.Gly130Arg) and exon 14 (c.5219G>A and p.Arg1740Lys) were evaluated *in silico* (Table 3; Fig. 2A,C). In all cases, the predicted weakening or abolishment of the corresponding splice site suggested an alteration of the splicing outcome (Table 3). Therefore, p.Gly130* can be considered to be a nonsense/splicing variant, the p.Gly130Arg a missense/splicing alteration, and the new p.Arg1740Gly, previously predicted to be a benign missense variant, a putative causative splicing variant (Table 3). Among patients carrying splicing variants, four had detectable trace of FVIII:Ag up to 1.6% (Table 3).

Large deletions

All of the large deletions listed in Table 4 were previously described in patients with severe hemophilia A, four of them having been reported in several patients. In our cohort, 203 patients (87.9%) had FVIII:Ag plasma levels below 1%, and 23 patients (10%) had measurable FVIII:Ag levels ranging from 1% to 16.5%. Among the 16 patients with large deletions, five (31.3%) had small but measurable amounts of plasma FVIII:Ag, ranging from 1.7% to 13.5%. In particular, these patients shared the deletions of exon 6 and of exons 5 + 6 (Table 4). As exons 5 and 6 have 69 and 117 nucleotides, respectively, skipping of these exons could cause in-frame deletions and synthesis of FVIII proteins with an A1 domain lacking 39 (exon 6 deletion) and 62 (exon 5 + 6 deletion) amino acids.

Inversions of intron 1 and intron 22

No traces of FVIII:Ag (<1%) were detected in the six patients with inversion of intron 1 or in 108 of 110 patients with inversion of intron 22. Residual traces of FVIII:Ag (1%) were found in one patient carrying the intron 22 inversion, and no plasma sample of the remaining patient was available.

Inhibitor occurrence and type of F8 mutation

Seventy-two of 231 patients with a variant identified as causing hemophilia A (31.2%) developed an FVIII inhibitor, with a cumulative inhibitor incidence of 36.3% (95% CI 29.4–43.2) during the 50 EDs of FVIII replacement therapy. In order to evaluate the association between *F8* variants and inhibitor development, all of the 231 causative variants were first grouped into low-risk and high-risk mutations according to Gouw *et al.* [28] (Fig. 3A). Only a small difference was observed in the cumulative inhibitor incidence (32.0% [95% CI 18.9–45.1] versus 37.9% [95% CI 29.9–45.9] for low-risk and high-risk mutations classified as above, corresponding to a hazard ratio [HR] of 1.35 [95% CI 0.78–2.35]) (Fig. 3A).

To evaluate the role of trace amounts of FVIII on immune tolerance to this protein and thus inhibitor incidence, all 231 variants were further classified as null and non-null according to their expected inability or ability to synthesize some dysfunctional FVIII protein, according to the criteria of Carcao *et al.* [13] (Fig. 3B). The cumulative inhibitor incidence rates were 38.5% (95% CI 31.1–45.9) for null mutations and 23.8% (95% CI 6.7–40.9) for non-null mutations, with an HR of 2.01 (95% CI 0.87–4.63) (Fig. 3B).

The same analysis was taken further on groups of null and non-null mutations reclassified according to their deleteriousness as predicted *in silico*. In particular, the c.1903+5G>A and c.6115+4delA variations, which failed to affect canonical splice site dinucleotides but were predicted to abolish the physiological ones, the c.388G>A transition, predicted to be either a missense or a splicing variant, and the c.5219G>A nucleotide substitution, predicted to be a splicing variant instead of a missense mutation, were all shifted from the non-null to the null-mutation category (Fig. 3C). The cumulative incidence of inhibitor development remained approximately similar:

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Table 3 *In silico* analyses of the identified variants localized in introns and exon–intron junctions

Variant	Ex/Int	In silico analysis SSF	MaxEnt	NNSplice	GeneSplicer	HSF	Cryptic ss score	Predicted effect	FVIII:Ag, %
Nonsense/Splicing									
p.Gly130*/c.388G>T	ex 3	86.37⇒73.76	9.25⇒6.96	0.99⇒0.80	2.14⇒—	93.03⇒82.16	70.29(HSF)	2-bp deletion of ex 3	<1
Missense/Splicing									
p.Gly130Arg/c.388G>A	ex 3	86.37⇒74.23	9.25⇒7.36	0.99⇒0.87	2.14⇒0.64	93.03⇒82.45	np	np	12.2
p.Arg1740Lys/c.5219G>A	ex 14	84.50⇒72.36	9.46⇒4.72	0.98⇒—	3.12⇒—	88.86⇒78.2	np	np	<1
Splicing									
(2x) c.389-1G>A	int 3	85.13⇒—	9.25⇒—	0.91⇒—	7.51⇒—	85.87⇒—	73.04(SSF) 3.75(MaxEnt) 76.92(HSF)	1-bp deletion of ex 4	1.1/1.6
c.601 + 2insC	int 4	99.69⇒72.64	11.00⇒1.99	1.00⇒—	4.46⇒—	99.97⇒81.53	67.29(HSF)	5-bp inclusion of int 4	1.3
c.670 + 1G>A	int 5	89.82⇒—	8.54⇒—	0.98⇒—	na	94.02⇒—	0.79(NNSplice)	4-bp inclusion of int 5	<1
c.766_787 + 1del	ex/int 6	89.83⇒—	9.80⇒—	1.00⇒—	6.64⇒—	100.00⇒—	np	np	<1
c.787+1G>A	int 6	89.83⇒—	9.80⇒—	1.00⇒—	6.64⇒—	91.19⇒—	np	np	<1
c.1537 + 1G>T	int 10	87.54⇒—	8.88⇒—	1.00⇒—	4.70⇒—	86.88⇒—	68.94(HSF)	1-bp deletion of ex 10	<1
c.1538-2A>G	int 10	76.54⇒—	6.83⇒—	0.42⇒—	na	76.61⇒—	np	np	1.3
c.1903+5G>A	int 12	74.28⇒—	8.34⇒—	0.83⇒—	4.24⇒—	82.56⇒70.39	np	np	<1
c.5220-1G>A	int 14	98.99⇒—	11.71⇒—	0.99⇒—	8.37⇒—	98.37⇒—	88.72(SSF) 7.37(MaxEnt) 0.98(NNSplice) 6.17(GeneSplicer) 87.61(HSF)	1-bp deletion of ex 15	<1
c.5374-2A>G	int 15	96.52⇒—	9.51⇒—	0.99⇒—	5.54⇒—	91.97⇒—	73.25 (SSF) 4.51 (MaxEnt)	11-bp deletion of ex 16	<1
c.5587-1G>A	int 16	82.12⇒—	12.41⇒—	0.96⇒—	11.40⇒—	88.34⇒—	71.28(SSF) 5.27(MaxEnt) 6.34(GeneSplicer) 80.80(HSF)	1-bp deletion of ex 17	<1
c.6115 + 4delA	int 19	74.68⇒—	6.97⇒—	0.91⇒—	2.85⇒—	82.56⇒—	70.80(HSF)	3-bp inclusion of int 19	<1
Polymorphism									
c.388 + 76C>T	int 3	86.37	9.25	0.99	2.14⇒2.23	93.0	68.87(HSF)	74-bp inclusion of int 3	/
c.1010-27G>A	int 7	84.76	7.11	0.46	5.73⇒5.87	87.89	np	np	/
c.5586 + 7A>G	int 16	85.71	8.69	1.00⇒0.99	3.76⇒4.28	93.07	np	np	/
c.6115+103T>C	int 19	74.68	6.97	0.91	2.85	82.56	np	np	/

FVIII:Ag, FVIII antigen levels expressed as percentage (%) of normal plasma level and reported for the splicing variants, and not (/) for polymorphisms. Scores of physiologic and altered splice site are reported on the left and on the right of the arrow, respectively. The — denotes an abolished splice site. Unreported variants and cDNA numbering are as in Table 2. Ex, exon; int, intron; SSF, Splice Site Finder-like; NNSplice, Neural Network Splice; HSF, Human Splicing Finder; ss, splice site; na, not available; np, not predicted. For each splicing prediction tool used, threshold and range of score are reported in the legend below.

	SSF	MaxEnt	NNSplice	GeneSplicer	HSF
Threshold	≥ 70	≥ 0	≥ 0.4	≥ 0	≥ 65
Score range	[0-100]	[0-12]	[0-1]	[0-15]	[0-100]

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Table 4 Identified large deletions and corresponding factor VIII antigen (FVIII:Ag) levels

Deletion	FVIII:Ag (%)
5′-UTR + exon 1	< 1
5′-UTR + exon 1	< 1
Exon 1	NA
Exons 1–6	< 1
Exons 2–6	< 1
Exons 2–12	< 1
Exons 5 and 6	1.7
Exons 5 and 6	1.8
Exons 5 and 6	2.7
Exon 6	4.2
Exon 6	13.5
Exons 7–10	< 1
Exons 7–10	< 1
Exon 14	< 1
Exons 20–22	< 1
Exon 26	< 1

NA, not available; UTR, untranslated region. FVIII:Ag level is expressed as a percentage (%) of normal plasma level.

38.2% (95% CI 30.9–45.5) and 24.1% (95% CI 5.1–43.1) for null versus non-null *in silico* mutation groups, with an HR of 2.08 (95% CI 0.84–5.17) (Fig. 3C).

An additional criterion was applied in order to distinguish the null and non-null mutations according to FVIII:Ag levels. In particular, the 23 variants found in patients with measurable FVIII:Ag levels (≥1%) were reclassified as non-null, and those with undetectable FVIII:Ag (<1%) as null (Fig. 3D). This led to a larger difference in risk: cumulative incidence rates of 38.3% (95% CI 31.1–45.6) and 10.1% (95% CI 3.0–35.0) for these revised no antigen versus antigen mutation groups, with a high HR of 3.61 (95% CI 0.89–14.74) (Fig. 3D).

Furthermore, both *in silico* and antigen criteria were applied together to the original null and non-null classification, and an approximately similar difference in cumulative incidence was found (39.2% [95% CI 31.6–46.8] and 23.0% [95% CI 7.5–38.5] for *in silico* + antigen null and non-null mutations), with an HR of 2.13 (95% CI 0.97–4.63) (Fig. 3E).

The cumulative incidence rates were further evaluated with omission of the recurrent inversion of intron 22, which is conventionally deemed to be a detrimental/null mutation. Despite larger CIs, owing to the reduced number of analyzed cases (121 instead of 231 mutations), the HRs for high-risk/null mutations were found to be similar to the previous ones: 1.25 (95% CI 0.64–2.46), 1.93 (95% CI 0.80–4.65), 1.98 (95% CI 0.77–5.12), 3.13 (95% CI 0.75–13.08) and 2.00 (95% CI 0.87–4.58) for Gouw *et al.* [28], Carcao *et al.* [13], *in silico*, antigen and *in silico* + antigen classifications, respectively.

The positive predictive value of a null mutation for inhibitor development was low for all models (33–34%), whereas the negative predictive value was 91% for the antigen model (Fig. 3).

Discussion

In a cohort of 231 pediatric patients with severe hemophilia A enrolled in the SIPPET study, mutation detection was highly successful (98.3%). This highlights the effectiveness of multistep screening, and suggests the usefulness of probing elusive variants in commonly unexplored deep-intronic regions. As expected, besides the canonical inversions of intron 22 (47.6%) and intron 1 (2.6%), which accounted for appriximately half of all identified causative variants, high allelic heterogeneity was found, as confirmed by the wide mutational spectrum and the high frequency (36%) of previously unreported variants. Moreover, some mutational hot spots were found (Fig. 2; Tables 3 and 4), with 13 of 16 recurrent point mutations and deletions (81.3%) being identified in patients from different countries.

According to the fully or partially expected impairment of protein synthesis, mutations are generally classified as null and non-null. However, the damaging effects and the causal role of variants are often predicted rather than experimentally determined. Programs enabling the prediction of the pathogenicity of the genetic variants are widely used, following the generation of massive data from next-generation sequencing [29]. The application of multiple algorithms for sequence variant interpretation may improve prediction [29]. We used multiple computational methods to predict the disease association of missense and splicing variants, allowing us to classify a variant as neutral or causative, and to predict the type of mutation (i.e. missense versus splicing) and the extent of the damage resulting from the splicing variants. Although these tools provide us with a good interpretation of splicing variants, they cannot predict the exact splicing outcomes (i.e. exon skipping, activation of cryptic splice sites, and intron retention), resulting in the synthesis of a varied pattern of aberrant FVIII proteins. *Ex vivo* approaches, based on the analysis of illegitimate *F8* transcripts and *in vitro* expression of the FVIII mutant proteins, are much more informative than *in silico* predictions, and can be used to more accurately investigate the consequences of missense or truncating variants [30–32]. Moreover, breakpoint mapping and transcript analysis should better characterize large deletions involving one or more exons. This is the case for exon 6 and exon 5 + 6 deletions, whose skipping should allow the synthesis of mutant FVIII proteins with in-frame deletions, described herewith in five patients with detectable levels of endogenous FVIII. However, these approaches are laborious and time-consuming, and are therefore not applicable to clinical practice.

To date, the *F8* mutation profile is the most promising inhibitor risk predictor in severe hemophilia [12], and is one of three factors included in the two inhibitor risk prediction models [33,34]. Furthermore, it is likely that residual amounts of endogenous FVIII in plasma

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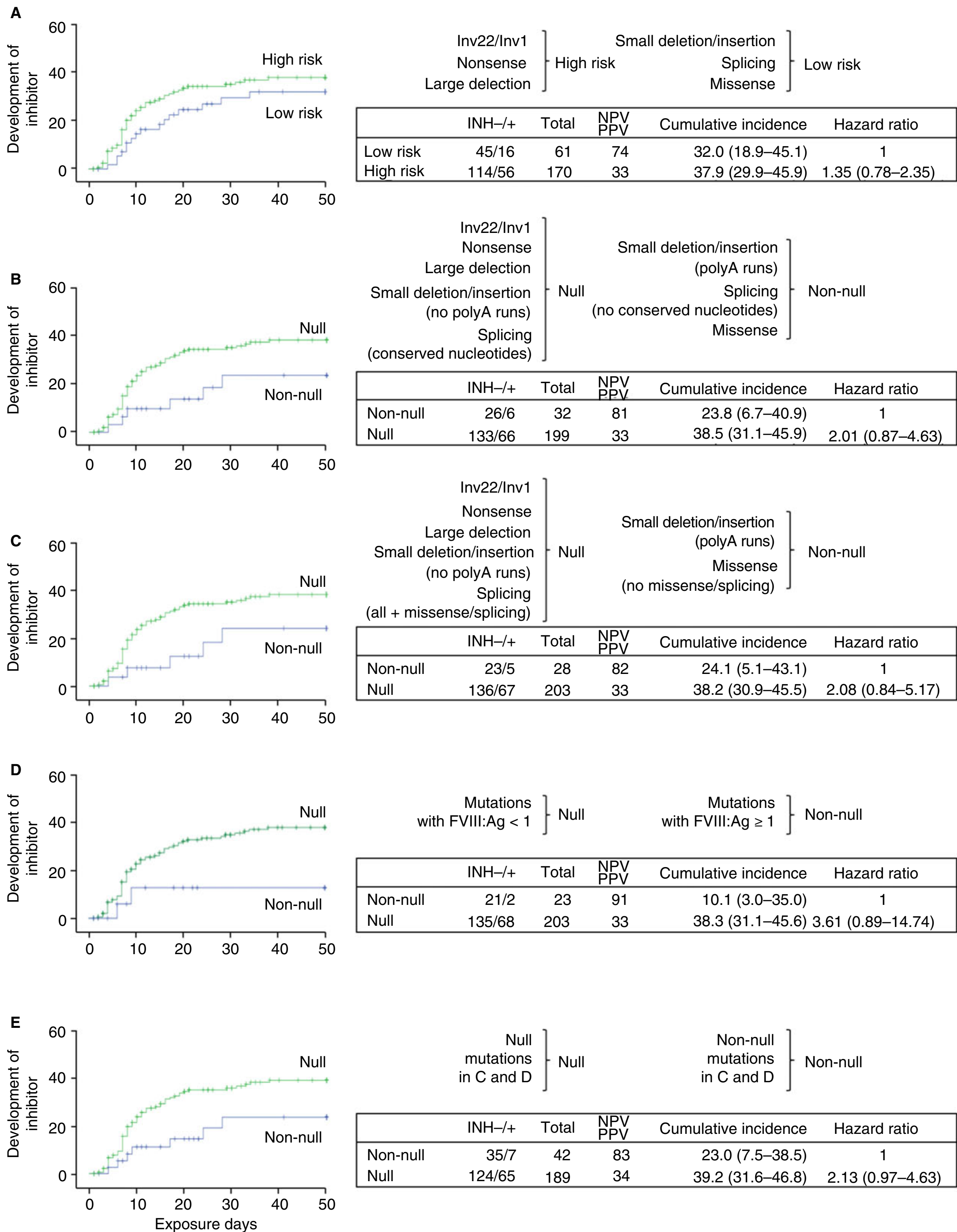

A	INH–/+	Total	NPV PPV	Cumulative incidence	Hazard ratio
Low risk	45/16	61	74	32.0 (18.9–45.1)	1
High risk	114/56	170	33	37.9 (29.9–45.9)	1.35 (0.78–2.35)

B	INH–/+	Total	NPV PPV	Cumulative incidence	Hazard ratio
Non-null	26/6	32	81	23.8 (6.7–40.9)	1
Null	133/66	199	33	38.5 (31.1–45.9)	2.01 (0.87–4.63)

C	INH–/+	Total	NPV PPV	Cumulative incidence	Hazard ratio
Non-null	23/5	28	82	24.1 (5.1–43.1)	1
Null	136/67	203	33	38.2 (30.9–45.5)	2.08 (0.84–5.17)

D	INH–/+	Total	NPV PPV	Cumulative incidence	Hazard ratio
Non-null	21/2	23	91	10.1 (3.0–35.0)	1
Null	135/68	203	33	38.3 (31.1–45.6)	3.61 (0.89–14.74)

E	INH–/+	Total	NPV PPV	Cumulative incidence	Hazard ratio
Non-null	35/7	42	83	23.0 (7.5–38.5)	1
Null	124/65	189	34	39.2 (31.6–46.8)	2.13 (0.97–4.63)

Fig. 3. Cumulative incidence of FVIII inhibitors according to the different mutation clusters. Kaplan–Meier curves of inhibitor development concerning high-risk and low-risk mutations grouped according to the classification reported by Gouw *et al.* [28] (A), null and non-null mutations according to Carcao *et al.* [13] (B), *in silico* analyses (C), FVIII antigen (FVIII:Ag) levels (D) and both *in silico* analyses and FVIII:Ag levels (E) are shown on the left side. Lists of mutation type (top) and of total and partial numbers of mutations identified in patients whose plasmas were negative (–) and positive (+) for the inhibitor (INH) (bottom) are shown on the right side beside the corresponding graphics. Negative predictive values (NPVs) and positive predictive values (PPVs) were based on the proportions, and are expressed as percentages. Inv1, intron 1 inversion; Inv22, intron 22 inversion. [Color figure can be viewed at wileyonlinelibrary.com]

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protect patients from the development of alloantibodies. Therefore, the detection of trace amounts of FVIII could improve our insights into the genotype–phenotype association (i.e. *F8* mutation type–inhibitor risk association). Up to now, the lack of systematic measurements of immunoreactive FVIII in the plasma of severe hemophilia A patients and of *in vitro* experimental assays that are able to reveal the real effect of each *F8* variant have hampered this evaluation. In order to address these gaps in our knowledge, we collected data on FVIII:Ag and *F8* mutation in the SIPPET study patients [14], and analyzed their associations with inhibitor development. To attain this goal, we chose to cluster the *F8* genetic defects into null and non-null mutations, not only according to the expected degree of FVIII synthesis, but also on the basis of *in silico* prediction of their pathogenic role and plasma levels of FVIII:Ag. In line with previous results, our findings confirm that *F8* mutations that are conventionally classified as null are more prone to causing inhibitor development than conventional non-null mutations (HR 2.01; 95% CI 0.87–4.63; Fig. 3B), demonstrating a protective role of those mutations that allow some residual synthesis of FVIII protein. A deeper investigation and reclassification of variants according to their deleteriousness as predicted *in silico* yielded similar results (HR 2.08; 95% CI 0.84–5.17; Fig. 3C). Moreover, the mutation classification based exclusively on measurable or unmeasurable immunoreactive FVIII in plasma showed the clearest difference (HR 3.61; 95% CI 0.89–14.74; Fig. 3D), confirming the protective effect of minute amounts of FVIII. The mutation reclassification taking into account both *in silico* analyses and FVIII:Ag levels did not change the overall predictivity (HR 2.13, but with a narrower CI, i.e. 0.97–4.63; Fig. 3E).

In addition to the plasma expression of FVIII, the intracellular expression of FVIII observed with inversion of intron 22 has been recently proposed as a mechanism of inhibitor protection [35]. Accordingly, this gene inversion would cause a lower risk of inhibitor formation than other null mutations associated with no secretion of FVIII. However, our exclusion of the intron 22 inversion from all of the described cumulative incidence analyses reduced to half the number of analyzed mutations but failed to affect the results. These findings emphasize the importance of investigating the plasma levels of residual FVIII:Ag for each causative variant in order to really understand its clinical relevance. However, our data are limited by the small sample size, particularly regarding missense and splice site variants and measurable FVIII:Ag, which led to wide CIs. Therefore, more patients with severe hemophilia A should be evaluated in order to strengthen our findings.

A minor limitation of this study concerns the use of a mAb for the FVIII:Ag measurement, which could result in some aberrant FVIII peptides being missed. The use of polyclonal antibodies could probably improve the detection of a trace amount of any circulating FVIII:Ag. Nevertheless, whichever model was used, the predictive power for inhibitor development remained low, indicating the presence of other acquired determinants of inhibitor development. Detection of trace amounts of FVIII in patient plasma proved to have a high negative predictive value, but this concerned only a minority (10%) of patients.

In conclusion, with the aforementioned limitations, the analytical methods employed by us of in-depth investigation of the pathogenic role of *F8* mutations may help to better predict the risk of inhibitor development.

Addendum

S. Spena designed the research, carried out *in silico* analyses, interpreted the results, and wrote the manuscript. I. Garagiola participated in study design and critically reviewed the manuscript. A. Cannavò performed statistical analyses. M. Mortarino performed detection of genetic variants. P. M. Mannucci and F. R. Rosendaal interpreted the results and critically reviewed the manuscript. F. Peyvandi designed the research, interpreted the results, and critically reviewed the manuscript. Members of the SIPPET study group enrolled the patients and collected data. All authors read the manuscript and approved the final version.

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Disclosure of Conflict of Interests

P. M. Mannucci reports receiving honoraria for participating as speaker at satellite symposia and educational meetings organized by Alexion, Baxalta/Shire, Bayer, CSL Behring, Grifols, Kedrion, LFB, and Novo Nordisk. He is member of the following scientific advisory boards: Bayer and Kedrion. F. Peyvandi reports receiving honoraria or consultation fees from Freeline, Kedrion Biopharma, LFB, and Octapharma. She has received honoraria for participating as a speaker at educational meetings organized by Ablynx, Bayer, Grifols, Novo

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Nordisk, and Sobi. She is member of the following advisory boards: Ablynx and F. Hoffmann-La Roche. P. Eshghi reports receiving personal fees from Aryogen, Baxter, CSL, and Novo Nordisk; and grants from Aryogen and Novo Nordisk. G. Young reports receiving personal fees from Baxalta, Biogen, Bayer, Grifols, Kedrion, and Novo Nordisk. E. Santagostino reports receiving personal fees from Baxalta, Baxter, Bayer, Biogen, CSL Behring, Grifols, Kedrion, Novo Nordisk, Octapharma, Pfizer, Roche, and Sobi; and grants from Pfizer. M. E. Mancuso reports receiving personal fees from Baxter, Bayer, CSL Behring, Novo Nordisk, Pfizer, and Sobi/Biogen. J. N. Mahlangu reports receiving personal fees from Amgen, Biogen, Biotest, Novo Nordisk, Pfeizer, and Roche; and grants from Bayer, Biogen, CSL Behring, and Novo Nordisk. M. Cerqueira reports receiving personal fees from Baxter and Novo Nordisk. N. P. Ewing reports receiving personal fees from Alpha Therapeutic, Baxter, Bayer, Biogen Idec, and Novo Nordisk. C. Male reports receiving personal fees and non-financial support from Baxter, Bayer, Biotest, CSL Behring, Novo Nordisk, and Pfizer; and grants from CSL Behring. T. Owaidah reports receiving honoraria for participating as speaker at satellite symposia and educational meetings organized by Alexion, Bayer, CSL Behring, Novo Nordisk, Octapharma, and Sobi; and he has received research grant funding from Novo Nordisk and consulting fees for serving on scientific advisory boards from Alexion and Novo Nordisk. M. Simpson reports receiving personal fees from Baxter, Biogen Idec, CSL Behring, and Octapharma. E. Zanon reports receiving personal fees from Baxalta, Bayer, CSL Behring, Grifols, Novo Nordisk, and Pfizer; and grants from Bayer. K. Kavakli reports receiving personal fees from Biotest; personal fees and non-financial support from Baxalta, Bayer, and Pfizer; and non-financial support from Octapharma and LFB. M. J. Manco-Johnson reports receiving personal fees from Baxter, Bayer, Biogen Idec, CSL Behring, and Novo Nordisk; and grants from Bayer. M. G. Mazzucconi reports receiving personal fees from Bayer, Baxter, Kedrion, Novo Nordisk, and Pfizer. D. Neme reports receiving personal fees from Novo Nordisk and Pfizer. B. M. Wicklund reports receiving personal fees from Baxalta, Bayer, Biogen, CSL Behring, and Novo Nordsisk, and other support from the American Thrombosis and Hemostasis Network and the National Hemophilia Foundation. B. Zulfikar reports receiving grants and personal fees from Bayer, Eczacibasi Baxter, Erkim LFB, Novo Nordisk, and Pfizer. The other authors state that they have no conflict of interest.

Appendix

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