Research paper

Inhibitors to factor VII and in congenital factor VII deficiency

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Running title: FVII inhibitors in congenital FVII deficiency: the STER
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Summary

Congenital factor VII (FVII) deficiency is a rare bleeding disorder, and few anecdotal reports are available regarding FVII inhibitors. Using data from the Seven Treatment Evaluation Registry (STER; 225 patients, 312 treatments), we have prospectively evaluated the occurrence of FVII inhibitors using a standardized method over a period of 8 years. The central laboratory screened 115 paired samples; FVII inhibitors were detected in 3/115 (2.6%) patients (one *de novo* inhibitor and two pre-existing inhibitors). A fourth inhibitor was detected in a patient who had been screened locally. All four patients had high-responding inhibitors (10–72 BU) and had previously received factor replacement therapy. In three patients, the inhibitors appeared before the age of 6 months during prophylaxis following central nervous system or gastrointestinal bleeds; however, treatments were continued without apparent loss of efficacy. In one patient, an inhibitor developed in adulthood after replacement therapy for minor surgery. No anaphylactoid reactions or renal complications were reported during or after prophylaxis. In conclusion, inhibitor development is rare in patients with congenital FVII deficiency (~2%), with an incidence similar to that in patients with haemophilia B. FVII inhibitors display immunological (anamnesis) and kinetic features similar to those in patients with haemophilia.

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Introduction

Antibodies to clotting factors have been consistently reported in the medical literature; the first was described in a patient with haemophilia A treated numerous times with blood products (Lawrence & Johnson, 1941). These antibodies are considered '*allo*-antibodies' if they arise after the administration of a therapeutic protein during replacement therapy in patients with congenital bleeding disorders, or '*auto*-antibodies' if a congenital bleeding disorder is not present. The latter usually occur in the elderly and can be associated with a variety of comorbidities. Clinically, only those antibodies that affect clotting activity are termed 'inhibitors' and are therefore considered relevant, because they may render patients refractory to treatment.

In patients with haemophilia A or B, inhibitors can occur after replacement therapy with factor VIII (FVIII) or factor IX (FIX) concentrates, respectively. The risk of developing inhibitors is particularly high in patients with severe deficiencies (FVIII or FIX <1% of normal) and in those with null mutations (Coppola *et al*, 2010; Oldenburg & Pavlova, 2006). Furthermore, inhibitors are far more frequent in patients with haemophilia A (typically 15–20%; range, 8–52%) than in patients with haemophilia B (~1–3%) (DiMichele, 2007; Mariani *et al*, 2012a). This difference is considered attributable, at least in part, to the larger size and complexity of FVIII compared with FIX. In any event, the ensuing immunological triggers and mechanisms associated with inhibitor development are not yet fully understood.

The development of an inhibitor to a clotting factor is associated with increased rates of morbidity and mortality, an increased cost of care and more complicated treatment regimens (Bolton-Maggs, 2006; Franchini *et al*, 2013). When compared with haemophilia A, the immune response in haemophilia B has several distinctive features: (i) an increased risk of developing anaphylaxis after the administration of FIX-containing concentrates to a patient with a FIX inhibitor; (ii) a risk of developing nephrotic syndrome after exposure to FIX replacement products; and (iii) a lower success rate of immune tolerance therapy for the eradication of FIX inhibitors (Coppola *et al*, 2010; DiMichele, 2012).

Congenital FVII deficiency is a rare bleeding disorder and there are few data regarding FVII inhibitors. FVII shares considerable homology with FIX, both at the gene and protein levels (Furie & Furie, 1992), and displays a comparable distribution of disease-

causing mutations, with a large predominance of missense changes (Bernardi *et al*, 2009). Anecdotal reports of inhibitors to FVII have been previously published in the literature, including reports from members of our group (Nicolaisen *et al*, 1996; Mariani *et al*, 1999; Ingerslev *et al*, 2005; Pruthi *et al*, 2007, Batorova *et al*, 2007; Tokgoz *et al*, 2012). Here, we report a prospective study in which screening for FVII antibodies was performed according to a specific protocol within the frame of the Seven Treatment Evaluation Registry (STER), which also collected data on the treatment of spontaneous bleeding episodes, surgical interventions and prophylaxis in patients with congenital FVII deficiency over an 8-year period (Mariani *et al*, 2011; Mariani *et al*, 2012b; Mariani *et al*, 2013; Napolitano *et al*, 2013). The inhibitor screening procedure was carried out centrally and data were analyzed in a standardized clinical and laboratory context (Ingerslev *et al*, 2005).

Methods

STER database

The STER (www.targetseven.org) is a multicentre, prospective, observational, Web-based registry that collected structured and detailed data on the management of FVII deficiency. STER followed the strictly controlled data collection procedures established by the International FVII Deficiency Study Group (IF7SG) (Bernardi *et al*, 2009; Mariani *et al*, 2005; Mariani *et al*, 2011; Mariani *et al*, 2012b; Mariani *et al*, 2013; Mariani & Bernardi, 2009; Napolitano *et al*, 2013). The STER protocol is published on clinicaltrials.gov (NCT01269138). Blood samples for centralized inhibitor determination (Ingerslev *et al*, 2005) were collected at enrolment, 30 d following administration of one or more replacement therapies, and as needed to check titres after multiple administrations at other times. Clinical and laboratory information was collected using Web-based forms and stored in a Siemens mainframe. Another form captured previous treatments and the actual regimen adopted (type of product, dosage and schedule), treatment efficacy, complications and adverse events. Investigators were also asked to report any bleeding episodes that occurred during the observation period.

The registry database was closed on 28 February 2012, by which time all inhibitor assays had been completed, and the data were checked for consistency.

Plasma collection and laboratory procedures

Platelet-free plasma samples were collected locally by a contract research organization, centrifuged, transferred to a repository, and stored at –80°C before being sent on dry ice to the central laboratory. Provisions were made to ensure that delivery of each shipment was pre-announced and at receipt, and that all samples were stored at –80°C until analysis, which occurred within four weeks. Each sample was assayed for FVII:C; assays were performed on the Thrombolyzer instrument (Benkh, Norderstedt, Germany). No clinical data were submitted to the central laboratories that received the plasma samples.

Inhibitors to FVII were detected using a modified Bethesda Assay that used a high-sensitivity thromboplastin as an 'activator' (Innovin, Siemens Diagnostica, Germany) (Ingerslev *et al*, 2005). The FVII-deficient substrate plasma was a natural deficient plasma obtained from a severely affected patient (FVII:C <1%; homozygous Arg100GLN mutation), and the diluent was an Imidazole buffer (50 mmol, pH 7.3). The calibrator was a Verify Reference Plasma (Lot 113029, Organon-Teknika, Durham, NC, USA) with an assigned FVII:C value of 1.12 IU/ml. The 1:1 mixture incubation period was 2 hours. An inhibitor titre of <0.6 BU was considered negative. Results were considered valid if the coefficient of variation (CV) of double determinations did not exceed 25% and if at least two diluted sample results were between 25% and 75% of the reference sample result. From the beginning of the study until May 2010, samples were screened at the Skejby Haemophilia and Thrombosis Laboratory, Skejby University Hospital, Denmark; after that date, and until February 2012, samples were screened at the St. Thomas' Central Laboratory, London, UK, using the same protocol and laboratory procedures. Only plasma samples with phenotypic baseline FVII:C levels at <4% of normal were screened for inhibitors.

Validation of the analytical procedures was conducted as follows (Bionalysis Report: STER internal document [4 October 2012]):

- FVII:C assay: complete linearity was observed between 1% and 259% ($r^2 = 0.99$), with an intra-assay CV of 1.9–2.6% and an inter-assay CV of 45–56%.

- Stability of FVII:C at 37%: no loss of FVII:C was observed, but there was a small increment in activity from hours 1–6.

- External control: during 12 External Quality Control for Assays and Tests (ECAT), quarterly exercises showed a minimal deviation from the mean of 117–136 laboratories (z-score ± 1 in 20 exercises and slightly above1 in 4).

- Inhibitor assay intrinsic variation: using an inhibitor with an expected potency of 1 BU, the mean ± SD of 31 controls was 0.9 ± 0.1 BU.

The research proposed by the STER Study Group was approved by the Ethics Committee of L'Aquila University (the STER coordinator's institution) and, in parallel, by the respective committees of the participating institutions.

Results

In total, 312 treatments administered to 225 patients with FVII deficiency were reported to STER. Among these, 78.5% of treatments were with recombinant activated FVII (rFVIIa; NovoSeven®, Novo Nordisk A/S, Denmark), 9.9% were with plasma-derived (pd)-FVII concentrates (Facteur VII™ LFB, Courtaboef, France, or Provertin-UM TIM3™, Baxter-Immuno, Vienna, Austria), 9.6% used fresh frozen plasma (FFP), 1.6% were with prothrombin complex concentrates (PCCs; Prothromplex T™, Baxter-Immuno, Vienna, Austria) and 0.3% were with pd-aPCC (FEIBA™, Baxter-Immuno, Vienna, Austria).

Overall, 115 paired samples suitable for screening were received by the central laboratories. Only those samples with FVII:C levels <4% were considered for the inhibitor assay. Samples screened between baseline and day 30 showed that 3/115 (2.6%) individuals were inhibitor positive. One was a *de novo* inhibitor (first sample negative, second sample positive; patient 1), and two were 'pre-existing' inhibitors (both samples positive; patients 2 and 4) (Table I). All three of these patients had low FVII:C levels and had previously been exposed to replacement therapy with at least one product (rFVIIa, pd-FVII or FFP). In one patient with a history of repeated bleeds and treatments, an inhibitor was detected in adulthood after a surgical intervention (multiple dental extractions; patient 1), while inhibitors occurred in the other two patients after prolonged rFVIIa prophylaxis for central nervous system (CNS) or gastrointestinal (GI) bleeds (patients 2 [Tokgoz *et al*, 2012] and 4, respectively) (Table I). An inhibitor was also detected in a patient who had been screened locally for logistical reasons (patient 3); this inhibitor was detected during rFVIIa prophylaxis for CNS bleeds. The three patients who developed inhibitors following

prophylaxis before the age of 6 months (patients 2, 3 and 4) all had severe clinical phenotypes; however, treatments were continued without apparent loss of efficacy. No anaphylactoid reactions or renal complications were observed during or after prophylaxis.

Clinical inhibitor-related characteristics are shown in Table I. Overall, maximum inhibitor titres varied from 10.4 to 72 BU. The occurrence of the first three inhibitors has been reported in previously published studies from the STER (Batorova *et al*, 2007, Mariani *et al*, 2011; Mariani *et al*, 2012b; Mariani *et al*, 2013; Napolitano *et al*, 2013).

Data on mutations in the FVII gene were available for two individuals: a missense mutation plus one codon deletion (Ala294Val + Del C) (Batorova *et al,* 2007) and a homozygous nonsense mutation (p.Ser112-Stop) (Tokgoz *et al*, 2012) were detected in patients 1 and 2, respectively (Table I).

The inhibitor time course for patient 1 over 6 years is illustrated in Fig 1. Patient 1 was also evaluated kinetically over 24 hours when two different inhibitor titres were present (Fig 2). At the highest inhibitor titre, the area under the curve (AUC) for FVII was approximately 100 times lower, and the clearance approximately 70 times higher, than in patients with FVII deficiency but without inhibitors (unpublished data from STER).

Discussion

We performed a prospective study of FVII inhibitor occurrence in a large number of patients with congenital FVII deficiency who had previously received replacement therapy for spontaneous or traumatic bleeding episodes (Mariani *et al*, 2013), for major or minor surgical interventions (Mariani *et al*, 2011; Mariani *et al*, 2012b), or for prophylaxis (Napolitano *et al*, 2013). All inhibitors that developed were high-titre, although very high titres (>100 BU) were never recorded during follow-up. The method used to detect FVII inhibitors was a modified Bethesda Assay (Ingerslev *et al*, 2005), with very reproducible analytical features. Although it was not possible to screen all patients of the registry, the prospective study by protocol collected 115 sets of plasma samples, enabling analysis of the incidence of inhibitors following sufficient significant exposure to FVII product..

With regard to the samples tested in the central laboratories, inhibitor development was detected in 2.6% (3/115) of patients; if all inhibitors observed during the 8-year STER were included, the prevalence was 1.7% (4/225). Among patients enrolled according to

protocol, the incidence of *de novo* inhibitors was 0.8% (1/115). Thus, in this rare bleeding disorder, the development of FVII inhibitors is as rare as that reported in patients with haemophilia B (inhibitor prevalence of 2–3%) (DiMichele 2007; Astermark *et al*, 2008; Mariani *et al*, 2012a). Comparison of FVII deficiency with haemophilia B is plausible given the similar gene and protein structures of FVII and FIX. There are some differences, however; for example, while severe gene defects (such as large or total deletions) have not been reported in patients with FVII deficiency (Mariani *et al*, 2005), they have been found in patients with haemophilia B with inhibitors. In addition, the immune response in FVII deficiency is apparently not complicated by anaphylactoid reactions, a severe clinical issue in patients with haemophilia B (Chitlur *et al,* 2009; Mariani *et al*, 2012a). The absence of this clinical complication in FVII deficiency was confirmed in the present study, in which prophylaxis was continued without any safety concerns in the presence of inhibitors in three patients (patients 2, 3 and 4) (Table I) (Napolitano *et al*, 2013).

The decrease in FVII inhibitors over time, as demonstrated by patient 1 (Fig 1), is comparable to the time course for FVIII and FIX inhibitors and displays sharp anamnesis following replacement therapy. The kinetics of FVII investigated in this patient in the presence of different inhibitor titres (0, 4 and 20 BU) (Fig 2) also confirmed the presence of a strong, titre-dependent, inhibiting activity. In the presence of the highest inhibitor titre, AUC was about 100 times lower and the clearance about 70 times faster than in FVII-deficient patients without an inhibitor (STER unpublished data).

As described in patients with haemophilia A or B (Christophe *et al,* 2001; Astermark *et al*, 2008; Mariani *et al*, 2012a), the immune reaction in FVII deficiency displays a restricted heterogeneity, with a clear-cut prevalence for the immunoglobulin G4 subclass, although with some variability related to the sample tested .

In the present study, three inhibitors were detected before the age of 6 months (Table I) in patients with a very severe bleeding phenotype; all of these patients received FFP before prophylactic rFVIIa was instituted in response to life-threatening CNS or GI bleeds. The fourth inhibitor developed in patient 1, who had a history of numerous severe gynaecological bleeds and repeated treatments with FFP, PCC, pd-FVII and rFVIIa. The inhibitor appeared after a surgical intervention covered by rFVIIa; in total, nine bolus injections were administered in 3 days. Considering the clinical treatment histories, no

clear-cut conclusions can be drawn concerning which replacement therapy actually triggered the immune response.

Gene mutations, evaluable for only two patients in our study (patients 1 and 2), account for low to very low FVII levels and severe clinical phenotypes, especially in the presence of a nonsense mutation (as in patient 2, who had CNS bleeding at 3 months of age). With regard to the frequent p.A354V-p.P464Hfs† mutation, no other inhibitors have been reported in the combined International Registry on Factor VII Deficiency (IRF7)/STER genetic databases, which contain data on homozygous (12) or compound heterozygous (64) genotypes. Consequently, this peculiar, frequent mutation probably does not represent a high risk for FVII inhibitor development. In contrast to haemophilia B with inhibitors (Mariani *et al*, 2012a; Collins *et al*, 2013), FVII deficiency has not been associated with large or complete gene deletions, and nor is the immune response complicated by rare but life-threatening anaphylactoid reactions or renal complications (Chitlur *et al*, 2009).

Investigators who reported the occurrence of an inhibitor to STER noted that prophylaxis with rFVIIa may still be efficacious (Napolitano *et al*, 2013). This supports a previous observation of patients with severe FVII deficiency and a very short FVII half-life (<1 hour) who clearly benefited from prophylaxis with rFVIIa (Mathijssen *et al*, 2004 Tokgoz *et al*, 2012). These observations may be explained by the rapid clearance of rFVIIa from the circulation through binding to extravascular tissue factor (Hoffman *et al,* 2007; Gopalakrishnan *et al*, 2010), as this could exert a protective effect from inhibitory antibodies.

In conclusion, FVII inhibitors in patients with FVII deficiency represent a rare complication (~2% of treated patients), with biochemical and kinetic features similar to those reported in patients with haemophilia A or B. Knowledge of the occurrence and characteristics of inhibitors to FVII is of great interest because this is a rare event in a rare disorder, and an increased understanding of inhibitor development may improve patient management and treatment outcomes.

Acknowledgements

Table I. Demographic and clinical data for patients with FVII deficiency with inhibitors.

Variable	Patient number			
	1	2	3	4
Gender/age	Female/53 years	Male/5 years	Male/5 months	Male/1 month
Weight	55.0 kg	20.0 kg	6.5 kg	9.1 kg
Age at inhibitor screening	59 years	3 months	5 months	1 month
FVIIc level	<1%	2%	1.3%	<1%
FVII gene mutation	p.A354V-p.P464Hfs†	p.Ser112-Stop (homozygous)	NA	NA
First symptom/age at first symptom	Epistaxis/3 years	CNS/3 months	CNS and GI/birth	Tongue haematoma/1 month
Symptoms reported*	Br-Ep-Gum-Hp-Me	CNS-Br-Ep-GI-Gum	CNS-GI-Gum	CNS-Br-Ep-Gum-Mu-Sc
Previous treatments	FFP, pd-FVII, rFVIIa	FFP	FFP	FFP
Event at inhibitor discovery	Multiple dental extraction	Prophylaxis (CNS)	Prophylaxis (CNS)	Prophylaxis (GI)
Pre-inhibitor treatment†	rFVIIa	rFVIIa	rFVIIa	rFVIIa
Schedule	Initial bolus 30 µg/kg plus 8 consecutive boluses 10 µg/kg	30 µg/kg × 3 weeks	65 µg/kg × 1 week	31 µg/kg × 3 weeks
Inhibitor titres, minimum–maximum	10–20 BU	38–68.3 BU	5.5–60 BU	32–72 BU
Concomitant medications	Tamoxifen, antifibrinolytics	None	Ciprofloxacin and fluconazole	None

*Br, easy bruising; Ep, epistaxis; Gum, gum bleeding; Me, menorrhagia; Hp, haemoperitoneum; CNS, central nervous system bleeding; GI, gastrointestinal bleeding; Mu, muscle haematoma; Sc, subcutaneous haematoma; FFP, fresh frozen plasma; pd-FVII, plasma-derived FVII concentrate; rFVIIa, recombinant activated FVII.
†Treatment upon inhibitor discovery.

Fig 1. The time course of the inhibitor titre and the effect of re-exposure to FVII in inhibitor patient 1 with severe FVII deficiency (baseline FVIIc, 0.8 IU/dl). Arrows indicate administration of pd-FVII and rFVIIa.

[Ideally, fig to be formatted so that: pd-FVII not pdFVIII; x-axis legend lower case; use ml (not mL)]
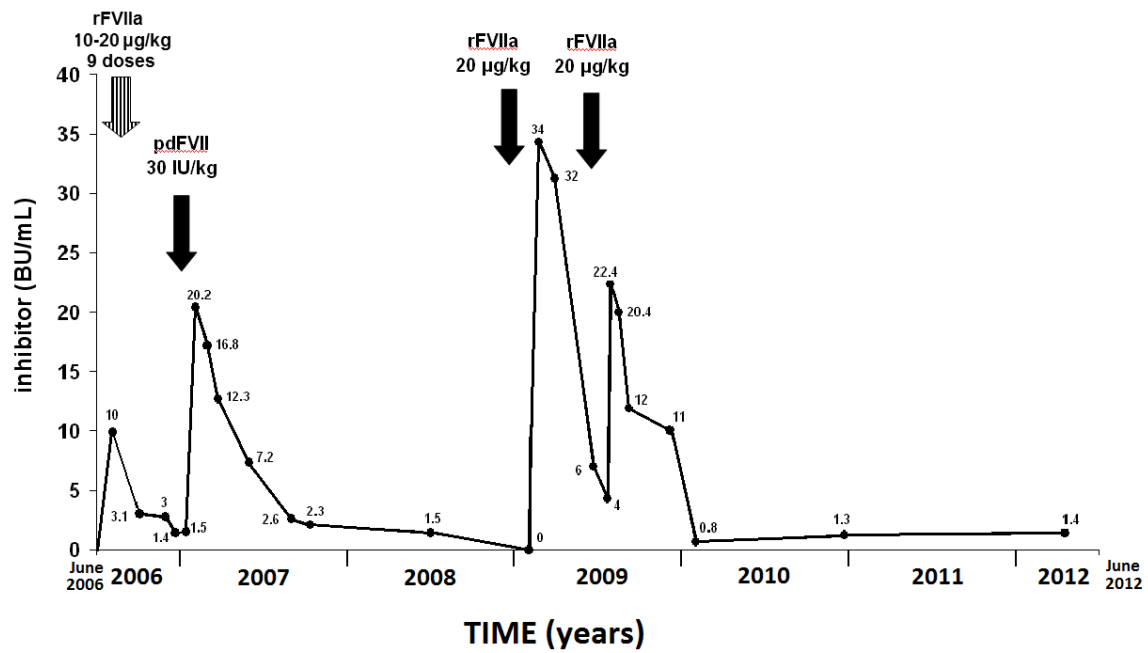
Fig 2. Pharmacokinetics of FVIIc after rFVIIa 20 µg/kg in a non-inhibitor patient with severe FVII deficiency and in Patient 1 with inhibitor, investigated at two occasions at different inhibitor levels (4.0 BU/ml and 20.4 BU/ml).
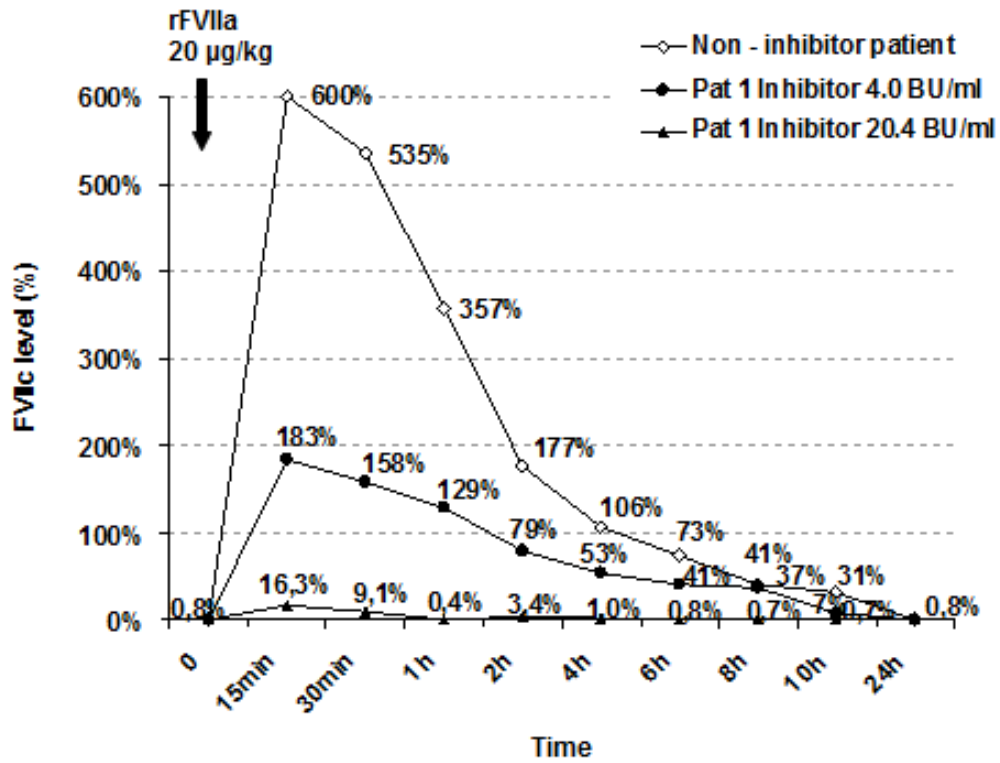
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