Inhibitors in Severe Hemophilia A: 25-Year Experience in Slovakia

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Abstract

We present 25-year experience with inhibitors in previously untreated patients (PUPs) with severe hemophilia A in Slovakia, where safe factor VIII (FVIII) concentrates have been used since 1990. A prospective study focused on inhibitor incidence in PUPs was established in 1997. Out of a total 61 PUPs born between January 1997 and October 2015, 59 were eligible for evaluation; 50 and 9 were treated with $>$ 20 exposure days (ED) of plasma-derived FVIII (pdFVIII) and recombinant FVIII (rFVIII) products, respectively. In the entire group 13/59 (22%) PUPs developed inhibitors; i.e. 7/50 (14%) and 6/9 (67%) treated with pdFVIII and rFVIII, respectively. Univariate analysis of inhibitor risk factors in patient groups with and without inhibitors showed the rFVIII and serious/recurrent infections within the first 50 EDs to be associated with inhibitor development (OR of 12.3 [95% CI 2.48–60.83; $p = 0.002$] and 5.0; [95% CI 1.16–21.9; $p = 0.03$), respectively]). Also, in multivariate Cox regression analysis, peak treatment $\geq$ 5 EDs reached statistical significance. The hazard ratio (HR) was 7.15 (95% CI 1.65–31.36) $p = 0.0086$ for rFVIII and 4.38 (95% CI 1.02–18.67) $p = 0.046$ for intensive treatment. Between 1993 and 2015, 21 immune tolerance inductions (ITIs) in 19 inhibitor patients were performed in the two largest hemophilia centers in Slovakia. In all but one ITI courses pdFVIII containing von Willebrand factor (FVIII/VWF) was used with preferred use of high-dose ITI (HD ITI) in high responders (HRs). Complete or partial success was achieved in 17/19 (89.5%) patients. Evaluating only the patients who already completed ITI, the success rate was even higher (15/16; 94%), including 7/7 low responders and 8/9 HR. Conclusion: Our national prospective study comprising entire group of PUPs with

Keywords
- severe hemophilia A
- PUPs
- inhibitors incidence
- immune tolerance induction

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severe hemophilia A showed higher incidence of inhibitors in patients treated with rFVIII and those with intensive therapy within first 50 EDs. However, our experience is limited to small numbers of patients; thus, our results must be interpreted cautiously. High success rate of the ITI in our inhibitor patients has been achieved with FVIII/VWF concentrates and preferred use of HD ITI in HR patients.

Remarkable progress in hemophilia care in the last two decades in Slovakia with current level of factor VIII (FVIII) supply of 6.5 IU/capita/y and expanding use of prophylaxis in children and adults resulted in significant improvement of quality of life in persons with hemophilia. Today's generation of young hemophiliacs has a real chance to reach the life expectancy of the normal healthy population. Alloantibodies neutralizing FVIII (inhibitors) are a most challenging complication of hemophilia therapy and always cause a big step-back from the advanced care attained. Patients with inhibitor require radical change in treatment strategy, including the use of less effective alternative hemostatic therapy[1–4] and demanding treatment aimed at eradicating of inhibitors and reinducing the tolerability of FVIII.

According to literature, FVIII inhibitors affect approximately 20 to 30% of patients with severe hemophilia A[5,6] however, most recently a higher incidence in previously untreated patients (PUPs) was reported, approaching even 38 to 42%.[7,8] For many years extensive research has been conducted aimed at unveiling the reason why some patients with severe hemophilia develop inhibitors while a larger proportion of patients remain inhibitor free. Intensive debate is ongoing especially on the impact of the type of FVIII product on inhibitor development and, in particular, on the potential for recombinant FVIII (rFVIII) concentrates to be more immunogenic than plasma-derived FVIII (pdFVIII).[9–14] Recently, also different immunogenicity of various types of rFVIII has been suggested.[15] However, the reports on the role of treatment products in inhibitor development are often contradictory and remain inconclusive.[7,8,15–17]

The only effective treatment for eradication of inhibitors is immune tolerance induction (ITI) with a rate of success of 75 to 94% reported for primary ITI and 44 to 73% for rescue ITI.[18–20] The role of treatment protocol and optimal dosing regimens is still not clear. However, the first prospective randomized international study (IITI) demonstrated that high-dose protocols used in HR with a good prognosis resulted in reduced bleeding frequency during ITI and faster achievement of success.[21] The high success rates with high-dose protocols were observed also in HRs with poor prognostic factors.[19,22] Several studies demonstrated a potential for FVIII concentrates containing von Willebrand factor (VWF) to achieve successful inhibitor eradication in a high proportion of patients, even in those with poor prognosis.[23–26] However, recently also rVIII products were shown to be highly effective in ITI,[27–29] and because of their wider safety margin, they are recommended as the preferred products for ITI by some Authors.[30,31]

In Slovakia since 1974 all patients with hemophilia have been registered in the National Hemophilia Registry kept by the National Hemophilia Centre (NHC). Safe pdFVIII concentrates were introduced into hemophilia treatment in 1990, and retrospective surveys comprising a 25-year period showed a cumulative incidence of inhibitors with these products in PUPs with severe hemophilia A ranging between 10.3 and 14% (high-titer inhibitors 7.4%). In 1997 the NHC established a prospective study to monitor systematically inhibitor incidence and potential risk factors in all PUPs with hemophilia A born in Slovakia from this date. Increasing factor supply in the 1990s also permitted introduction of ITI therapy. All patients, either PUPs or previously treated patients (PTPs), developing clinically relevant inhibitors after 1990 were indicated for ITI. In the present article we report the interim results of this ongoing prospective inhibitor study and our experience with ITI performed in patients with inhibitors in the period 1993–2015.

Materials and Methods

Prospective Study on Inhibitors Incidence in PUPS

Patients

All consecutive patients with hemophilia A born in Slovakia since 1997 have been involved in a prospective, open-label nationwide ongoing study focused on inhibitor development in PUPs. Inhibitor status was tested every 4 to 5 exposure days (EDs) during the first 20 EDs, then every 10 and 20 EDs up to 50 and 150 EDs, respectively. Potential risk factors for developing inhibitor were followed: severity of hemophilia, *F8* gene mutation, family history of inhibitors, age at the first bleeding and first therapy with FVIII, reason for the first therapy, vaccination concurrent with FVIII, and the type of product (pdFVIII, rFVIII). Severe bleeding, surgery, red blood cells transfusion, severe infection, and FVIII replacement during 3 to 4 days and $\geq$ 5 days within the first 50 ED were also recorded.

Treatment with FVIII Concentrates

Between 1997 and 2008 exclusively pdFVIII concentrates were used with a majority comprising FVIII/VWF products. In 2004 prophylaxis in children was introduced and vaccination without concurrent FVIII was preferred. rFVIII products started to be used in PUPs in 2008, and the choice of product (pdFVIII or rFVIII) was based on the discussion with parents and their preference. Intensive treatment was defined as administration of FVIII during $\geq$ 5 consecutive days. Only

patients who received > 20 EDs of FVIII and patients developing inhibitors before reaching 20 EDs were eligible for the evaluation of inhibitor incidence.

Laboratory Methods

Both standard Bethesda method and Nijmegen modification were used for inhibitor testing and the titers of > 0.6 BU/mL and > 0.5 Nijmegen BU/mL (NBU/mL) were considered as positive. The diagnosis of inhibitor was based on two consecutive positive results. *F8* genotyping was performed by standard techniques, such as long-distance polymerase chain reaction (PCR), multiple ligation-dependent probe amplification (MLPA), and DNA sequencing methods. Inversion of intron 22 and intron 1, large deletions, and nonsense mutations were classified as high-risk mutations, and small deletions/insertions and missense mutations as low-risk mutations for inhibitor development.

Immune Tolerance Therapy in Patients with Inhibitors

Patients and Immune Tolerance Induction Protocols

Since 1993 all consecutive ITI courses performed in the two largest hemophilia comprehensive care centers (HCCC), the NHC in Bratislava and the Regional HCCC in Banska Bystrica, have been evaluated. The data on the history of inhibitor development, the course of ITI, and the treatment outcomes are precisely recorded. The first patient was treated with Malmö protocol[32] with immunosuppression (corticosteroids, cyclophosphamide, and intravenous immune globulin G [IVIgG]). Low responders (LR) were treated with a modified low-dose protocol (LD ITI) using initial neutralizing phase with FVIII 50 IU/kg twice a day during 2 to 3 weeks, followed by 50 IU/kg every other day or three times a week. A high-dose (HD ITI) protocol (2 × 100 IU/kg/d) was recommended for high responders (HRs) with inhibitor levels > 5 BU/mL confirmed by both Bethesda and Nijmegen methods. Administration of a high-dose IVIgG and anti-CD20 antibodies (rituximab) during ITI was reserved for patients with a poor response to primary ITI and for rescue therapy. After confirmation of a complete success, FVIII dose was tapered down slowly by 20 IU/kg/d every 4 to 6 weeks toward prophylactic regimen 50 IU/kg every other day. In children with poor venous access, a central venous device (Port-A-Cath, Smiths Medical ASD, Inc., Dublin, OH) was implanted under the cover of recombinant FVIIa (rFVIIa).

Laboratory Monitoring

Inhibitor status was tested frequently upon the start of ITI to capture anamnestic peak titer and afterwards once monthly. After reduction of inhibitor < 1 NBU/mL in vivo recovery was monitored, and when negativity of inhibitor was achieved, investigation of FVIII pharmacokinetics was performed to determine FVIII clearance and half-life by standard method.[33]

Definition of Immune Tolerance Induction Outcome

Complete success (CS) was defined as negative inhibitor titer confirmed by both methods (< 0.6 BU/mL and < 0.5 NBU/mL), in vivo recovery > 66% and half-life > 6 hours. Partial success (PS) was determined by negative inhibitor without full normalization of recovery or half-life, however, enabling prophylaxis with FVIII, in the absence of anamnestic response. Treatment failure was defined as inability to eradicate inhibitor and install an effective prophylaxis within 36 months of ITI. Reappearance of inhibitor and/or treatment ineffectiveness in patients with previous CS and PS was classified as an inhibitor relapse.

Ethical Considerations

Both studies were conducted in accordance with the ethical principles according to the Declaration of Helsinki with the informed consent signed by patients and/or parents.

Statistical Analysis

Quantitative variables were expressed in means ± 1 standard deviation (SD) and medians (interquartile ranges [IQRs]) and compared by unpaired *t*-test or nonparametric Mann-Whitney test. Categorical parameters were evaluated by chi-squared test and Fischer's exact test. Kaplan-Meier method was used for analysis of inhibitor-free survival up to 150 EDs. Univariate and bivariate analysis as well as multivariate Cox regression were performed to assess the risk of inhibitor development using StatsDirect 2.8.0 software (StatsDirect Ltd., Cheshire, United Kingdom).

Results

►**Fig. 1** shows increasing cumulative incidence of inhibitors in PUPs with severe hemophilia in Slovakia observed within the past 25 years in three different time periods: 1990–1996, that is, from the introduction of purified FVIII concentrates to the start of prospective study: 10.3% (95% confidence interval [CI] 3.6–26.4%); 1997–2007, that is, a period with increased FVIII supply and nationwide introduction of prophylaxis in children:14.3% (95% CI 6.3–29.4%); and 2008–2015, that is, the period with introduction of rFVIII: 33.3% (95% CI 17.9–53.2%).

Prospective Study on Inhibitor Incidence and Risk Factors

Ninety patients with hemophilia A were born in Slovakia between January 1997 and October 2015, 61 with severe, 17 moderate, and 12 mild hemophilia. Fifty-nine of 61 patients with severe hemophilia A were treated with FVIII concentrates and were eligible for evaluation (►**Table 1**). All patients used the same brand of product up to at least 100 EDs or until development of inhibitor, whichever came first. All inhibitors developed within 35 EDs. Forty-six patients did not develop an inhibitor, of them 11% and 80% had > 50 and > 100 EDs, respectively. Ten patients switched from pdFVIII to rFVIII after > 100 EDs and they were analyzed in the pdFVIII group. In the given time period 13/59 (22%) PUPs with severe hemophilia A developed an inhibitor at a median age of 17 months (IQR 14–20) after 18 EDs (12–25 EDs), with historical peak of inhibitor 7.5 BU/mL (range 1.0–500 BU/mL). Eight (61.5%) and five (38.5%) inhibitor patients were high and low responders, respectively.

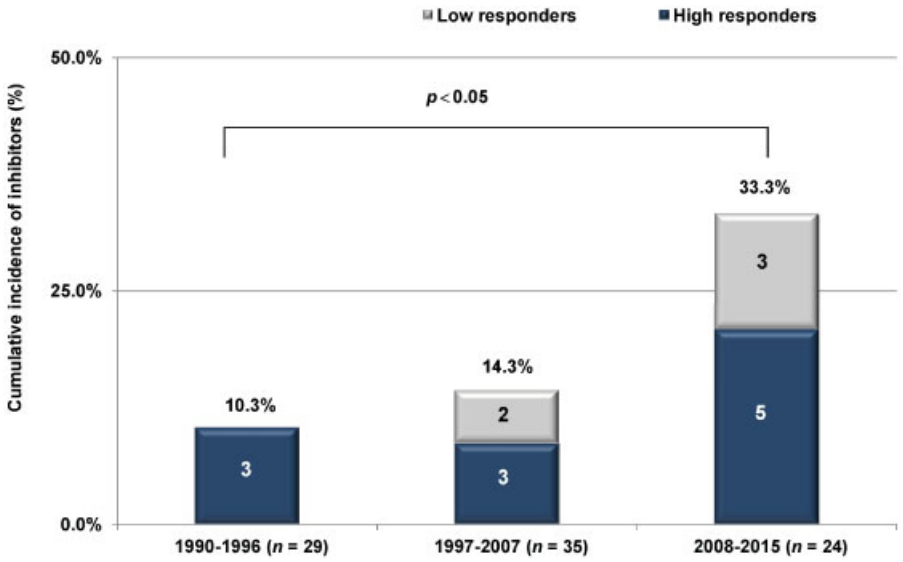

Fig. 1 Retrospective evaluation of cumulative incidence of inhibitors in previously untreated severe hemophilia A patients in Slovakia in three periods between 1990 and 2015. Period 1990–1996 with a use of purified plasma-derived FVIII (pdFVIII) concentrates and before the start of prospective study; period 1997–2007 characterized with increased access to pdFVIII and nationwide introduction of secondary prophylaxis in children; period 2008–2015: primary prophylaxis and recombinant FVIII (rFVIII) were introduced and either pdFVIII or rFVIII concentrates have been used in previously untreated patients (PUPs).

Table 1 Inhibitor prospective study in previously untreated patients born between 1997 and 2015: characteristics of 59 patients

Characteristic	Number (%)
Age (y)[a]	12.5 (4.5–12.5); 1.2–18
Family history of inhibitor	10 (17)
Gene mutation	
High-risk mutation	27 (46)
Low-risk mutation	13 (22)
Unknown	19 (32)
No of ED	
$\leq$ 20	8 (14)
$>$ 20–50	9 (15)
51–100	5 (8)
$>$ 100	37 (63)
First bleeding (mo)[a]	8 (5–12); 1 d—24
First exposure to FVIII (mo)[a]	11 (6–13); 1 d—24
Type of product	
pdFVIII	50 (85)
rFVIII	9 (15)
Switch for rFVIII after $>$ 100 ED of pdFVIII	10/50 (17)
Number of patients developing inhibitor	13 (22)
High responders	8(14)
Low responders	5 (8)
Age at inhibitor development (mo)[a]	17 (14–20); 11–60
No. of ED at inhibitor development[a]	18 (12–25); 6–35
Maximum inhibitor titer (BU/mL)[a]	7.5 (2.8–13); 1.0–500

Abbreviations: BU/mL, Bethesda unit per milliliter; ED, exposure days; FVIII, factor VIII; pdFVIII, plasma-derived FVIII; rFVIII, recombinant FVIII.
High-risk mutations: large deletions, intron 22 inversion, intron 1 inversion, nonsense mutations.
Low-risk mutations: small deletions, missense mutations.
[a]Values expressed in median (interquartile range); range.

Risk Factors and Inhibitor Development

The proportion of putative risk factors in 13 and 46 PUPs with and without inhibitors, respectively, is shown in the ►**Table 2**. In univariate analysis none of the risk factors showed significant association with inhibitor development except for serious/recurrent infections within the first 50 EDs (odds ratio [OR] = 5.0; 95% CI 1.16–21.9; $p = 0.03$) and the initial treatment with rFVIII (OR = 12.3; 95%CI 2.48–60.83; $p = 0.002$). Also, in bivariate analysis, high-risk mutations and positive family history of inhibitor were associated with inhibitor development ($p < 0.05$). The risk of rFVIII was significantly higher also in multivariate Cox regression analysis (hazard ratio [HR] 7.15; 95% CI 1.65–31.36; $p = 0.0086$), in which also intensive treatment reached significant association with inhibitor development (HR 4.38; 95% CI 1.02–18.67; $p = 0.046$). In contrary to expectations, the number of patients vaccinated with concurrent FVIII was apparently lower in inhibitor group (albeit nonsignificantly) compared with noninhibitor patients: 38.5 versus 54.3% (OR = 0.52; 95% CI 0.14–1.85; $p = 0.36$). No significant differences in the distribution of the risk factors were observed between the high and low responders.

Treatment Product and Inhibitor Development

Out of the 50 PUPs initially treated with pdFVIII, 7 (14%) developed inhibitor of whom 4 patients had a high-titer inhibitor. In the group of nine PUPs treated with rFVIII six (66.7%) patients developed inhibitors of whom four patients had high-titer inhibitors. None of the 10 patients switching from pdFVIII to rFVIII developed inhibitor. There was no significant difference in the distribution of putative confounders between pdFVIII and rFVIII treatment groups (►**Fig. 2**), except for a higher proportion of the family history of inhibitor in the rFVIII group: 4/9; 44.4% versus 6/50; 12% (OR = 5.86; 95% CI 1.22–28.12; $p = 0.03$). However, two PUPs born in 2012 were from one hemophilia family and they had negative history before they began therapy with rFVIII. The number of patients with > 50 EDs was significantly lower in the rFVIII group than in pdFVIII group due to a higher proportion of inhibitors within first 35 EDs. A subanalysis of 24 patients treated from 2008 onward, that is, when rFVIII and pdFVIII were concomitantly used, showed inhibitors development in 6/9 (66.7%) patients treated with rFVIII (4 HRs) versus 2/15 (13.3%) patients treated with pdFVIII concentrates (1 HR). ►**Table 3** shows the characteristics of inhibitors in patients treated with rFVIII and pdFVIII. The first bleeding and first treatment were recorded earlier in the pdFVIII inhibitor group, whereas the proportion of HRs and the inhibitor titers were higher in the rFVIII group (the difference was not statistically significant). As to the type of rFVIII, inhibitors developed in 4/4 PUPs treated with the second-generation full-length rFVIII; one of three patients treated with the third-generation rFVIII and one of two PUPs with a second-generation B-domain deleted FVIII (BDD FVIII).

All but one inhibitor patient, who had historical peak of 500 BU/mL and is still waiting for his inhibitor level to drop below 10 BU/mL, underwent ITI.

Immune Tolerance Induction

Between 1993 and 2015, 21 ITI treatments were performed in 19 severe hemophilia A patients with inhibitors in the NHC in Bratislava (15 primary and 3 rescue ITIs) and in regional HCCC in Banska Bystrica (3 primary ITIs). ►**Table 4** summarizes the main clinical characteristics of patients and ITI procedures. Six patients were PTPs who developed inhibitors after the switch from cryoprecipitate to purified FVIII at the median age 20 years (IQR 14–36) after a median of 28 exposures to FVIII concentrate (25–200 EDs). In one patient, inhibitors developed after major surgery and successfully eradicated with ITI; however, the patient had two relapses, each after the next consecutive operation. The PUPs treated exclusively with FVIII concentrates developed inhibitor earlier (median age 1.5 years; IQR 1.4–1.9 years) after 18 EDs (12–25 EDs). In one PUP high-titer inhibitor developed at the age of 1.5 years and was eradicated by 36 months ITI in another center. Inhibitor relapsed at age 10 years and the patient underwent a rescue ITI in our center at the age of 17 years. Patient with concomitant Down syndrome and Fallot tetralogy with early perspective of major cardiac surgery was put on early prophylaxis with rFVIII at the age of 4 months. He developed low-titer inhibitor after 6 EDs. He was treated with high doses of FVIII, which successfully covered also emergency heart surgery. However, ITI was not completed as he died on day 10 post-surgery due to heart failure.

In the entire group, a total of 11 patients were HRs, of whom 9 were treated with HD ITI and 2 with historical titer ≤ 20 BU/mL and negative pre-ITI titer were treated with LD ITI. Out of eight LRs, seven were treated with LD ITI and one with HD ITI. As shown in ►**Table 5**, pdFVIII/VWF concentrates were used in all but one ITI therapy. CS was achieved in 15/19 (79%) patients and PS in one (5.3%). Treatment failure in patient 10 was supported by poor prognostic factors and patient's noncompliance. HD ITI is ongoing in two HRs, including the patient with PS and still subnormal FVIII half-life.

Rescue therapy was used for inhibitor relapses in two patients. In patient 6 the LD ITI was given concomitantly with a standard cycle of rituximab (four doses of 375 mg/m^2) with a CS and the sustained remission after the first and second treatment with rituximab, respectively. Despite delayed rescue, ITI patient 7 achieved CS after 24 months of HD ITI. Patient 13 achieved an early PS after 3 months of ITI; however, each of the three infection complications of Port-A-Cath resulted in significant inhibitor increase with a peak of even 70 BU/mL despite continuing ITI. Addition of rituximab to the ITI at the month 40 resulted in complete inhibitor eradication with normalization of FVIII pharmacokinetics.

Port-A-Cath was used in eight patients (age range 1.5–6.0 years). In four patients 1 to 3 removal procedures and reimplantations were required, in two of them Port-A-Cath was switched for tunneled catheter Broviac (Bard Access Systems Inc.).

We analyzed the ITI outcome with regard to inhibitor titer and treatment regimen. All patients with pre-ITI inhibitor titer < 5 BU/mL (range 0–3.5 BU/mL) achieved a CS, while the ITI failed in one patient with pre-ITI titer of

Table 2 Inhibitor prospective study: risk factors in previously untreated patients developing inhibitors ($n = 13$) and without inhibitors ($n = 46$)

Characteristics	W/o inhibitor	Inhibitor	OR	*p*	High responders	Low responders	OR	*p*
	$n = 46$	$n = 13$	(95% CI)		8	5	(95% CI)	
	N (%)	*N (%)*			*N* (%)	*N* (%)		
Age (y)[a]	9 (5–12)	5 (3–9)		0.35	5 (3–6)	7 (5–7)		0.6
Range	1–18	2–18			2–18	2–17		
Family history of inhibitor	6 (13%)	4 (31%)	2.96 (0.7–12.7)	0.2	3 (40)	2 (40)	0.9 (0.09–8.9)	1
Gene mutation tested	27	13						
High-risk mutation (% of tested)	16 (59)	11 (85)	3.78 (0.69–20.51)	0.16	6 (75)	5 (100)		
Low-risk mutation (% of tested)	11(41)	2 (15)	0.26 (0.04–1.43)	0.16	2 (25)	0		
No of ED								
≤ 20	0	8 (62)			4 (50)	4(80)	0.25 (0.02–3.34)	0.56
> 20–50	4 (9)	5 (38)	6.5 (1.44–29.9)	0.02	4 (50)	1 (20)	4.0 (0.29–53.47	
51–100	5 (11)	0		–				
> 100	37 (80)	0		–				
Type of product								
pdFVIII	43 (94)	7 (54)	0.08 (0.01–0.4)	0.002	4 (50)	3 (60)	0.66 (0.06–6.4)	1
rFVIII	3 (6)	6 (46)	12.3 (2.5–60.9)	0.002	4 (50)	2 (40)	1.5 (0.15–14.42)	1
Switch for rFVIII after > 100 ED of pdFVIII	10 (22)	–			–	–		
First bleeding (mo)[a]	8 (6–12)	6 (4–10)		0.15	9 (4–11)	5 (4–6)		0.6
Age at first exposure to FVIII (mo)[a]	12 (6–13)	10 (5–13)		0.6	13 (12–13)	5 (4–6)		0.052
Day 1–1 mo	8 (17)	2 (15)	0.9 (0.15–4.67)	1.0	1 (12,5)	1 (20)	0.57 (0.02–11.8)	1.0
> 1–6 mo	12 (26)	5 (39)	1.77(0.48–6.48)	0.49	1 (12,5)	4 (80)	0.03 (0.00–0.76)	0.03
> 6–12 mo	11 (24)	2 (15)	0.57 (0.11–3.01)	0.71	2 (25)	0	1.5 (0.16–14.4)	
Risk factors within first 50 ED								
1st treatment 3–4 d	6 (13)	3 (23)	1.63 (0.8–3.4)	0.31	2 (25)	1 (20)	1.5 (0.16–14.4)	1
1st treatment ≥ 5 d	7 (15)	4 (31)	2.47 (0.59–10.3)	0.23	1 (12,5)	3 (60)	0.09 (0.006–1.49)	
Surgery	2 (4)	2 (15)	4.0 (0.5–31.64)	0.20	1 (12,5)	1 (20)	0.57 (0.02–11.8)	1
Vaccination concurrent with FVIII	25 (54)	5 (39)	0.52 (0.14–1.85)	0.36	3 (37.5)	2 (40)	0.9 (0.09–8.9)	1
Severe/recurrent infections	5 (11)	5 (39)	5.0 (1.16–21.9)	**0.03**	2 (25)	3 (60)	0.22 (0.02–2.45)	0.29
RBC transfusion	8 (17)	3 (23)	1.43 (0.31–6.38)	0.69	1 (12,5)	2 (40)	0.21 (0.01–3.36)	0,51
Patients with inhibitors								
Age at inhibitor diagnosis (mo)[a]	–	22 (15–18)		–	19 (18–21)	15 (11–16)		0.14
No of ED at inhibitor development	–	18 (8–18)		–	21 (14–22)	12 (12–15)		0.27

Abbreviations: ED, exposure days; FVIII, factor VIII; OR (95% CI), odds ratio (95% confidence interval); pdFVIII, plasma-derived FVIII; RBC, red blood cells; rFVIII, recombinant FVIII.
[a]Median, IQR.

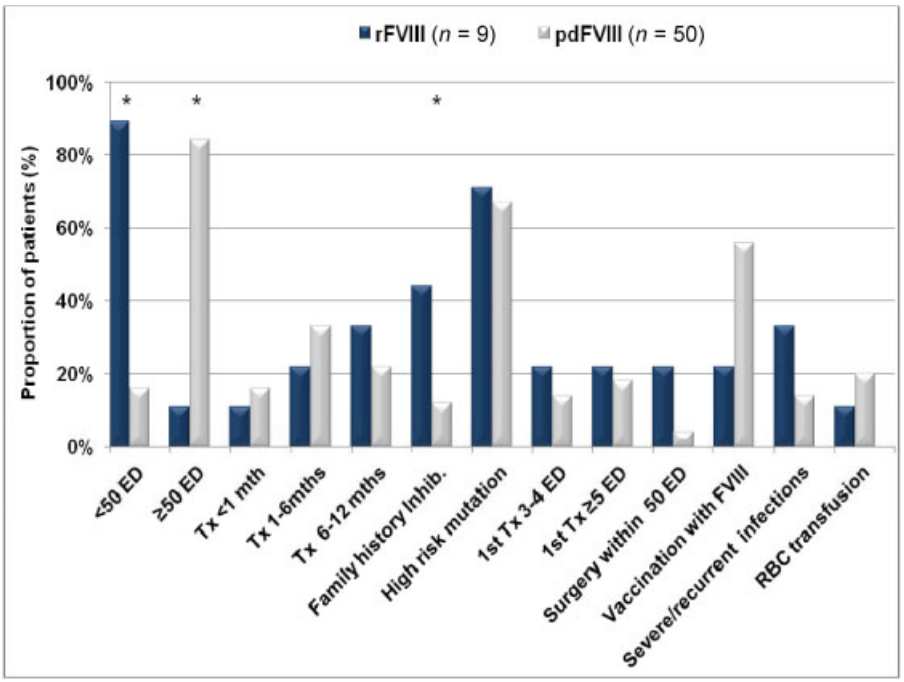

Fig. 2 Inhibitor prospective study: proportion of risk factors in PUPs treated initially with recombinant FVIII ($n = 9$) and plasma-derived FVIII ($n = 50$). $^{*}p < 0.05$. 1st Tx, first therapy with FVIII concentrate; ED, exposure days; Inhib, inhibitor; pdFVIII, plasma-derived FVIII; RBC, red blood cells; rFVIII, recombinant FVIII.

10.8 BU/mL. The median time to negativity of inhibitor and to CS was significantly shorter in LR than in HR: 2.8 (IQR 2–3) versus 6.0 months (2–11.3), $p < 0.001$; and 7.5 (IQR 3–6) versus 20.0 months (13.5–24.0), $p < 0.001$, respectively. The LD ITI resulted in the inhibitor negativity and CS earlier than HD ITI. However, the HD ITI was used in patients with less favorable prognosis (HRs and poor prognosis factors). Despite this, the success rate in HD ITI group was 5/6 (83.3%) when all completed HD ITI treatments were evaluated.

Discussion

Concomitantly with the progress in hemophilia care including an improved access to replacement therapy and prophylaxis, we are witnessing an increase in the incidence of inhibitors, today the most challenging complication of hemophilia therapy. Increased morbidity, less effective hemostatic therapy with bypassing agents, limited access to prophylaxis, and demanding ITI inevitably bring a huge burden on patients, their families, and hemophilia treaters as well. The

Table 3 Inhibitor prospective study: characteristics of previously untreated patients who developed inhibitors after rFVIII ($n = 6$) and pdFVIII ($n = 7$)

	rFVIII	pdFVIII	*p*
	***n* = 6**	***n* = 7**	
Age (y)	4.5 (3.3–5.0)	9 (5.5–16)	0.22
High-risk mutation (*n*/%)	4/67%	7/100%	
1st bleed (mo)	7(5.3–9.5)	5 (0.03–9.5)	0.1
1st therapy (mo)	12 (7.5–14)	5 (2.5–5.5)	0.15
Age at inhibitor (mo)	16 (14.3–17.8)	20 (17.5–35)	0.17
No. of ED at inhibitor	18 (11–24)	18 (12–24)	0.88
HR (*n*/%)	4/67%	4/57%	0.55
LR (*n*/%)	2/33%	3/43%	
Maximal titer (BU/mL)	10.2 (3.5–12.5); 0.8–500	6.8 (3.3–13.9); 3.6–27	0.76

Abbreviations: BU/mL, Bethesda unit per milliliter; ED, exposure days; HR, high responder; LR, low responder; pdFVIII, plasma-derived FVIII; rFVIII, recombinant FVIII.
Note: Values expressed in median (IQR); range.

Table 4 Characteristics of patient with inhibitors undergoing Immune tolerance therapy

Characteristic	*n* (%)
All	19 (100)
Family history of inhibitor	6 (31.6)
Age at 1st infusion (y)[a]	1.0 (0.6–1.4)
Therapy before inhibitor	
PTPs (cryoprecipitate > 100 ED) switched for pdFVIII	5 (26.3)
PTPs (cryoprecipitate > 100 ED) switched for rFVIII	1 (5.3)
PUPs treated with pdFVIII	8 (42.1)
PUPs treated with rFVIII	5 (26.3)
PTPs	6 (31.6)
Age at inhibitor development (y)[a]	20 (14 - 36)
No. of ED of FVIII concentrate before inhibitor[a]	28 (25 - 200)
PUPs	13 (68.4)
Age at inhibitor development (y)[a]	1.5 (1,4 - 1,9)
No. of ED before inhibitor[a]	18 (12 - 25)
Inhibitor category	
Low responders (< 5 BU/mL)	8 (42.1)
High responders (> 5 BU/mL)	11 (55.9)
Age at the start of ITI (y)[a]	6.0 (2.0–17.0)
Total No. of ITI	21 (100)
Primary ITI	18 (85.7)
Rescue ITI for relapse	3 (14.3)
Low-dose regimen	11 (52.4)
High-dose regimen	10 (47.6)
Central venous device (Port-A-Cath)	8 (42.1)

Abbreviations: BU/mL, Bethesda unit per milliliter; ED, exposure days; ITI, immune tolerance induction; pdFVIII, plasma-derived FVIII; PTPs, previously treated patients; PUPs, previously untreated patients; rFVIII, recombinant FVIII.
[a]Median (interquartile range).

situation is further aggravated by the fact that this complication arises mostly in the youngest children with severe hemophilia soon after initiation of therapy.

Several potential risk factors for inhibitor development has been recognized and several models for inhibitor prediction based on the combinations of these risk factors were developed.[34,35] Since so-called "nonmodifiable" genetically determined risk factors[5,36] of inhibitors cannot be avoided, to prevent an impact of "modifiable" environmental and treatment-related risk factors, various preventive approaches were recommended such as early prophylaxis, avoidance of early surgery and intensive treatment with high doses of factor concentrate, vaccination without concomitant factor administration, etc.[36–38] However, an early or intensive treatment and emergency surgery cannot be fully avoided in all patients and early prophylaxis is not feasible for all; thus the potential role of the type of FVIII product in inhibitor development becomes increasingly important. In patients using rFVIII cumulative inhibitor, incidence of 30% and even higher has been reported, with high-titer inhibitors reaching 19 to 24%.[7,8,17,39] Recently several studies pointed on a higher immunogenicity of some brands of rFVIII.[7,8,15] However, the large observational multicenter studies,[15,40,41] national and international surveys,[10,11,16,42,43] and, in particular, a growing number of meta-analyses of numerous, mostly retrospective PUPs studies,[17,44–48] provide contradictory and still inconclusive information. Gouw et al reported the results of RODIN (Research Of Determinants of INhibitor development among PUPs with haemophilia) study showing comparable incidence of inhibitors in patients treated with pdFVIII a rFVIII.[15] This contrasts with the results of other studies[10,11,14,49] as well as with the situation in our country. The differences in the incidence of inhibitors in different studies are usually attributed to the frequency of inhibitor testing, laboratory method used, and/or the different design of the studies. Nevertheless, the probability of overlooking the clinically relevant inhibitors in experienced center is extremely low and more frequent testing only improves revealing of very low-titer or transient inhibitors. Our prospective nationwide inhibitor study showed in 59 PUPs born

Table 5 Immune tolerance induction and treatment outcomes in 19 patients with inhibitors

Pat. No	Mutation	Pre-Inh. therapy	Age at 1st FVIII (y)	Age at Inh. Dg (y)	No. of ED FVIII before Inh.	Historical peak (BU/mL)	Age at ITI (y)	pre-ITI Inh. titer (BU/mL)	ITI protocol	Product	Adjuvant Tx	Peak titer on ITI BU/mL	Time to Inh. neg-ve (mo)	Time to CS (mo)	Out-come	FVIII Half-life (h)	Follow-up since CS (y)
1	Missense	Cryo-pdFVIII	1.5	13	26	6.5	13	0.6	LD	pdFVIII		1.2	5	6	CS	14	22
2	Small del.	Cryo-pdFVIII	1.0	15	20	17.0	15	0.9	Malmö	pdFVIII		4.6	2	10	CS	9.2	20
3	int 22 inv	Cryo-pdFVIII	1.1	13	25	2.8	13	1.0	LD	pdFVIII		3.0	1	8	CS	10.4	18
4	Small del.	Cryo-pdFVIII	1.5	40	> 100	3.5	40	3.5	LD	pdFVIII		3.5	1	6	CS	10.0	9
5	Small del.	Cryo-rFVIII	1.0	24	30	20.0	31	0.6	LD	pdFVIII		3.0	3	9	CS	7.7	10
6	Nonsense	Cryo-pdFVIII	2.0	57	> 100	3.5	57	3.5	LD	pdFVIII		3.8	1	3	CS→Rel	8.0	1
„	„	pdFVIII	„	58	1st Rel.	3.5	58	3.3	LD[a]	pdFVIII	Ritux	22.0	2	6	CS→Rel	8.5	2
„	„	pdFVIII	„	60	2nd Rel.	22.0	60	2.5	LD[a]	pdFVIII	Ritux	2.5	3	4	CS	9.4	5
7	Missense	pdFVIII	0.7	1.5	15	2800	17	2.0	HD[a]	pdFVIII		4.0	15	24	CS	8.4	2
8	Missense	pdFVIII	1.0	3	25	500.0	15	1.7	HD	pdFVIII		0.7	10	24	CS	10.4	2.5
9	int 22 inv	pdFVIII	2.0	4	18	2.8	5	0.0	LD	pdFVIII		1.2	3	6	CS	7.2	11
10	int 22 inv	pdFVIII	3.0	5.5	34	138	6.0	10.8	HD	pdFVIII	IVIgG	2100	–	–	Failure	–	–
11	Missense	pdFVIII	day 1	1.8	10	2.4	2.4	0.5	LD	pdFVIII		1.9	6	12	CS	8.9	13
12	Missense	pdFVIII	0.9	1.5	35	7.8	2.7	0	HD	pdFVIII		45.0	2	18	CS	10.3	4
13	int 22 inv	rFVIII prophyl	1.2	1.4	32	8.4	1.5	7.4	HD	pdFVIII	Ritux	70.0	42	43	CS	7.4	0.9
14	int 22 inv	rFVIII prophyl	0.3	0.9	25	1.8	1.0	1.2	LD	rFVIII		1.9	2	3	CS	7.9	5
15	int 1 inv	rFVIII prophyl	0.4	0.6	8	0.8	0.6	0.8	HD	pdFVIII		1.0	NA	NA	NA	–	Died[b]
16	int 22 inv	rFVIII	1.0	1.4	20	12.6	1.5	3.0	HD	pdFVIII		3.8	9	13	CS	8.4	2
17	Small del.	rFVIII prophyl	0.4	1.5	15	13.0	2.0	3.2	HD	pdFVIII		3.6	2		PR	5.4	ITI ongoing
18	Nonsense	pdFVIII	1.1	1.9	9	20.0	2.8	3.4	HD	pdFVIII		50.0	ongoing 3 mo		PR	NA	ITI ongoing
19	Nonsense	pdFVIII	0.5	0.9	12	4.6	1.5	0.5	LD	pdFVIII		4.5	2	6	CS	8.0	0.5
	Median		1.0	3.0	20	8.1	6	1.5				**3.6**	**3**	**8**		**8.5**	**4.5**
	IQR		0.6–1.4	1.5–15	15–26	3.5–20	2–17	1.5–3.4				**1.9–4.6**	**2.6**	**6.18**		**7.9–10**	**2–11**

Abbreviations: Adjuvant Tx, adjuvant therapy; cryo, cryoprecipitate; cryo-pdFVIII, switch from cryoprecipitate to plasma-derived FVIII; cryo-rFVIII, switch from cryoprecipitate to recombinant FVIII; CS, complete success; Dg, diagnosis; ED, exposure days; HD, high-dose regimen; Inh, inhibitor; int 1 inv, intron 1 inversion; int 22 inv, intron 22 inversion; IQR, interquartile range; ITI, immune tolerance induction; IVIgG, high-dose intravenous immune globulin G; LD, low-dose regimen; NA, not applicable; pdFVIII, plasma-derived FVIII; PR, partial remission; prophyl, prophylaxis; Rel., relapse; rFVIII, recombinant FVIII; Ritux, rituximab; small del, small deletion.

[a]Rescue ITI after relapse.

[b]Concomitant Down syndrome and Fallot tetralogy; died due to heart failure after surgery.

in Slovakia between January 1997 and October 2015 a cumulative incidence of all and high-titer inhibitors 22% (95% CI 11.4–32.6%) and 13.5% (95% CI 4.3–22.2%), respectively. A slight increase compared with previous retrospective evaluation may be partially attributed to increased access to FVIII concentrates during the 1990s. However, a stable factor supply since 2000 and treatment policy according to the national guidelines[50] cannot explain a notable increase in inhibitors, especially since 2008, when prophylactic therapy was common and rFVIII started to be used in PUPs. Moreover, inhibitor incidence in patients treated with pdFVIII remained stable (7/50; 14%), while inhibitors appeared in six (67%) of nine PUPs initially treated with rFVIII (OR 12.3; 95% CI 2.5–60.9). Inhibitors were diagnosed in all four PUPs treated with the second-generation full-length rFVIII (3 and 1 high and low titer, respectively); one of three patients treated with the third-generation rFVIII and one of two PUPs with a second-generation BDD FVIII. Univariate analysis of putative risk factors in entire group of patients showed significant association of inhibitors with rFVIII products ($p = 0.002$) and recurrent infections ($p < 0.03$), in multivariable analysis in addition to rFVIII also intensive treatment reached statistical significance ($p = 0.046$). There was no difference in the distribution of risk factors between inhibitor patients treated with rFVIII or pdFVIII, except for a higher frequency of family history of inhibitor in rFVIII group ($p = 0.04$). All PUPs were obligatory vaccinated. In agreement with literature,[12,51] vaccination concurrent with preventive FVIII administration was not a risk factor for inhibitor development.

Our observations of higher incidence of inhibitors in PUPs treated with rFVIII is in concordance with so much awaited results of the first randomized prospective PUPs study (SIPPET [Survey of Inhibitors in Plasma-Products Exposed Toddlers]) presented at the 57th Annual Meeting of the American Society of Hematology and so far preliminary reported in abstract form. This study demonstrated the cumulative incidence of inhibitors 26.7% (95% CI 18.3–35.1%) for pdFVIII and 44.5% (95% CI 34.7–54.3%) for rFVIII, representing HR 1.87 (95% CI 1.18–2.97) or 87% higher inhibitor incidence after rFVIII compared with pdFVIII. For high-titer inhibitors, the HR was 1.70 (95% CI 0.96–2.99) which, however, did not reach statistical significance.[52]

The major limitation of our study is the small sample size. On the other hand, the advantage is a homogenous patient population from one country with similar treatment conditions. The occurrence of eight new, clinically relevant inhibitors in a 7-year period, without any change in treatment policy other than type of product, is a reality pointing out a major safety issue in hemophilia therapy. In theory, to achieve an incidence of 14% as observed with pdFVIII, another 33 patients treated with rFVIII would have to remain free of inhibitor. As reported by Franchini et al, because of still vivid memory of the immense tragedy of human immunodeficiency virus (HIV) infection transmitted by plasma-derived non-virally inactivated concentrates, the choice of the type of product in Italy is still dictated by safety concerns rather than immunogenicity of products.[53] However, Slovakia has not been confronted with HIV infection as none of our hemophiliacs was infected, so an increasing incidence of inhibitors is perceived as a major safety issue. Tremendously challenging management of inhibitors, especially in little children, with potential risk of the ITI failure has an impact on the product choice. In the current era of availability of virologically safe pdFVIII concentrates, most of our hemophilia treaters are reluctant to put the PUPs on rFVIII products. This has been also a major reason for so far low number of subjects in our rFVIII study group.

The only effective treatment for eradication of inhibitors is ITI. Depending on the protocol, success can be achieved in 60 to 90% of patients.[21,31,54–57] Relevant predictors of a good prognosis of ITI include historical inhibitor titer < 200 BU/mL, pre-ITI titer < 10 BU/mL, younger age, and shorter interval from inhibitor diagnosis to ITI start.[56–58] Type of *F8* gene mutation also seems to have an impact on the outcome of ITI.[59] Several studies and previous international registries demonstrated conflicting data regarding to the ITI dosing protocols and success rate.[54,56,57] The results of the first prospective randomized international study (IITI) did not demonstrate the difference in the success between the LD and HD ITI in HRs with good prognosis; however, a high-dose strategy using FVIII $\geq$ 100–200 IU/kg/d resulted in reduced bleeding frequency during ITI and faster achievement of success.[21] This may justify the use of HD ITI protocols in all HRs, with either good or poor prognostic factors. Controversy remains also regarding the treatment product for ITI and the impact on ITI success.[54] Several centers demonstrated a potential for FVIII concentrates containing VWF to achieve a high rate of successful inhibitor eradication.[22–26,60] In vitro and in vivo experiments showed that pdFVIII/VWF provides better protection against inhibitor neutralization than rFVIII, which results in prolonged persistence of FVIII in the circulation.[61] Most of current guidelines for ITI commonly recommend the switch for FVIII/VWF concentrates in patients who failed to achieve ITI success with rFVIII as well as for the rescue ITI.[30,31,54,62]

Our study included all consecutive patients with FVIII inhibitors undergoing ITI in two HCCC and showed a high success rate in inhibitor eradication. The CS and PS was achieved in 17/19 (89.5%) patients, including four patients with poor prognosis factors. Evaluating only the patients who completed ITI, the success rate was even higher (15/16; 94%), comprising 7/7 LRs and 8/9 HRs.

High success rate in our study was associated with (1) an early start of ITI in most patients; (2) low historical and pre-ITI inhibitor titers; (3) preference of the HD protocol for the high responders; and (4) possibly to preferred use of FVIII/VWF concentrates. Our ITI policy is based on the experience with FVIII/VWF in the ITI from other centers.[22–26] We do not wait for the failure of ITI with products missing VWF, but we prefer to start with FVIII/VWF concentrates as we consider this approach more biologically plausible. The use of FVIII/VWF concentrates is supported also by the most recent results of prospective Observational ITI study (OBSITI) demonstrating a high rate of CS (70.8%) even in the HRs with poor prognostic factors.[24] Our three inhibitor patients successfully participated in this study.

Use of adjuvant immunosuppressive therapy, especially anti-CD 20 antibodies in patients with hemophilia and

inhibitors, is a matter of intensive debate. The use of rituximab is mostly supported by several case reports or small series, with a success rate ranging between 25 and 53%.[63–67] Several ITI guidelines recommend this treatment as a second-line therapy after the ITI failure or in patients refractory to ITI.[30,31,55,62] We used rituximab in two patients as an adjuvant to the rescue ITI therapy with a good response and CS.

Conclusion

We present 25-year experience with FVIII inhibitors in patients with severe hemophilia A. Our prospective study shows that rFVIII products are significant risk factors for inhibitor development in our PUPs with severe hemophilia A. We realize that because of a small sample size in our study, the power of statistical evaluation is limited and the results must be interpreted with a caution. However, several new inhibitors observed in a recent period resulting in increased demands on the resources for therapy reflects a real world, which cannot be ignored. Our experience with ITI using FVIII/VWF concentrates and proper use of high-dose protocol showed favorable results with a high success rate.

Recombinant products undoubtedly represent a major progress in the treatment of hemophilia, and new-generation, longer-acting recombinant factors will probably replace current drugs soon. With regard to this fact it is of paramount importance to have the products with a wider margins of safety in terms of inhibitor development, which will be safe in all situations of hemophilia management and not only in "ideal" low-risk patients.

Authors' Contribution

A.B. and E.B. designed the study. A.B. and A.M. collected and evaluated the data and wrote the manuscript. A.B., E.B., D.J., T. P., A.M., and M. Mi. participated in the patients follow-up and management of ITI. J.H. and J.C. provided comprehensive inpatient pediatric care at the initiation of the ITI, and M. Ma. took care of the central venous access in outpatients. J.CH. and D.B. arranged the genetic testing. All authors revised the manuscript and approved its final version.

Conflict of Interest

A.B., E.B., D.J., T.P., and A.M. participated in the OBISTI Study sponsored by Octapharma.

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