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Brazilian Immune Tolerance BrazIT Study

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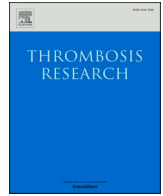
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Full Length Article

Large deletions and small insertions and deletions in the factor VIII gene predict unfavorable immune tolerance induction outcome in people with severe hemophilia A and high-responding inhibitors

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ABSTRACT

Introduction: Hemophilia A is an inherited bleeding disorder caused by pathogenic variants in the factor VIII gene (F8), which leads to factor VIII (FVIII) deficiency. Immune tolerance induction (ITI) is a therapeutic approach to

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eradicate alloantibodies (inhibitors) against exogenous FVIII in people with inherited hemophilia A. Few studies have evaluated the role of *F8* variants on ITI outcome.

Material and methods: We included people with severe hemophilia A (FVIII < 1 international units/dL) and high-responding inhibitors (≥ 5 Bethesda units/mL lifelong) who underwent a first course of ITI. Socio-demographic, clinical and laboratory data were collected. ITI outcomes were defined as total, partial successes, and failure. Detection of intron 1 and 22 inversions was performed by polymerase-chain reaction, followed by *F8* sequencing. **Results:** We included 168 people with inherited hemophilia A and high-responding inhibitors, median age 6 years at ITI start. Intron 22 inversion was the most prevalent variant (53.6 %), followed by nonsense (16.1 %), small insertion/deletion (11.3 %), and large deletion (10.7 %). In comparison with intron 22 inversion, the odds of ITI failure were 15.5 times higher (odds ratio [OR] 15.50; 95 % confidence interval [95 % CI] 4.59–71.30) and 4.25 times higher (95 % CI, 1.53–12.3) among carriers of *F8* large deletions and small insertions and deletions, respectively.

Conclusion: *F8* large deletions and small insertions/deletions predicted ITI failure after a first course of ITI in patients with severe hemophilia A and high-responding inhibitors. This is the first study to show *F8* large deletions and small insertions/deletions as predictors of ITI failure.

1. Introduction

Hemophilia A (HA; OMIM #306700; <http://www.omim.org>) is an X-linked inherited bleeding disorder caused by pathogenic variants in the factor VIII (FVIII) coding gene (*F8*), which leads to the deficiency of FVIII [1]. *F8* encompasses 186 kilobases at the distal arm of the X chromosome (Xq28) and comprises 26 exons that encodes a six-domain protein of 2332 amino acids [2]. About 45 % of patients with severe hemophilia A (FVIII < 1 international units (IU)/dL) carry a homologous recombination event within the intron 22 of *F8* and its extragenic inversely oriented copies (Inv22–1 and Inv22–2) [3,4]. Other *F8* pathogenic alterations reported in people with severe hemophilia A are large deletions, nonsense and, less frequently, missense, and splice-site variants [5].

Despite the advent of non-factor replacement therapy (emicizumab) for hemophilia A [6], exogenous FVIII still remains the mainstay of hemophilia A treatment in most parts of the world. FVIII replacement can lead to the development of neutralizing alloantibodies (i.e., inhibitors) against FVIII in up to 35 % of people with severe hemophilia A [7]. Ultimately, inhibitors render FVIII replacement ineffective for both episodic and prophylactic treatment, demanding alternative treatments [8]. As result, people with hemophilia A and high-responding inhibitors have worse outcomes due to increased morbidity and mortality [9,10].

Immune tolerance induction (ITI) is the recommended therapeutic approach to eradicate inhibitors and is based on regular and sustained administration of FVIII concentrates to reestablish tolerance [11]. The success of ITI is variable, ranging from 60 % to 80 % across studies [12]. Determinants of ITI outcome are largely unknown, of which mostly are of non-genetic basis. Indeed, a systematic review and meta-analysis confirmed that lower historical peak titer, pre-ITI titer and peak titer during ITI were associated with a higher chance of ITI success [13]. However, few studies have evaluated the role of *F8* variants on ITI outcome [14–17]. These studies were either descriptive or reported the variants in block and not individually [14–17].

In the present study, we evaluated the association of *F8* pathogenic variants on ITI outcome in people with severe hemophilia A and high-responding inhibitors (≥ 5 Bethesda units [BU]/mL) among well-characterized patients who underwent ITI in the HEMFIL or the Brazilian Immune Tolerance (BrazIT) cohorts [18,19].

2. Methods

2.1. Population

We included people with severe hemophilia A and high-responding inhibitors and participants of the HEMFIL ($n = 15$) and the BrazIT ($n = 228$) cohort studies who underwent ITI [18,19]. Both studies were approved by local Committees on Ethics Research (CAAE 05648412.2.2002 and CAAE 52812415.8.0000.5149). Written

informed consent was obtained from all participants or their guardians. Data were collected from medical records and using standardized forms.

People with severe hemophilia A and high-responding inhibitors were enrolled at 16 Hemophilia Treatment Centers in Brazil. All patients were treated with ITI according to the protocol of the Brazilian Ministry of Health [20]. For this study, the inclusion criteria were: (a) inherited, unrelated people with hemophilia A and (b) residual plasma FVIII activity < 1 IU/dL before inhibitor development, (c) inhibitor titer ≥ 5 BU/mL at least once during lifetime (i.e., high-responding inhibitor) [21], (d) requirement of bypassing agents (recombinant factor VII activated [rFVIIa] or activated prothrombin complex concentrates [aPCC]) for episodic treatment or prophylaxis, and (e) completion of a first course of ITI (Supplementary Fig. 1). All individuals were included regardless of sex, age, or time between inhibitor development and ITI start.

Inhibitor positivity was defined as a titer of ≥ 0.6 BU/mL at two measurements taken at least 2 weeks apart using a Nijmegen-modified Bethesda assay [21]. Historic peak inhibitor titer was the highest inhibitor titer detected before the start of ITI. Pre-ITI titer referred to inhibitor titer immediately before start of ITI. Inhibitor peak during ITI corresponded to the highest inhibitor titer during ITI. Race/ethnicity were self-reported as skin color. Information on date of birth, date of hemophilia A and inhibitor diagnosis, date of ITI start and end were also collected.

2.2. Immune tolerance induction protocol

The Brazilian ITI Protocol is described elsewhere [22]. In brief, FVIII type (whether recombinant or plasma-derived) was the same against which the patient with hemophilia developed inhibitor. ITI regimen was 50 IU/kg of FVIII concentrate 3 times weekly. If inhibitor titers did not reduce by 20 % in 3–6 months, the regimen was incremented 100 IU/kg of FVIII daily. The maximal ITI duration was approximately 33 months.

ITI outcomes were assessed using the criteria published elsewhere [23,24]. In brief, total success was defined as a persistently negative inhibitor titer (< 0.6 BU/mL), and normal FVIII pharmacokinetics (i.e., recovery ≥ 66 % of the expected value and half-life ≥ 6 h). Partial success was defined as a negative inhibitor titer but persistently abnormal FVIII pharmacokinetics, without an anamnestic response. Failure was defined as presence of high-responding inhibitor titer of 5 BU/mL or higher. No patient received emicizumab before or during ITI.

2.3. Detection of *F8* variants

For DNA extraction, peripheral blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Detection of inversion of intron 1 (Inv1) was carried out as previously described with slight modifications [25]. Intron 22 inversion was detected by inverse-shift polymerase chain reaction (PCR) technique, according to previous report with minor adaptation [26]. To unveil *F8* genetic variants

Table 1
Characteristics of the study population (n = 168).^a

		Missing
Age, in years, at diagnosis of hemophilia A, median (IQR)	0.8 (0.5–1.4)	9
Ethnicity, n (%)		2
White	94 (56.6)	
Pardo	61 (36.7)	
Black	11 (6.6)	
F8 variant type, n (%)		0
Intron 22 inversion	90 (53.6)	
Inv22-1	68 (40.5)	
Inv22-2	22 (13.1)	
Nonsense	27 (16.1)	
Small insertion/deletion	19 (11.3)	
Poly-A run ^b	2 (10.5)	
Large deletion	18 (10.7)	
Multiple exons/UTR	16 (88.9)	
Missense	5 (3.0)	
Splice site	5 (3.0)	
Conserved nucleotide ^c	5 (100)	
Intron 1 inversion	4 (2.4)	
Inhibitor characteristics		
Age at inhibitor diagnosis, in years, median (IQR)	2.0 (1.2–5.8)	0
Age at ITI start, in years, median (IQR)	6 (2–16)	2
Interval between diagnosis of hemophilia A and inhibitor development, in years, median (IQR)	1.5 (0.6–5.8)	15
Historic inhibitor peak, in BU/mL, median (IQR)	45 (19–131)	8
Inhibitor titer immediately before ITI, in BU/mL, median (IQR)	7 (3–14)	8
Inhibitor peak during ITI, in BU/mL, median (IQR)	33 (6–162)	8
ITI duration, in years, median (IQR)	2.6 (1.8–3.1)	8

BU, Bethesda Units; F8, factor VIII gene; FVIII, factor VIII; IQR, interquartile range; ITI, immune tolerance induction; IU, International Units; UTR, untranslated region.

^a All included patients have severe (factor VIII activity levels <1 %) hemophilia A and high-titer inhibitor.

^b Poly-A run was defined as at least 6 adenines in a row.

^c Conserved splice site was defined as those affecting the +1, +2, −1, or −2 nucleotide position at a splice site.

Table 2
Characteristics of the study population according to immune tolerance induction outcome.

Characteristics	ITI outcome (n = 168)		P
	Success ^a (n = 107)	Failure (n = 61)	
Age, in years, at diagnosis of hemophilia A, median (IQR)	0.8 (0.5–1.4)	0.9 (0.6–1.1)	0.800
Ethnicity, n (%)			0.026
White	59 (62.8)	35 (37.2)	
Pardo	37 (60.7)	24 (39.3)	
Black	11 (100.0)	0 (0.0)	
F8 variant type, n (%)			
Intron 22 inversion	68 (75.6)	22 (24.4)	<0.001
Inv22-1	50 (73.5)	18 (26.5)	0.033
Inv22-2	18 (81.8)	4 (18.2)	0.061
Nonsense	16 (59.3)	11 (40.7)	0.510
Small insertion/deletion	8 (42.1)	11 (57.9)	0.037
Poly-A run ^b	0 (0.0)	2 (100.0)	0.130
Large deletion	3 (16.7)	15 (83.3)	<0.001
Multiple exons/UTR	2 (12.5)	14 (87.5)	<0.001
Missense	5 (100.0)	0 (0.0)	0.160
Splice site	4 (80.0)	1 (20.0)	0.654
Conserved nucleotide ^c	4 (80.0)	1 (20.0)	0.654
Intron 1 inversion	3 (75.0)	1 (25.0)	1.000
Inhibitor characteristics			
Age at inhibitor diagnosis, in years, median (IQR)	2.4 (1.4–6.8)	3.6 (1.4–7.7)	0.300
Age at ITI start, in years, median (IQR)	6 (2–16)	7 (2–16)	0.500
Interval between diagnosis of hemophilia A and inhibitor development, in years, median (IQR)	1.2 (0.6–4.8)	2.7 (0.5–6.3)	0.500
Historic inhibitor peak, in BU/mL, median (IQR)	30 (14–57)	128 (48–461)	<0.001
Inhibitor titer immediately before ITI, in BU/mL, median (IQR)	4 (3–9)	17 (7–57)	<0.001
Inhibitor peak during ITI, in BU/mL, median (IQR)	13 (4–46)	173 (61–659)	<0.001
ITI duration, in years, median (IQR)	2.2 (1.6–2.9)	2.8 (2.3–3.4)	<0.001

BU, Bethesda Units; F8, factor VIII gene; FVIII, factor VIII; IQR, interquartile range; ITI, immune tolerance induction; IU, International Units; UTR, untranslated region.

^a Success represents the sum of total and partial successes.

^b Poly-A run was defined as at least 6 adenines in a row.

^c Conserved splice site was defined as those affecting the +1, +2, −1, or −2 nucleotide position at a splice site.

Table 3

Association of factor VIII gene variants with immune tolerance induction outcome.

F8 variant, n (%)	Success ^a (n = 107)	Failure (n = 61)	OR (95 % CI)	P
Intron 22 inversion	68 (75.6)	22 (24.4)	1.00 (Reference)	–
Nonsense	16 (59.3)	11 (40.7)	2.13 (0.85–5.25)	0.1
Small insertion/deletion	8 (42.1)	11 (57.9)	4.25 (1.53–12.30)	0.006
Large deletion	3 (16.7)	15 (83.3)	15.50 (4.59–71.30)	<0.001
Missense	5 (100.0)	0 (0.0)	–	–
Splice site	4 (80.0)	1 (20.0)	0.77 (0.04–5.57)	0.8
Intron 1 inversion	3 (75.0)	1 (25.0)	1.03 (0.05–8.52)	>0.900

CI, confidence interval; F8, factor VIII gene; ITI, immune tolerance induction; OR, odds ratio; 95 % CI, 95 % confidence interval.

^a Success represents the sum of total and partial successes.**Table 4**

Characteristics of the included population stratified to factor VIII gene large deletions and other variants.

Characteristics	Overall (n = 168)	Large deletion (n = 18)	Other variants ^a (n = 150)	P
Ethnicity, n (%)				0.700
White	94 (100.0)	11 (11.7)	83 (88.3)	
Pardo	61 (100.0)	7 (11.5)	54 (88.5)	
Black	11 (100.0)	0 (0.0)	11 (100.0)	
ITI outcome, n (%)				<0.001
Total success	48 (100.0)	1 (2.1)	47 (97.9)	
Partial success	59 (100.0)	2 (3.4)	57 (96.6)	
Failure	61 (100.0)	15 (24.6)	46 (75.4)	
ITI and inhibitor titer				
Age at ITI start in years, median (IQR)	6 (2–16)	12 (5–20)	6 (2–14)	0.150
Interval between diagnosis of hemophilia A and inhibitor development in years, median (IQR)	1.5 (0.6–5.8)	3.5 (1.0–6.3)	1.4 (0.6–5.6)	0.400
Historic inhibitor peak in BU/mL, median (IQR)	45 (19–131)	122 (47–298)	43 (17–95)	0.024
Inhibitor titer immediately before ITI in BU/mL, median (IQR)	7 (3–14)	29 (11–73)	6 (3–13)	0.001
Inhibitor peak during ITI in BU/mL, median (IQR)	32 (6–162)	144 (54–792)	30 (6–139)	0.002
ITI duration in years, median (IQR)	2.55 (1.75–3.13)	2.74 (2.26–3.15)	2.52 (1.71–3.12)	0.300

^a Other variants correspond to the sum of intron 1 and 22 inversion, nonsense, small insertion/deletion, missense and splice site variants. BU, Bethesda Units; IQR, interquartile range; ITI, immune tolerance induction.

beyond inversions, two different high-throughput sequencing approaches were used. The first approach detected *F8* variants in people with hemophilia A from the HEMFIL Study. A customized gene panel (Illumina, San Diego, United States of America [USA]) was designed to target all exonic and intronic flanking regions, as well the 5' and 3' untranslated regions (UTRs), of *F8* (chrX:154,835,788–155,026,940, GRCh38/hg38). The libraries were prepared using the AmpliSeq Custom DNA Panel (Illumina) and were sequenced on the Miseq system (Illumina). Quality metrics, variant calling, and annotation were performed using the DNA Amplicon application (Sequence Hub platform, Illumina). The second approach was used for people with hemophilia A included in the BrazIT Study. A whole exome sequencing (WES) approach was performed at DASA Laboratory (São Paulo, Brazil) using xGen Exome Research kits Panel v2, xGen CNV Backbone Panel (IDT, Coralville, US). Sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina). The bioinformatics analysis was performed using the platform StrandOmics (<https://clinical.strandomics.com/>), based on the GRCh37/hg19. Annotation was performed with ANNOVAR [27] on the *F8* region (chrX:154,064,064–154,250,998).

2.4. Classification of *F8* variants

F8 variants were classified following the guidelines for variant interpretation and pathogenicity established by the American College of Medical Genetics and Association for Molecular Pathology (ACMG/AMP) [28], Varsome (<https://varsome.com/>), and ClinVar databases (<https://www.ncbi.nlm.nih.gov/clinvar/>). The transcript numbering was based on Ref Seq NM_000132.3 cDNA, following the guidelines of Human Genomic Variation Society (HGVS) (<http://varnomen.hgvs.org/>).

The category of *F8* variants were classified based on their deleterious impact on FVIII activity [28]. *F8* genotype was categorized as follows: (i)

large deletions (> 50 base-pairs [bp]), affecting a single exon or multiple exons/regions; (ii) nonsense and missense variants, occurring in either the light or heavy chain of FVIII; (iii) small deletions/insertions, found either within or outside poly-A runs (defined as sequences of at least 6 adenines in a row); (iv) intron 1 and 22 inversions, and (v) splice site, classified into conserved (affecting +1, +2, –1, or –2 nucleotide position at a splice site) and non-conserved nucleotides (all other types of splice site).

2.5. Statistical analysis

Continuous variables were reported as medians and interquartile range (IQR), and median differences were assessed using Mann-Whitney's *U* test or Kruskal-Wallis' *H* test. Categorical variables were reported as numbers and relative frequencies in percentages. Associations between categorical variables were evaluated using χ^2 test or Fisher's exact test. Logistic regression was used to estimate odds ratios (OR) using the intron 22 inversion variant as reference. Missing data were excluded from the analysis. Statistical analysis was conducted using R version 4.1.0 (<https://www.R-project.org/>).

3. Results

3.1. Study population

We included 168 people with severe hemophilia A and high-responding inhibitors, median age 6 years (IQR 2–16) at ITI start. The majority self-declared as white (56.6 %). Intron 22 inversion was the most prevalent variant (53.6 %), followed by nonsense (16.1 %), small insertion/deletion (11.3 %), and large deletion (10.7 %). Detailed information on the characteristics of the participants is shown in Table 1.

Table 5Main characteristics of the large deletion variants^a and respective immune tolerance induction outcome^b.

Deletion range (n = 18)	F8 exon/region	FVIII domain	FVIII chain	ITI outcome
One exon	Exon 12	A2	Heavy	Total success
	Exon 14	B	Heavy	Failure
Multiple exons/regulatory region	5'UTR - exon 1	A1	Heavy	Partial success
	5'UTR - exon 6	A1	Heavy	Partial success; failure ^c
	5'UTR - exon 14	A1-B	Heavy	Failure
	5'UTR - exon 22	A1-C1	Light	Failure ^d
	Exon 2–13	A1-A2	Heavy	Failure
	Exon 4–13	A1-A2	Heavy	Failure
	Exon 4–6	A1	Heavy	Failure
	Exon 7–10	A1-A2	Heavy	Failure
	Exon 7–11	A1-A2	Heavy	Failure
	Exon 7–12	A1-A2	Heavy	Failure§
	Exon 14–3'UTR	B-C2	Heavy/light	Failure
	Exon 23–25	C1-C2	Light	Failure

F8, factor VIII gene; FVIII, factor VIII; ITI, immune tolerance induction; UTR, untranslated region.

^a >50 base-pairs.^b Success represents the sum of total and partial successes.^c Two unrelated individuals carried the same mutation, although responded differently to ITI.^d Three unrelated individuals carried the same mutation and presented the same ITI outcome (failure). § Two unrelated individuals carried the same mutation and presented the same ITI outcome (failure).

3.2. Characteristics associated with immune tolerance induction outcome

Since the characteristics of the groups with total and partial successes were similar (Supplementary Table 1), these groups were merged and considered as success ITI in the subsequent analyses.

Black people with severe hemophilia A were more likely to respond successfully to ITI. Intron 22 inversion was associated with successful ITI (75.6 % success vs. 24.4 % failure; $p < 0.001$), while large deletions (16.7 % success vs. 83.3 % failure; $p < 0.001$) and small insertion/deletion (42.1 % success vs. 57.9 % failure; $p = 0.037$) were associated with ITI failure. Except for two participants, 14 patients with severe hemophilia A who carried deletion of multiple exons/UTR had ITI failure (Table 2).

Few people with severe hemophilia A had missense and splice site variants (3 % each). This confirms the scarcity of this type of F8 variants in individuals with severe hemophilia A and high-titer inhibitors. Interestingly, except for one patient with hemophilia A who carried a splice site variant and had ITI failure, the remaining people with severe hemophilia A carrying missense and splice site variants (9 out of 10; 90 %) had ITI success (Supplementary Table 2). Despite small numbers, this suggests that F8 missense and splice site variants associated with a favorable ITI outcome (Table 2).

We found three missense variants (c.1 A > G, c.1456 A > G and c.2020G > A) which had not been previously reported with inhibitor development. Furthermore, the missense variants c.1456 A > G and c.2020G > A are newly reported variants. The splice-site variants c.2113 + 1G > T and c.5998 + 1G > A are also reported here for the first time in association with inhibitor development. Of all missense and splice-site variants found, only the variant c.5815 + 1G > A associated with ITI failure (Supplementary Table 2).

Table 2 presents ITI success according to patient and inhibitor characteristic and according to FVIII mutation variants. Higher inhibitor titer as opposed to lower inhibitor titers were associated with unfavorable ITI outcome. This applied to historic inhibitor peak, inhibitor titer immediately before ITI and inhibitor peak during ITI (all $p < 0.001$). A longer ITI duration was also associated with ITI failure ($p < 0.001$). Age at inhibitor diagnosis, age at ITI start, and interval between diagnosis of hemophilia A and inhibitor development were not associated with ITI outcome (Table 2).

3.3. Factor VIII gene variants and immune tolerance induction outcome

Table 3 presents the OR for the association between ITI success and F8 variants. In comparison with people with severe hemophilia A carrying intron 22 inversion, the ones carrying F8 large deletions had an odds of 15.5 (95 % CI, 4.59–71.3) times higher of failing ITI. Small insertion/deletion variants also conferred an odds of 4.25 (95 % CI, 1.53–12.3) times higher of ITI failure when compared with intron 22 inversion (Table 3).

Three out of 18 (17 %) patients with hemophilia A carrying a large deletion had successful ITI as opposed to 104 out of 150 (69 %) carrying the other variants ($p < 0.001$). People with severe hemophilia A carrying large deletions had higher levels of historic inhibitor peak (median 122 BU/mL [IQR 47–298] vs 43 [IQR 17–95]; $p = 0.024$), inhibitor titer immediately before ITI (median 29 BU/mL [IQR 11–736] vs 6 [IQR 3–13]; $p = 0.001$) and inhibitor peak during ITI (median 144 BU/mL [IQR 54–792] vs 30 [IQR 6–139]; $p = 0.002$) in comparison with the ones who carried other variants, respectively (Table 4).

Most of the large deletions identified was in the FVIII heavy chain, either in the A1, A2 or A1-A2 domains of FVIII (Table 5). We found no association between the location of non-structural F8 variants (missense, small insertions/deletions, nonsense, and splice site) and immune tolerance induction outcome (Supplementary Table 3).

4. Discussion

In this study, we evaluated the role of F8 genotype in predicting the outcome of ITI in a large cohort of people with severe hemophilia A and high-responding inhibitors who completed one course of ITI. We found that, in comparison with intron 22 inversion, F8 large deletions and small insertion/deletions associated with about 15-fold and 4-fold higher odds of failure in inhibitor eradication following ITI. To our knowledge, this is the first study to report an association of these variants as predictors of ITI failure and the largest cohort investigating the association of F8 genotyping of people with severe hemophilia A and high-responding inhibitors with ITI outcome reported to date.

Pathogenic variants in F8 are a well-established risk factor for inhibitor development in people with hemophilia A [29]. However, few studies have investigated the role of F8 variants on ITI outcome [14–16]. In our study, 18/168 (10.7 %) people with severe hemophilia A presented F8 large deletions, of whom 16/18 (88.9 %) carried deletion

encompassing multiple exons. Carrying *F8* large deletions increased the odds of ITI failure about 15 times. Salviato et al. were the first to suggest the association between *F8* large deletions and ITI unresponsiveness [16]. However, this suggestion was based on a small study, reporting on 16 people with severe hemophilia A and high-responding inhibitors, of whom 2/16 carried *F8* large deletion and failed ITI [16]. Peyvandi et al. also reported that only 1/8 patients with hemophilia A and high-responding inhibitors carrying *F8* large deletion had successful ITI [17]. However, these studies were only descriptive, and did not compare the outcome with other *F8* variants. Furthermore, the studies did not inform on whether the deletions involved one or more *F8* exons or UTR.

Coppola et al. evaluated *F8* genotype and ITI outcome in 86 people with severe hemophilia A and high-responding inhibitors from the Italian PROFIT Study [14]. They found that low-risk mutations for inhibitor development (small insertions/deletions and missense mutations) were associated with higher rate of ITI success on a multivariate analysis (adjusted OR 6.2; 95 % CI 1.1–36.0) when compared with those carrying high-risk mutations [14]. These findings were confirmed in an update of the PROFIT Study which included 137 people with severe hemophilia A and high-responding inhibitors. In this follow-up, the authors confirmed that low-risk mutations predicted total inhibitor eradication ($p = 0.027$) [15]. However, both studies merged *F8* variants into high- and low-risk mutations and, therefore, were unable to test the effect of individual variants on ITI outcome [14] [15]. In our study, despite the low numbers of *F8* missense and splice site variants, 9 out of 10 (90 %) people with severe hemophilia A carrying these variants had successful ITI. The small numbers of these variants is expected in our study, because our cohort was composed by people with severe hemophilia A and high-responding inhibitors, in whom low-risk mutations are expected to be less prevalent, such as demonstrated in a recent report from the PedNet Group [30].

ITI can eradicate inhibitors in 60 %–80 % of people with hemophilia A and high-responding inhibitors [12]. However, it is a demanding and costly treatment. Furthermore, with the advent of non-factor therapies, the use of ITI to eradicate inhibitors is debatable. Despite this, predicting ITI response may guide therapeutic options based on a personalized approach. It is conceived that the main ITI predictors are anti-FVIII inhibitor titers [13]. However, despite cut-offs of inhibitor titers have been proposed as criteria for ITI outcome and implementation of different ITI regimens [11–13], a robust prediction tool for ITI outcome is not yet available. The results of our study add knowledge of genetic determinants (*F8* variants) of ITI outcome, which can in the future, be incorporated in a prediction tool for ITI outcome. This could tailor the therapeutic approach based on individual *F8* variant profile to maximize treatment efficacy.

We did not find any association between the position of *F8* non-structural variants in the FVIII domains, or light/heavy chains with ITI outcome. A study conducted with a European cohort [14] corroborates our findings, also presenting a lack of relationship between the location of *F8* mutations (and nonsense variants occurring in coding for the heavy or light chain of FVIII) and ITI outcome [14]. Previous studies on epitope mapping have demonstrated that domain's specificity of FVIII alloantibodies could influence ITI outcome [31–35]. People with hemophilia A with successful ITI presented inhibitory alloantibodies exclusively directed towards the light chain of FVIII [31], and plasma levels of antibodies against A1 and A2 domains prior to ITI were seemed to predict who were successfully tolerized [32]. Despite remarkably, these studies enrolled a small number of patients with hemophilia A and, therefore, validation of their findings in larger cohorts is needed.

The main strength of our study refers to its large population size comprising two well-characterized cohorts undergoing the same ITI protocol and outcome definitions. This enabled us to perform a more refined analysis, accounting for the investigation of specific types of *F8* variants and ITI outcome instead of grouping them into mutation categories, such as has been performed in previous studies [14,15,36]. Other strengths worth mentioning are that ITI outcome was centrally reviewed

and *F8* genotyping was centrally performed in the coordinating center. Finally, to our knowledge, this is the first study to investigate an admixed population of people with severe hemophilia A and high responding inhibitors, adding to the understanding of ITI outcome in a more diverse population.

Some limitations of our study should be mentioned. Firstly, the prevalence of people with severe hemophilia A and high-responding inhibitors carrying some types of *F8* variants was low, precluding robust analyses regarding ITI outcomes. This was particularly the case of missense and splice-site variants, which are observed in low frequencies in people with severe hemophilia A worldwide and accounted for 6 % of the variants in our study. Secondly, the assessment of FVIII levels and inhibitors was performed in the local laboratories of the HTCs. However, these laboratories have external quality control programs in place. Finally, additional mutations (*de novo* inversions, complex rearrangements, or variants located in introns and regulatory regions) could not be covered by the whole exonic sequencing approach used in the study.

We conclude that *F8* large deletion and small insertion/deletion are predictors of ITI failure in patients with severe hemophilia A and high-responding inhibitors after a first course of ITI.

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CRediT authorship contribution statement

Luciana Werneck Zuccherato: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Renan Pedra Souza:** Writing – review & editing, Writing – original draft, Validation, Formal analysis. **Ricardo Mesquita Camelo:** Writing – review & editing, Visualization, Validation, Data curation. **Maise Moreira Dias:** Writing – review & editing, Validation, Data curation. **Letícia Lemos Jardim:** Writing – review & editing, Validation, Data curation. **Marcio Antônio Portugal Santana:** Writing – review & editing, Validation, Data curation. **Andrea Gonçalves Oliveira:** Writing – review & editing, Data curation. **Claudia Santos Lorenzato:** Writing – review & editing, Data curation. **Monica Hermida Cerqueira:** Writing – review & editing, Data curation. **Vivian Karla Brognoli Franco:** Writing – review & editing, Data curation. **Rosângela de Albuquerque Ribeiro:** Writing – review & editing, Data curation. **Leina Yukari Etto:** Writing – review & editing, Data curation. **Maria do Rosario Ferraz Roberti:** Writing – review & editing, Data curation. **Fábia Michelle de Araújo Callado:** Writing – review & editing, Data curation. **Maria Aline Ferreira de Cerqueira:** Writing – review & editing, Data curation. **Ieda Solange de Souza Pinto:** Writing – review & editing, Data curation. **Andrea Aparecida Garcia:** Writing – review & editing, Data curation. **Tania Hissa Anegawa:** Writing – review & editing, Data curation. **Daniele Campos Fontes Neves:** Writing – review & editing, Data curation. **Doralice Marvulle Tan:** Writing – review & editing, Data curation. **Rafael Pereira Tou:** Writing – review & editing, Data curation. **Daniel Gonçalves Chaves:** Writing – review & editing, Data curation. **Johanna van der Bom:** Writing – review & editing, Methodology. **Suely Meireles Rezende:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

RMC received speaker fees from Bayer, NovoNordisk, Hoffman-La Roche, and Takeda, consultancy fees from Hoffman-La Roche and Takeda, and sponsorship to attend scientific event grants from Bayer, NovoNordisk, Hoffman-La Roche, and Takeda. AGO received speaker fees and scientific event grants from NovoNordisk and Takeda. MRFR received speaker fees from NovoNordisk, scientific event grants from Hoffman-La Roche, Takeda and NovoNordisk, and consultancy fees from the Brazilian Ministry of Health. FMRAC received sponsorship to attend

scientific event grants from Hoffman-La Roche and NovoNordisk. LYE received scientific event grants from Hoffman-La Roche. MAFC received sponsorship to attend scientific event grants from Hoffman-La Roche and NovoNordisk. ISSP received speaker fees from Hoffman-La Roche, NovoNordisk and Takeda, sponsorship to attend scientific event grants from Hoffman-La Roche, Takeda and NovoNordisk, consultancy fees from the Hoffman-La Roche, NovoNordisk, Bayer and Biomarin, and grants for scientific publication from Takeda. AAG received speaker fees from Takeda and NovoNordisk, sponsorship to attend scientific event grants from Takeda and NovoNordisk, and consultancy fees from NovoNordisk. THA received sponsorship to attend scientific event grants from Hoffman-La Roche and Takeda. DCFN received sponsorship to attend scientific event grants from Takeda. LWZ, RPS, MMD, MAPS, LLJ, RAR, MHC, CSL, DMT, VKBF, RPT, DGC, JVDB and SMR declare they have no competing interests which might be perceived as posing as a conflict.

Data sharing statement

The data that support the findings of this study are available from the corresponding author (SMR), upon reasonable request due to ethical restrictions.

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