Haemophilia
The Official Journal of the World Federation of Hemophilia,
European Association for Haemophilia and Allied Disorders and
the Hemostasis & Thrombosis Research Society

Haemophilia (2013), **19**, 511–518 DOI: 10.1111/hae.12105

ORIGINAL ARTICLE *Clinical haemophilia*

Factor VIII genotype characterization of haemophilia A affected patients with transient and permanent inhibitors: a comprehensive Argentine study of inhibitor risks

L. C. ROSSETTI,*† I. SZURKALO,*† C. P. RADIC,*† M. M. ABELLEYRO,*† L. PRIMIANI,‡§ D. NEME,‡§ M. CANDELA,†‡§ R. P. BIANCO,†‡§ M. DE TEZANOS PINTO,†‡§ I. B. LARRIPA*† and C. D. DE BRASI*†

**Instituto de Medicina Experimental (IMEX), CONICET-Academia Nacional de Medicina; †Instituto de Investigaciones Hematológicas Mariano R Castex, Academia Nacional de Medicina; ‡Fundación de la Hemofilia Alfredo Pavlovsky; and §Grupo Argentino de Estudio de Inhibidores en Hemofilia (GADEI), Buenos Aires, Argentina*

Summary. Inhibitor development against exogenous factor VIII is a severe impairment of replacement therapy affecting 18% of Argentine patients with severe haemophilia A (HA). To study the molecular predisposition for inhibitor development, we genotyped 260 HA patients with and without inhibitors, countrywide. The inhibitor-positive population (19 transients, 15 low responders, LR and 70 high responders, HR) of 104 severe-HA patients showed 59 Inv22 (intron 22 inversions), 18 small ins/del-frameshifts, 12 gross deletions, 12 nonsense, one splicing defect and two missense, p.Arg531Pro and p.Leu575Pro, both LR and thought to impair FVIII A2 domain secondary structure. In addition, a patient with mild HA and HR showed the missense p.Glu1704Lys associated with two neutral intronic substitutions potentially affecting the A3 domain. A case/control study (84/143) permitted estimation of *F8* genotype–specific inhibitor risks [OR; prevalence (CI)] in severe-HA patients classifying a high-risk group including multi-exon deletions [3.66; 55% (19–100)], Inv22 [1.8; 24% (19–100)] and nonsense in FVIII-LCh [1.2; 21% (7–59)]; an average risk group including single-exon deletions, indel frameshifts and nonsense-HCh; and a low-risk group represented by missense defects [0.14; 3% (0.6–11)]. Analysis of inhibitor concordance/discordance in related patients indicated additional genetic factors other than *F8* genotype for inhibitor formation. No significant inhibitor-predisposing factors related to FVIII product exposure were found in age- and *F8* genotype–stratified populations of severe-HA patients. In conclusion, the Argentine HA patient series presents similar global and mutation-specific inhibitor risks than the HA database and other published series. This case-specific information will help in designing fitted therapies and follow-up protocols in Argentina.

Keywords: *F8*, FVIII inhibitors, HEMA, mutation characterization

Introduction

Haemophilia A (HA) (OMIM 306700) is an X-linked coagulopathy that affects one in 5000 human males worldwide. In 2009 the Argentine population corresponded to 41.343.201 inhabitants including 1842 registered people with HA and 227 who developed clinically identified factor VIII inhibitors [1]. HA is classified by the residual clotting activity of FVIII in severe, moderate and mild affecting about 40%, 10% and 50%, respectively, of patients with HA [2].

HA is caused by heterogeneous molecular defects in the coagulation FVIII gene (*F8*). Due to a complex structure of 26 exons spread over 186 kb of genomic DNA on Xq28 [3], *F8* still challenges mutation detection in many laboratories, particularly those from developing countries. Most patients with HA are efficiently treated with FVIII concentrates. However, about 20–30% of severe cases with HA from different ethnicity developed FVIII neutralizing antibodies (inhibitors) making replacement therapy ineffective [4].

The pathogenesis of FVIII inhibitors is influenced by host-related and therapy-related factors. Most inhibitors occur in persons with severe HA – those with

Correspondence: Liliana C. Rossetti, Molecular Genetics of Haemophilia Section, Academia Nacional de Medicina. Pacheco de Melo 3081, Buenos Aires (1425), Argentina.
Tel.: 5411 4805 8803; fax: 5411 4803 9475;
e-mail: rossetti@hematologia.anm.edu.ar

GADEI members are listed in Appendix

Accepted after revision 14 January 2013

baseline levels of FVIII of <0.01 U/mL – and several studies have shown that the causative mutation is the most decisive risk factor for inhibitor formation. Those mutations that prevent endogenous synthesis of FVIII (null mutations), such as large deletions, nonsense mutations and the prevalent intron 22 inversions (Inv22), are associated with the highest risk of inhibitor formation. These findings indicated that the presentation of a novel antigen to the patient's immune system is the main driving force of inhibitor formation, representing a frequent event in about one-third of severe-HA patients [5].

However, other host-related factors have been shown to influence inhibitor development in HA, such as the ethnic origin – for example, persons of African ancestry with HA tend to show higher inhibitor risk than Caucasian [6] – indicating the importance to report studies on population-specific estimations of the inhibitor risk factors worldwide.

Most patients develop inhibitors in early childhood after relatively few exposure days to FVIII (median 9–11 days). Some patients are high responders (≥ 5 BU/mL) with a rapid anamnestic response to FVIII, whereas others are low responders whose inhibitor concentration never exceeds few Bethesda units (typically <5 BU/mL). Some inhibitors (10–30%) are transient and thus can be missed if testing is not carried out at frequent intervals. In many patients with HA, inhibitors can be completely suppressed or eradicated with an appropriate immune tolerance induction (ITI) regimen [7].

This study describes a comprehensive and multicentre countrywide *F8* genotype characterization study of Argentine patients with HA and inhibitors using a cost-effective laboratory scheme, particularly useful for developing countries. The analysis between the type and location of *F8* mutation allows us to associate specific HA patients with locally estimated inhibitor risks.

Materials and methods

Studied populations

A total of 260 patients with HA from different regions of Argentina were enrolled in this study, 107 with inhibitors and 153 without. Inhibitor population included 106 severe and one mild HA patients classified by their inhibitor status in T (transient, <6 months), LR (low responders, 1–5 BU/mL) and HR (high responders, >5 BU/mL).

Since 1995–2008 an inhibitor-unbiased population of 107 Argentine familiar probands with severe HA was *F8* genotyped, prior to the start point of the protocol for *F8* genotype characterization of patients with inhibitors (2009–11), was used to state the natural *F8* mutation type/location frequencies and to estimate the absolute inhibitor risks (Table 1, SHA). A comprehensive population of 227 severe-HA patients (including the 107 unbiased population mentioned above) was analysed to estimate the relative mutation-specific inhibitor risks. Thirty-three Argentine patients with moderate (17) and mild (16) HA were also studied.

Our institutional Ethics Committee approved the study and a written informed consent was obtained in all cases.

Inhibitor testing was performed once every 6 months and every 3 months in inhibitor-positive patients before a surgery or other medical procedure. For inhibitor screening, samples of 3.2% sodium citrate-anticoagulated venous blood were drawn. Inhibitor testing was performed by Bethesda assay using the Nijmegen modification [8,9]. A positive inhibitor titre was defined as equal to or more than 0.6 BU/mL.

Table 1. FVIII inhibitor development vs. *F8* mutation type/location in Argentine patients with severe HA.

Mutation type	SHA[†] (%)	Cases[‡] $n = 84$	Controls[§] $n = 143$	OR[¶] (CI 95%)	IP[††] (CI 95%)	*P* value[‡‡]
INV22	44.0	48	60	1.84 (1.07–3.18)	24 (18–29)	0.0287*
INV1	1.9	0	2	0.33 (0.02–7.07)	5.9 (0.3–100)	0.5317
MED	6.5	8	4	3.66 (1.07–12.55)	55 (19–100)	0.0604
SED	3.7	3	5	1.02 (0.24–4.39)	18 (4–69)	1.0000
NS.LCH	6.5	5	7	1.23 (0.38–4.01)	21 (7–59)	0.7642
NS.HCH	3.7	4	9	0.74 (0.22–2.50)	13 (4–41)	0.7719
FS.I/D	15.9	13	27	0.79 (0.38–1.62)	14 (7–26)	0.5905
IF.I/D	1.9	0	2	0.33 (0.02–7.07)	5.9 (0.3–100)	0.5317
MS	12.2	2	21	0.14 (0.03–0.62)	3 (0.6–11)	0.0025**
SPD	3.7	1	6	0.27 (0.03–2.33)	5 (0.6–39)	0.2641

[†]SHA (%): Natural (unbiased) mutational prevalence in severe HA showing an absolute inhibitor prevalence estimation of 17.6% ($n = 107$), similar to those previously reported in the same population [11].
[‡]Cases: Permanent inhibitor-positive cases (high and low responders).
[§]Controls: Inhibitor negative or transient.
[¶]OR: Inhibitor likelihood odds ratio (Mutation positive/Mutation negative); (CI 95%): Confidence interval of 95%.
[††]IP (%): Absolute inhibitor prevalence; (CI 95%).
[‡‡]*P* value: Fisher exact test *P* value; *$P < 0.05$ significant; **$P < 0.01$ highly significant.
MED: Multi-Exon Deletion; SED: Single-Exon Deletion; NS.LCH: Nonsense Light Chain; NS.HCH: Nonsense Heavy Chain; FS.I/D: Frameshift Indel; IF.I/D: In-Frame Indel; MS: Missense; SPD: Splicing Defect.

Clinical data related to therapy and blood product exposure for the analysis of an age-stratified subgroup of 151 severe-HA patients were available: 81 cases with inhibitors and 70 controls without inhibitors. This group encompassed patients with the Inv22 (98), nonsense (22) and Ins/del frameshifts (31).

F8 mutation characterization

The molecular protocol was applied on genomic DNA prepared from peripheral-blood leucocytes using standard phenol–chloroform or salting-out methods. Inv22 and intron 1 inversions (Inv1) were analysed by inverse shifting polymerase chain reaction (PCR) [10]. All the relevant sequences of *F8* (37 products) were screened for large deletions and small mutations by multiplex PCR amplification and CSGE (conformation sensitive gel electrophoresis) respectively [11]. Large deletions were defined as a consistent specific amplification failure in a group of contiguous exons. Small mutation screening was performed by heteroduplex analysis using high-resolution CSGE, and DNA sequencing of specific PCR amplification product/s that showed anomalous CSGE patterns [11].

Bioinformatics

DNA sequence management was performed using files mapping to Xq28 spanning *F8* (ChrX: 154064063–154250998 complement). *F8* mutation nomenclature followed the Human Genome Variation Society (HGVS) recommendations [12] and those for genetic variants in haemostasis [13]. However, to be consistent with other studies, p. notation followed FVIII legacy amino acid (aa) numbering (i.e. start point of mature FVIII). Amino acidic changes were examined for their conservation in murine, porcine and canine FVIII using the sequence alignment line-up provided by HADB (Haemophilia A mutation database, http://hadb.org.uk/) [14].

Analysis of ESEs (Exonic Splicing Enhancers) was performed online using ESE-Finder software (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) [15]. The NNSPLICE software was used to score new potential splicing sites associated with intronic substitutions (http://www.fruitfly.org/seq_tools/splice.html) [16].

Data analysis

Relative and absolute *F8* genotype–specific inhibitor risks were estimated as odds ratio (OR) and inhibitor prevalence, respectively; by a case–control study of severe-HA patients using the Fisher exact test.

Clinical data on FVIII product exposure were compared between cases and controls populations by Mantel–Haentszel (MH) common odds ratio on three layers stratified by age (2–12 years; 12–34 years and >34 years; each associated with a peak of high probabilities to start inhibitor response). Stratification was performed because case and control groups differ on average age (cases: 17.3 years, controls: 20.9 years; Student's *t*-test $P = 0.043$). MH statistics included calculation of the MH common OR, homogeneity analysis of *stratum*-specific ORs (Breslow–Day test) and the Cochran–MH (CMH) test of conditional independence (Ho: CMH OR $\neq 1$).

Results and discussion

F8 *genotype characterization of HA patients with inhibitors*

We found the HA-causative mutation in 107 Argentine patients with FVIII inhibitors: 72 HR (67%), 16 LR (15%) and 19 T (18%). The 2009 WFH Annual Survey informed that Argentina presented 227 HA patients with clinically identified inhibitors [1] indicating that we could characterize about one half of these cases countrywide.

Among them, 59 patients (55%) showed the Inv22 (51 type 1 and eight type 2 patterns) (40 were HR, 9, LR, and 10, T) and 12 (11%) showed large deletions, three single-exon deletion (all HR) and nine multi-exon deletions (eight HR or one T) (Fig. 1). Four multi-exon deletions were novel, whereas exon 10 and 5 single-exon deletions were reported in HADB but without inhibitors (Fig. 1). Twelve severe-HA patients with inhibitors (11%) showed nonsense mutations: six affected the FVIII Light Chain (LCh) (four HR, one LR and one T) and six, the Heavy Chain (HCh) (four HR and two T) (Fig. 1). Our highly reported nonsense mutations p.Arg2147* (7+ /24 entries) and p.Arg 1966* (8+ /19) associated with hypermutable CpG transitions. Among our nonsense mutations with HR inhibitors, p.Ser488*, p.Tyr586* and p.Gln796* were not associated with inhibitors in the HADB, and p.Gln2222* and p.Gln2235* were novel (Fig. 1).

Small insertions/deletions frameshifts (indel) were the most represented group of small mutations (18 cases, 17%) (nine HR, four LR and five T) (four in the LCh and 14 in the HCh). Among them, six patients showed four novel indels: p.Pro451Leufs*12 (three cases from two unrelated families), p.Ala811Alafs*15, p.Arg1233Serfs*4 and p.Lys1012Leufs*9. Consistent with the immunogenic and functional profiles of FVIII domains, most indels in B domain were associated with LR or T inhibitors, whereas indels in A2 or A3 with HR inhibitors (Fig. 1).

Three missense mutations (3%) were found with inhibitors (Fig. 1). All of them involve highly conserved residues as indicated in orthologous FVIII line-ups of porcine, murine, canine and human FVIII. Interestingly,

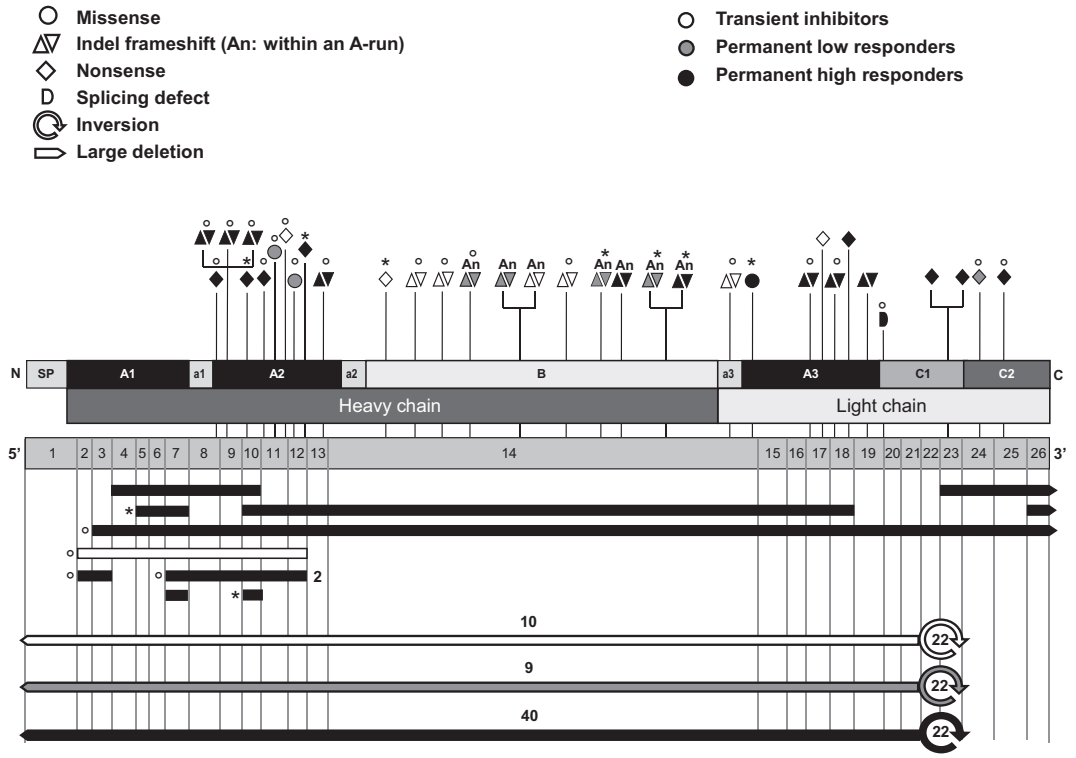

Fig. 1. Spectrum and molecular distribution of HA causative in Argentine patients with HA and with permanent or transient inhibitors (n = 105). Icons for each type of mutation are shown on the upper panel. °indicates unreported mutation in the HADB, *indicates reported but inhibitor status unmatched, SP indicates signal peptide, A1, A2, A3, B, C1 and C2, major FVIII domains, and a1, a2 and a3, minor acidic peptides.

a patient with mild HA and HR inhibitors showed that p.Glu1704Lys affecting the LCh, linked to two reported intronic single nucleotide polymorphisms (SNP) (c.389-9C > T in IVS3, rs35621875 and c.2114-100A > G in IVS13, rs36044968). Although theoretical analysis of potential putative splice sites of these SNPs predicted no changes, splicing defects could not be completely excluded without analysis of patient's *F8* mRNA.

Mutation p.Glu1704Lys is a non-conservative change, acidic negative to basic positive and was once reported in a severe-HA patient without inhibitors [17]. The molecular basis of the dissimilar phenotype of p.Glu1704Lys found in the HADB remains unclear. Perhaps, the plasticity of missense defects to accommodate charges modifying local structure may play a role to explain this inconsistency.

In addition, two patients with HA and LR inhibitors showed novel missense mutations affecting the HCh, p.Arg531Pro and p.Leu575Pro (Fig. 1). These changes to proline have stronger stereochemical constraints [18] that might disrupt the regular secondary structure of the A2 domain. Missense affecting Arg531 had 69 reports in the HADB (Arg > His and Arg > Cys associated with CpG transitions) but only two presented inhibitors. Studies in recombinant FVIII with p.Arg531His demonstrated that after thrombin activation an increased disassociation of the A2 subunit of mutated FVIII associated with an accelerated rate of spontaneous inactivation and reduction in FVIII activity [19].

Only one patient with HR inhibitors showed a novel splice site defect affecting the IVS19 acceptor splice site (c.6116-1G > A), which is consistent with the low-inhibitor risk of splicing mutations found in most reports, beyond some controversy [20].

Our series included two patients (2%) with inhibitors (HR and LR), who remained uncharacterized after completing our *F8* genotyping scheme showing that our approach is highly effective. These two severe-HA mutations may be undetected intronic mutations, or large duplications in which detection was more difficult to achieve.

Inhibitor-positive HA patients with small mutations (n = 34) (i.e. missense, nonsense and indels) showed no significant differences in their FVIII location (LCh vs. HCh P = 0.56). Consistent with the immunogenic characteristics of A2, its functional binding areas (e.g. Arg484-Ile508 peptide), but despite the homology between A domains (32–34%), differences in the density of inhibitor-positive mutations were observed in our series (A2 > A3 ≫ A1) (Fig. 1). Although C2 domain had shown inhibitory antibody targets overlapping critical procoagulant functions, which made these inhibitors clinically significant [21], we found a relatively

low density of small mutations affecting C2 and C1 domains (Fig. 1).

F8 *genotype–specific inhibitor risks in Argentine patients with severe HA*

To estimate the risks for developing FVIII inhibitors associated with each *F8* mutation type/location, we considered an Argentine unbiased group of patients with severe HA ($n = 107$) who showed an Inhibitor Prevalence (IP) of 17.6%, similar to those previously reported [11]. The comprehensive population of Argentine patients with severe HA ($n = 227$, 84 cases and 143 controls) was considered to estimate relative inhibitor risks (inhibitor OR) associated with each mutation type/location (Table 1).

Table 1 shows IP values for each *F8* mutation type/location. The Inv22 showed significant high IP of 24% (18–29) and OR of 1.84 (1.07–3.18) ($P = 0.0287$) predicting nearly twice inhibitor risk in patients with Inv22 than patients without Inv22, which classified Inv22 to the intermediate-risk group consistent with the literature. Our patients with multiexon deletions, a known high-risk mutation type, showed an elevated OR of 3.66 (1.07–12.55) and IP of 55% (19–100) ($P = 0.06$).

On the other hand, missense mutations showed significantly reduced inhibitor risk ($P = 0.0025$), an OR of 0.14 (0.03–2.33) predicting more than seven times less risk for this mutation type and an IP of 3% (0.6–39).

Nonsense mutations in the FVIII LCh have been significantly associated with inhibitors as compared with those in HCh [22,23]. Analysis of HADB nonsense mutations ($n = 311$) showed a relative risk OR of 5.5 (3.1–9.6) LCh/HCh ($P<0.0001$) for inhibitor development, whereas the same analysis in our series ($n = 25$) showed no significant differences (OR LCh/HCh of 1.6 (0.3–8.3)) (Figure S1). Our nonsense mutations showed an apparent inhibitor risk heterogeneity within LCh (L1 residues 1649_2124 vs. L2 2125_2332) (OR L2/L1 of 24.0 (1.1–505.6), $P = 0.0728$). To investigate the molecular basis of this observation, a hypothesis based on the independent expression of *F8B* gene [24], '*F8B* hypothesis', was designed and tested using HADB data of nonsense mutations. This analysis indicated an OR L2/L1 of 1.1 (0.6–2.0) ($P = 1.000$, $n = 163$) and consequently the *F8B* hypothesis was excluded (Figure S1).

F8 *genotype characterization of patients with moderate and mild HA*

To complete the study, 33 patients with HA and FVIII:C levels above 1 IU/dL (16 with mild and 17 with moderate HA) were analysed and found only a case with HR inhibitors in a patient with mild HA caused by a missense mutation in the LCh, p.Glu1704Lys, in phase with two intronic nucleotide substitutions presented and discussed above [i.e. c.(5167G > A; 389-9C > T; 2114-100A > G)]. Although inhibitors occur in patients with mild and moderate HA they are believed to be uncommon. However, there have been few large studies to estimate the prevalence. Although some of the risk factors for inhibitor development are similar to those in severe HA, others are specific for mild/moderate haemophilia. The prevalence of these inhibitors has been estimated to be between 3% and 13% [25–27]. Missense mutations in the LCh are more often (12%) associated with inhibitors than are missense mutations in others parts of FVIIIa (3.9%) [22]. In patients with mild/moderate HA and inhibitors certain missense mutations seem to predispose to inhibitor formation, mainly in C1 and C2 domains and less frequently in A2 domain [28].

Inhibitor status concordance analysis in related patients with severe HA

To explore the influence of genetic factors other than *F8* genotype in our population, we analyse the degree of inhibitor status concordance or discordance in two populations of familiarly related patient pairs (consanguinity coefficients (CC) greater or equal to 1/8, e.g. first-degree cousins): (i) different mutations ($n = 46$) including 26 patients with Inv22, 10 with ins/del frameshifts, 6 with large deletions and 4 nonsense mutations; and (ii) the Inv22 ($n = 26$) (Table 2). Briefly, inhibitor

Table 2. FVIII inhibitor status concordance/discordance vs. related/expected-if-unrelated in patients with severe HA.

Patient group (Consanguinity[†])	Concordant[‡]	Discordant[§]	OR[¶] (CI 95%)	*P* value[††]
All mutations[‡‡]				
Related ($\geq$1/8) obs.	36	10	2.8(1.1–6.9)	0.0261*
Expected if Ho	26	20		
F8 intron 22 inversions				
Related ($\geq$1/8) obs.	20	6	3.3(1.0–11.0)	0.0438*
Expected if Ho	13	13		

[†]Consanguinity coefficients $\geq$1/8 included: monozygotic twins (1); full brothers (1/2); uncle–nephew, half brothers and Grandfather–grandson (1/4); and first-degree cousins (1/8).

[‡]Concordant: In related patients, cases with a concordant inhibitor status matching pair; in expected if Ho (null hypothesis: concordant/discordant matching pairs expected by chance in the same population, or 'apart from the causative mutation, no additional genetic factors influence the development of inhibitors'), the expected matching pair was calculated using the Binomial distribution and the frequency of patients with inhibitors (p) and without inhibitors (q) [i.e. ExpConcHo = $(2pq)n$] ($n = 46$ for the all mutations group and $n = 26$ for Inv22 patients).

[§]Discordant: In related patients, same as concordant inhibitor status matching pair; in expected if Ho, the discordant expected matching pair was calculated using the formula ExpDiscHo = n−ExpConcHo (i.e., ExpDiscHo = $n-(2pq)n$).

[¶]OR: Inhibitor concordance odds ratio (Related-obs/Expected-if-Ho); (CI 95%): Confidence interval of 95%.

[††]*P* value: Chi-square test. *P* value; *$P < 0.05$, significant differences.

[‡‡]All mutation groups totally included 46 cases: 26 Inv22, 10 ins/del frameshifts, 6 large deletions and 4 nonsense mutations.

Table 3. FVIII inhibitor risks and blood product exposure in age-stratified populations of Argentine patients with severe HA.

Exposure[†]	Inhibitor rate[‡] Cases/Cases & Controls (%)	MHc OR[§] (CI 95%)	CMH[¶] *P* value
Intron 22 inversions (n = 98), Indel frameshifts (n = 31) & Nonsense (n = 22)			
Number of expositions to FVIII (n = 146)			
>100	16/67 (24)	0.18(0.09–0.41)	0.000
≤100	49/79 (62)***		
Use of haemocomponents[††] (n = 146)			
Yes	29/75 (39)	0.78(0.38–1.58)	0.484
No	35/71 (49)		
Intensive treatment justification[‡‡] (n = 148)			
Yes	23/46 (50)	1.52(0.74–3.11)	0.251
No	43/102 (42)		
On demand vs. prophylaxis (n = 150)			
On Demand	62/130 (48)	2.89(0.98–8.52)	0.055
Prophylaxis	6/20 (30)		
Haemoderivative vs. recombinant FVIII (n = 150)			
Haemoderivative FVIII	62/140 (44)	0.70(0.18–2.67)	0.601
Recombinant FVIII	6/10 (60)		
Change in FVIII product brand (n = 151)			
>2	51/131 (44)	0.69(0.25–1.94)	0.481
≤2	12/20 (60)		
Intron 22 inversions (n = 98)			
Number of expositions to FVIII (n = 93)			
>100	11/40 (28)	0.26(0.10–0.66)	0.004
≤100	32/53 (60)**		
Use of haemocomponents[††] (n = 93)			
Yes	20/46 (43)	1.06(0.45–2.51)	0. 896
No	22/47 (47)		
Intensive treatment justification[‡‡] (n = 95)			
Yes	22/37 (59)*	2.85(1.17–6.90)	0.021
No	22/58 (38)		
On demand vs. prophylaxis (n = 97)			
On Demand	41/81 (51)	3.04(0.89–10.37)	0.077
Prophylaxis	5/16 (31)		
Haemoderivative vs. recombinant FVIII (n = 97)			
Haemoderivative FVIII	41/89 (46)	0.65(0.14–3.00)	0.581
Recombinant FVIII	5/8 (63)		
Change in FVIII product brand (n = 98)			
>2	40/88 (45)	0.43(0.10–1.85)	0.258
≤2	7/10 (70)		

[†]Clinical characteristics of therapeutic blood product exposure.

[‡]Inhibitor Rate indicates the number of cases with permanent inhibitors (low and high responders) over all patients including cases (inhibitor positive) and controls (patients without permanent inhibitors including transient and no inhibitors), and the resultant percentage of the row.

[§]MHc OR: Mantel-Haentszel common odds ratio. To study inhibitor risks associated with different therapeutic factors (Exposure), and taking into account that case and control groups differ on their average age (case mean: 17.3 years; control mean: 20.9 years; $P = 0.043$), the population was stratified by age into three layers, i.e., 2–12 years; 12–34 years and more than 34 (each cohort associated with the peaks of high probabilities to start inhibitor response to exogenous FVIII), and analysed by use of the Mantel–Haentszel statistics. Consequently, the MH common OR and its 95% CI were calculated along with the analysis of homogeneity of the three strata-specific ORs (Breslow–Day test) and the Cochran–Mantel–Haentszel (CMH) test of conditional independence (Ho: MHc OR $\neq 1$) to estimate the remnant association between the variables of the Exposure[1] after adjusting by age.

[¶]CMH P value of the test of conditional independence. P value significant differences associated with the increased risk factor; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

[††]Use of Haemocomponents includes, for example, platelet concentrates, whole blood transfusion, etc.

[‡‡]Intensive treatment justification includes, e.g. major surgical procedures, Central nervous system bleedings, etc.

status concordance was found significantly higher than expected by chance in populations (i) and (ii) with odds ratios of 2.8 (1.1–6.9) and 3.3 (1.0–11.0) respectively (Table 2). Our series showed an absolute concordance in a case of monozygotic twins, 83% in 24 full brothers (CC, 1/2), and 70% of concordance in other related cases (CC, 1/4–1/8) with an odds ratio of 2.4 (0.6–9.9). Although this latter result revealed a neat tendency to involved additional genetic factors in developing inhibitors in severe-HA affected patients, the null hypothesis cannot be statistically excluded perhaps due to the small size of our related population.

These results agree with those obtained from the literature in which monozygotic twins showed concordant inhibitor status, and the finding of some of these additional genetic factors influencing inhibitor formation tendency, such as polymorphisms of *IL10*, *TNFA* and *CTLA4* [29].

FVIII product exposure and inhibitor development in stratified populations of patients with severe HA

To explore possible non-genetic inhibitor-predisposing factors related to therapeutic FVIII product exposure,

a homogeneous group of 151 severe-HA patients (i.e. Inv22, nonsense mutations and ins/del frameshifts) or separately the 98 patients with Inv22 were age stratified (three strata) and analysed by the Mantel–Haentszel statistics.

Table 3 shows evidence indicating that a category of less than 100 expositions to therapeutic FVIII showed a significant association with the inhibitor groups of three null mutations and Inv22 as compared with more than 100 expositions ($P < 0.0001$ and $P = 0.004$ respectively). These results agree with the known evidence indicating that some predisposed patients developed inhibitors before the first 50 expositions to FVIII products [30]. In addition, the intensive treatment justification, associated with major surgical procedures, central nervous system bleeding, etc., showed an increased tendency to develop inhibitors in the group of three null mutations and a significant MH common OR of 2.85 (1.17–6.90) ($P = 0.021$) in the group of Inv22 (Table 3). None additional factors showed associations with inhibitor formation in both groups of severe HA-causative mutations, i.e. use of haemocomponents, on-demand treatment vs. prophylaxis, haemoderivated vs. recombinant FVIII and change in FVIII product brand (Table 3).

Despite our results related to FVIII product exposure, inhibitor formation in HA showed evidence of a multifactorial trait involving a complex net of interactions between leading genetic factors and environmental factors on a second plane.

In conclusion, our findings on inhibitor risks associated with host-related (HA-causative mutation and other genetic factors) and therapy-related factors showed a general agreement with the literature. In particular, our data on inhibitor status concordance and consanguinity suggested the involvement of further genetic factors beyond *F8* genotype as secondary inhibitor-predisposing factors. Because our population of patients with severe HA is properly stratified by age- and *F8* genotype, it is prepared to detect further genetic risk factors weaker than the causative mutation for inhibitor formation (e.g. allelic variants of the major histocompatibility complex).

It has been well established that inhibitors represent numerous antibodies directed against portions of FVIIIa involved in the procoagulant activity (epitopes on A2, C2 and A3/C1 domains with more than 80% of patients bearing antibodies against two or more of these) [29]. This evidence is highly consistent with the same distribution of inhibitor-predisposing point mutations in the FVIII [27]. However, this characteristic distribution could not be seen in our series of point mutations associated with inhibitors. This disagreement may be associated with the size of our study group, as there are no reasons to support alternative hypothesis about ethnical or geographical differences in our population.

Despite these considerations, our results provide valuable information to Argentinean haematologists on the locally specific risks for developing inhibitors associated with each *F8* genotype in patients with HA. Consequently, these data may help our haemophilia doctors to design improved treatment protocols and fitted follow-ups regimes.

Acknowledgements

We wish to thank patients, relatives and the haemophilia care staff; and the scientists Derrick Bowen, Anne Goodeve, Amy Curto and Eduardo Tizzano for their help in different phases of the work.

Author contribution

LCR: designed and performed research, analyse data and wrote the manuscript; IS: designed and performed research, and final approval of the manuscript; CPR, MMA: performed research, and final approval of the manuscript; LP: collected and analyse data; DN, MC: designed research, performed clinical evaluation of patients and final approval of the manuscript; RPB, MTP: performed clinical evaluation of patients and final approval of the manuscript; IBL: designed research and final approval of the manuscript; CDB: designed and performed research, analysed data and wrote the manuscript.

Fundings

This study was supported by grants from Novo Nordisk Argentina. Additional supporters were the René Barón Foundation, the Alberto J. Roemmers Foundation, the Florencio Fiorini Foundation, the National Research Council (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and the World Federation of Hemophilia.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

References

1 World Federation of Hemophilia. Report on the Annual Global Survey 2009. Montreal, Canada: WFH, 2011.
2 Graw J, Brackmann HH, Oldenburg J, Schneppenheim R, Spannagl M, Schwaab R. Haemophilia A: from mutation analysis to new therapies. *Nat Rev Genet* 2005; **6**(Suppl.6): 488–501.
3 Gitschier J, Wood WI, Goralka TM *et al.* Characterisation of the human factor VIII gene. *Nature* 1984; **312**: 326–30.
4 Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia* 2006; **12**(Suppl. 6): 15–22.
5 Hay CR. Why do inhibitors arise in patients with haemophilia A? *Br J Haematol* 1999; **105**(3): 584–90.
6 Viel KR, Ameri A, Abshire TC *et al.* Inhibitors of factor VIII in black patients with hemophilia. *N Engl J Med* 2009; **360**(16): 1618–27. Erratum in: N Engl J Med 2009; 361(5):544.
7 DiMichele DM, Hoots WK, Pipe SW, Rivard GE, Santagostino E. International workshop on immune tolerance induction: consensus recommendations. *Haemophilia* 2007; **13**(Suppl. 1): 1–22.

8 Kasper CK, Aledort L, Aronson D *et al.* Proceedings: a more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975; **34**(2): 612.
9 Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost* 1995; **73**(2): 247–51.
10 Rossetti LC, Radic CP, Larripa IB, De Brasi CD. Developing e new generation of tests dor genotyping hemophilia-causative rearrangements involving *int22 h* and *int1 h* hotspots in the factor VIII gene. *J Thromb Haemost* 2008; **6**(5): 830–6.
11 Rossetti LC, Radic CP, Candela M *et al.* Sixteen novel hemophilia A causative mutations in the first Argentinian series of severe molecular defects. *Haematologica* 2007; **92** (6): 842–5.
12 HGVS. Nomenclature for the description of sequence variations homepage. Available at http://www.hgvs.org/mutnomen. Accessed February 2, 2011.
13 Goodeve AC, Reitsma PH, McVey JH. Working group on nomenclature of the scientific and standardisation committee of the international society on thrombosis and haemostasis. Nomenclature of genetic variants in hemostasis. *J Thromb Haemost* 2011; **9**(4): 852–5.
14 Kemball-Cook G, Tuddenham EG, Wacey AI. The factor VIII structure and mutation resource site: HAMSTeRS version 4. *Nucleic Acids Res* 1998; **1: 26** (1): 216–9.
15 Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESE finder: a web resource to identify exonic splicing enhancers. *Nucl Acids Res* 2003; **31**(13): 3568–71.
16 Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in genie. *J Comp Biol* 1997; **4**(3): 311–23.
17 Paynton C, Sarkar G, Sommer SS. Identification of mutations in two families with sporadic hemophilia A. *Hum Genet* 1991; **87**(4): 397–400.
18 Richardson JS, Richardson DC. The de novo design of protein structures. *Trends Biochem Sci* 1989; **14**(7): 304–9.
19 Cid AR, Casaña P, Cabrera N, Haya S, Cortina V, Aznar JA. Inhibitor development in one patient and laboratory discrepancies in several families with both mild haemophilia and Arg531Cys mutation. *Haemophilia* 2007; **13**(2): 206–8.
20 Boekhorst J, Lari GR, D'Oiron R *et al.* Factor VIII genotype and inhibitor development in patients with haemophilia A: highest risk in patients with splice site mutations. *Haemophilia* 2008; **14**(4): 729–35.
21 Shen BW, Spiegel PC, Chang CH *et al.* The tertiary structure and domain organization of coagulation factor VIII. *Blood* 2008; **111**(3): 1240–7.
22 Goodeve AC, Peake IR. The molecular basis of hemophilia A: genotype-phenotype relationships and inhibitor development. *Semin Thromb Hemost* 2003; **29**(1): 23–30.
23 Astermark J, Oldenburg J, Escobar M, White GC II, Berntorp E. Malmö International Brother Study study group. The Malmö International Brother Study (MIBS). Genetic defects and inhibitor development in siblings with severe hemophilia A. *Haematologica* 2005; **90**(7): 924–31.
24 Levinson B, Kenwrick S, Gamel P, Fisher K, Gitschier J. Evidence for a third transcript from the human factor VIII gene. *Genomics* 1992; **14**: 585–9.
25 Lusher JM, Arkin S, Abildgaard CF, Schwartz RS. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N Engl J Med* 1993; **328**(7): 453–9.
26 Sultan Y. Prevalence of inhibitors in a population of 3435 hemophilia patients in France. French Hemophilia Study Group. *Thromb Haemost* 1992; **67**(6): 600–2.
27 Rizza CR, Spooner RJ. Treatment of haemophilia and related disorders in Britain and Northern Ireland during 1976–80: report on behalf of the directors of haemophilia centres in the United Kingdom. *Br Med J* 1983; **286**(6369): 929–33.
28 Hay CR, Ludlam CA, Colvin BT *et al.* Factor VIII inhibitors in mild and moderate-severity haemophilia A. UK Haemophilia Centre Directors Organisation. *Thromb Haemost* 1998; **79**(4): 762–6.
29 Astermark J. Basic aspects of inhibitors to factors VIII and IX and the influence of non-genetic risk factors. *Haemophilia* 2006; **12** (Suppl. 6): 8–13; discussion 13–4.
30 Hay CR, Palmer B, Chalmers E *et al.* Incidente of factor VIII inhibitors throughout life in severe hemophilia A in the United Kingdom. *Blood* 2011; **117**(23): 6367–70.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Analysis of *F8B* hypothesis 'Increased risk of inhibitor in nonsense mutations on *F8* exons 23–26 than remnant light chain and heavy chain'.

Appendix

GADEI members by region

City of Buenos Aires: Miguel Tezanos Pinto, Raul Perez Bianco, Miguel Candela, Daniela Neme, Laura Primiani. Province of Buenos Aires: Mónica Martinez, Gabriela Sliba, Susana Garbiero, Gustavo Aletti, Pablo Martinez. Catamarca: Maria S. Vides Herrera. Córdoba: María Williams, Raul Bordone, María Jose Lauría, Viviana Listello, Adriana Berreta. Corrientes: Emilio Lanari, Angeles Romero Maciel, Gabriela Erro. Chaco: Graciela Pujal. Formosa: Victoria Welsh. Jujuy: Susana Gastaldo. La Rioja: Gabriel Campreher. Mendoza: Silvia Yañez, Guillermo Arbesu. Misiones: Ramón Lucio Mariani, Maya Schweri, Sandra Borchichi, Ana Lia Mariani. Neuquén: Alejandra Cedola, Alejandra Kurchan. Salta: María del Carmen Morales, Rosana Quinteros. San Juan: Daniel Arias, Mara Melian. San Luis: Andrea Torresi. Santa Fe: Virginia Rescia, Mauro Dávoli. Santiago del Estero: María Elena Sanchez, Alba Ruiz. Tucumán: Virginia Guerrero, Graciela Negrete.

