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TOPIC: Inhibitors

INHIBITOR RISKS IN ARGENTINE PATIENTS WITH SEVERE HA. *F8* GENOTYPE, STATUS CONCORDANCE IN SIBLING PAIRS AND IMMUNE GENE POLYMORPHISMS STUDIES

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BACKGROUND

Haemophilia A (HA) is an X-chromosome inherited disorder associated with deleterious mutations in the coagulation factor VIII gene (*F8*). The development of inhibitory antibodies is a serious complication that occurs in 15-30% of patients with severe HA in response to replacement therapy with FVIII, and affects about 20% of Argentine cases with severe HA. As a multifactorial complex trait, both genetics and non-genetics factors have been implicated in inhibitor formation (Astermark, 2006). Among patient's genetics, the type and location of the haemophilia causative mutation have been considered as the most important factor for inhibitor development (Oldenburg et al, 2002), as well as other genetic factors such as family history and polymorphisms associated with interleukin-10 (*IL10*), tumour necrosis factor-ά (*TNFA*) and cytotoxic T-lymphocyte antigen-4 (*CTLA4*) genes.

This study involved the analysis of severe HA patients with and without inhibitors countrywide, and it is aimed to characterise the most relevant genetic factors associated with inhibitor formation described internationally so far, including the *F8* genotype and polymorphisms associated with immune genes in Argentinean patients with severe HA.

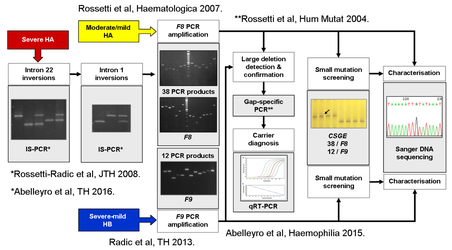
OBJECTIVES

- Stratified our locally specific risks for inhibitor development associated with the *F8* genotype in severe HA patients.
- Study the association of concordance/discordance status between siblings *vs* random pairs in patients with intron 22 inversions.
- Explore the influence of SNPs in *IL10*, *TNFA* and *CTLA4* on the risk of inhibitor development in Argentina.

METHODS

Studied populations: We studied DNA samples from 352 severe HA patients, classified by inhibitor status in INH positive [+] LR (low responders, 1-5 UB/dl) and HR (high responders, >5 UB/dl), or negative [-]. To estimate the risks for developing INH associated with each *F8* mutation type/location, we considered an Argentinean unbiased group of severe HA patients (n=107) showing an absolute Inhibitor Prevalence (IP) of 17.6% (Rossetti et al, 2007). Our comprehensive population with sHA (n=352, 107 cases, INH [+] and 245 controls, INH [-]) was applied to estimate relative inhibitor risks (OR) and 95% confident intervals (CI) of each *F8*-genotype including the group of 23 sib-pairs (14 pairs with the Inv22), subject of the INH status concordance study. A cohort of 164 patients was subjected to the investigation of immune gene polymorphisms.

Cost-effective laboratory algorithm for mutational analysis of *F8* and *F9* in Argentina.



The *F8* and *F9* gene analysis are represented in 38 and 12 amplimers, respectively. Three points to enter the scheme are indicated. Severe HA (red), moderate or mild HA (yellow), and severe, moderate or mild HB (blue).

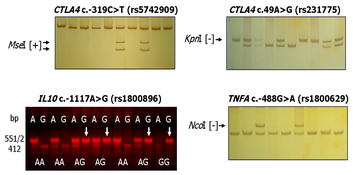
Statistics: Fisher exact test (FET) was applied to analyse contingency tables of Inhibitor risk studies (i.e., INH [+]/[-] *vs F8* genotype and immune-gene SNPs. Chi square test was applied to study inhibitor status concordance in sibling pairs vs a null hypothesis (Ho) consisting in forming random pairs. The IP of a specific mutation (e.g., Inv22) was calculated using the average prevalence of severe HA, $IP^{averageSHA}$ (17.6%), the natural frequency of the Inv22 in severe HA $Freq^{Inv22}$ (unbiased population), 44%, and the Inv22 specific OR (OR^{Inv22}). IP^{Inv22}= $IP^{average\text{-}SHA}$ x OR^{Inv22} / (1 + $Freq^{Inv22}$ x OR^{Inv22} – $Freq^{Inv22}$). These analysis were achieved by use of GraphPad Prism 5.0 software.

SNP analysis in IL10, TNFA and CTLA4. PCR approach details

This analysis included four single nucleotide polymorphisms (SNPs) in immune-modulatory genes, referred as recommended the Human Genome Variation Society (HGVS) nomenclature committee and Legacy (between brackets) to allow an easy comparison with other studies: (a) *IL10* rs1800896 NM_000572.2:c.-1117A>G (-1082A>G), (b) *TNFA* rs1800629 NM_000594.3:c.-488G>A (-308G>A), (c) *CTLA4* rs5742909 NM_001037631.2:c.-319C>T (-318C>T) and (d) *CTLA4* rs231775 NM_001037631.2:c.49A>G NP_001032720.1:p.Thr17Ala (+49A>G).

Primer	Primer sequence (5' > 3')	PCR product size (bp)	Analysis type	Genotypes	Restriction fragments (bp)	References
CTLA4			*Msd* RFLP	C/C	226 , 21	Deichmann et al, 1996
C-318F	AAATGAATTGGACTGGATGGT	247	T/TAA	C/T	226, 130, 96, 21	
C-318R	TTACGAGAAAGGAAGCCGTG			T/T	130, 96, 21	
CTLA4		195	*Kpn*I RFLP	G/G	195	Deichmann et al, 1996
C-49F	CAAGGCTCAGCTGAACCTGGGT		GGTAC/C	A/G	195, 173, 22	
C-49R	TACCTTTAACTTCTGGCTTTG			A/A	173, 22	
TNFAF	AGGCAATAGGTTTTGAGGGCCAT	107	*Nco*I A-RFLP	G/G	87, 20	Wilson et al, 1992
TNFAR	TCCTCCCTGCTCCGATTCCG		C/CATGG	A/G	107, 87, 20	
				A/A	107	
IL10GF	TACTAAGGCTTCTTTGGGAG	551	Allele	A/A		Zheng et al, 2001
IL10AF	CTACTAAGGCTTCTTTGGGAA	552	Specific	A/G	------	
IL10r	CAGCCCTTCCATTTTACTTTC		PCR	G/G		Rossetti et al, 2007
IP-18A	AGAGTATATCTGTGGGAGTG	412	Internal amplification control			
IP-18B	CTTAAGAGCATGGAGCTTGT					

Underlined allele indicates hypothetical increased risk/protective association with inhibitor development.



SNP genotyping approach. Gel electrophoresis analysis corresponding to DNA polymorphisms in *IL10*, *TNFA*, and *CTLA4*, in each case the allele associated with high risk of inhibitor development is shown by arrows (i.e., *IL10* (c.-1117; [G]), *CTLA4* (*Mse*I [+]; [T]), *CTLA4* (*Kpn*I [-]; [G]), *TNFA* (*Nco*I [-]; [A]).

RESULTS

We studied the HA causative mutation in 352 severe patients, including 107 cases with inhibitors INH [+] higher & lower responders and 245 without inhibitors INH [-]. An unbiased population of 107 Argentinean patients with severe HA showed a permanent inhibitor prevalence of 17.6%, which was assessed to calculate the natural distribution of *F8* mutation type/location in severe HA (Figure 1, upper panel).

The case/control study (107/245) in severe HA patients permitted estimation of *F8* genotype-specific inhibitor risks OR and IP(95%CI) classifying a high-risk group including multi-exon deletions MED of 6.21, 82%(32-100); the Inv22 of 1.8, 24%(19-28) and nonsense in the FVIII-light chain LCh [1.8; 31%(12-71)] and in the high chain HCh 1.6, 27%(11-63); an intermediate risk group including single-exon deletions SED and *indel* frameshifts FSH-I/D; and a low-risk group represented by missense defects MS 0.09, 2%(0.4-6) (Figure 1, lower panel).

To explore the influence of genetic factors other than the *F8* genotype, we analysed inhibitor status concordance or discordance in sib-pairs (n=28) *vs* random pairs of patients with the Inv22 as the causative mutation (*F8* genotype strata) (n=140) and found higher inhibitor status concordance than it was expected by chance: OR(95%CI) of 3.2(1.2-8.3), by Fisher exact test (FET) p=0.0201 (Table 1).

Immune gene regulatory polymorphisms' analysis in the genes encoding for *IL10*, *TNFA*, and *CTLA4* indicated a significantly higher inhibitor risk of those patients with the p.Thr17Ala allele of *CTLA4*: OR(95%CI) 2.11(1.18-3.76) p<0.02 in Inv22 strata and also including all sib mutational groups(Figure2).

F8 genotype Frequency & Inhibitor Prevalence

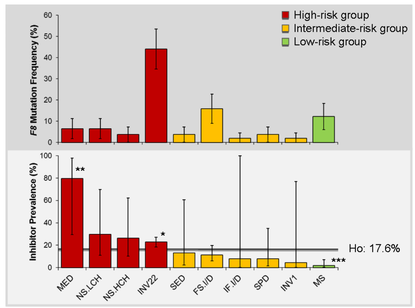


Figure 1. Upper panel: *F8* mutation frequencies of an unbiased Argentinean population of severe HA patients. Lower panel: Inhibitor prevalence risks of *F8* mutations in the comprehensive population of Argentine patients with sHA (n=352, 107 cases and 245 controls). MED: Multi-Exon Deletion; SED: Single-Exon 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.

FVIII inhibitor development *vs TNFA*, *CTLA4* and *IL10* polymorphisms in Argentine patients with severe-HA

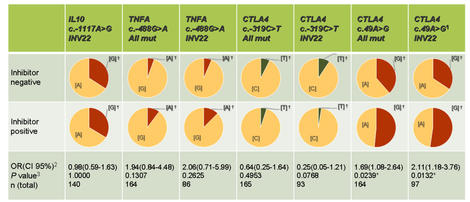


Figure 2. Risk of inhibitor development associated with SNPs in *IL10*, *TNFA* and *CTLA4* in severe HAs series. The OR and 95% confidence intervals are shown for each SNP; *IL10* c.-1117A>G (rs1800896), *TNFA* c.-488G>A (rs1800629), *CTLA4* c.-319C>T (rs5742909) and *CTLA4* c.49A>G (rs231775) alleles under analysis (n=164). 1 p.Thr17Ala. 2 OR: Inhibitor odds ratio; (CI 95%): Confidence interval of 95%. 3 *P* value: Fisher exact test, **P* < 0.05 significant. † Risk or Protective allele.

Table 1. FVIII inhibitor status concordance in siblings *vs* random pairs

Patient Group (Consanguinity[1])	Concordant[2]	Discordant[3]	OR[4](CI95)	P value[5]
***F8* Intron 22 Inversions**				
Related (≥ 1/2) Obs.	22	6	3.2 (1.21-8.31)	0.0201*
Expected-if-Ho	75	65		

[1] Consanguinity coefficient for full brothers ≥ 1/2. [2] Concordant: In related patients, cases with a concordant inhibitor status matching pair.[3] Discordant: In related patients, cases with a discordant status matching pair. [4] OR: Inhibitor Concordance Odds Ratio (Related-Obs/Expected-if-Ho); (95%CI): Confidence interval of 95%. [5] *P* value: Chi square test. * P<0.05, significant differences.

CONCLUSIONS

- The Argentine series of severe HA patients presents similar global and mutation-specific inhibitor risks than the international HA database and published series.
- The stratified analysis of inhibitor status concordance or discordance in sib-pairs *vs* random-pairs with the intron 22 inversion suggests the involvement of additional genetic factors other than the *F8* genotype for inhibitor development.
- *CTLA4* p.Thr17Ala polymorphism (Legacy +49A>G) contributes to increase the risk for inhibitor formation in Argentinean patients with severe HA.

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