F8 GENOTYPE AND NOT POLYMORPHISMS IN *IL10*, *TNFA*, AND *CTLA4* INFLUENCES INHIBITOR DEVELOPMENT IN ARGENTINE PATIENTS WITH SEVERE HA

Liliana Rossetti[1,2‡], Claudia Radic[1,2], Johanna Zuccoli[1,2], Irupé Szurkalo[1,2], Martín Abelleyro[1,2], Lilian Franzi[1,2], Laura Primiani[3], Miguel Candela[2,3], Raúl Pérez Bianco[2,3], Miguel de Tezanos Pinto[2,3], Irene Larripa[1,2], Carlos De Brasi[1,2].

[1]Instituto de Medicina Experimental -IMEX- (CONICET/ANM), [2]Instituto de Investigaciones Hematológicas -IIHEMA- Academia Nacional de Medicina (ANM), [3]Fundación de la Hemofilia *Dr. Alfredo Pavlovsky*.

‡Email: rossetti@hematologia.anm.edu.ar

BACKGROUND

Haemophilia A (HA) is an X-chromosome inherited disorder associated with deleterious mutations in the coagulation factor VIII (*F8*) gene. 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. Version 2009 of the WFH Global Annual Survey (WFH, Annual Survey, 2011) informed that 227 out of 1842 people with HA presented with clinically identified inhibitors in Argentina, indicating the significance of their comprehensive characterization in our patients. Both genetics and non-genetics factors have been implicated to influence 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), and others genetics factors like polymorphisms in interleukin-10 (*IL10*), tumour necrosis factor- alpha (*TNFA*) and cytotoxic T- lymphocyte antigen-4 (*CTLA4*) genes have been described as associated factors in the inhibitor development.

Due to the features of *F8*, and the heterogeneous nature of its molecular defects characterisation of haemophilia mutations still represents a challenge worldwide. This study involves the analysis of patients with and without inhibitors countrywide, and it is aimed to characterise influences of the most relevant genetics factors associated with inhibitor formation described so far, including the *F8* genotype and polymorphisms associated with immune genes in Argentine patients with severe HA.

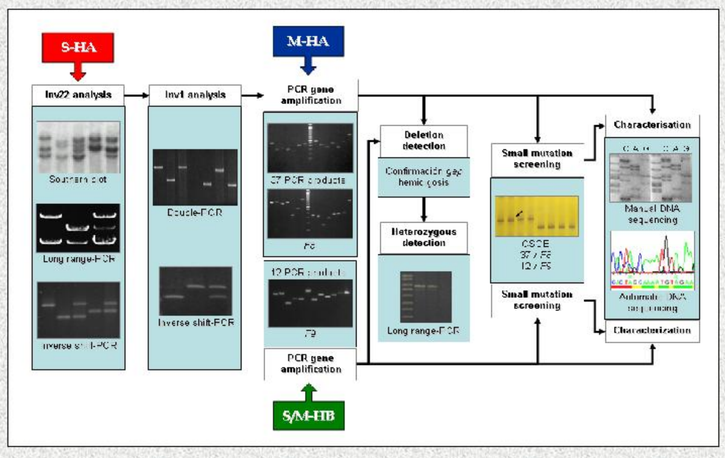
OBJECTIVES

- Characterise all haemophilia causative mutations associated with permanent and transient inhibitors in our population of patients with severe HA.
- Estimate the risk of inhibitor development associated with each specific mutation type and location.
- Estimate the influence of *IL10*, *TNFA* and *CTLA4* gene polymorphisms associated with the risk of inhibitor development.

METHODS

Studied populations: We studied DNA samples from 170 severe HA patients, classified by inhibitor status (INH): T (transient, <6 months), LR (low responders, 1-5 UB/dl) and HR (high responders, >5 UB/dl), or negative [-]. The population of patients with severe HA includes an important number of patients with inhibitor that were studied in our laboratory during 2008-2010 period while an specific protocol for mutation characterization of inhibitor positive patients (GADEI´s group) was performed. In addition, an unbiased population of 119 Argentine patients with severe HA was also analysed to show the natural frequency distribution of *F8* mutation type/location in severe HA.

Molecular algorithm analysis for *F8* and *F9* genes: *F8* intron 22 and intron 1 inversions were analysed by inverse shifting-PCR (Rossetti-Radic et al, 2008). All the relevant sequences of *F8* were represented in 37 PCR-amplifications that were designed for mutation screening by CSGE (conformation sensitive gel electrophoresis). Large deletions were defined as a consistent absence of PCR-product amplification of a contiguous group of exonic sequences. Small mutation screening was performed using CSGE and DNA-sequencing (Rossetti et al, 2007).



Molecular Algorithm for screening and characterisation of HA and HB causative mutations in Argentina. The *F8* and *F9* gene analysis are represented in 37 and 8 amplimers, respectively. Three points to enter the scheme are indicated. Severe HA is indicated as S-HA, moderate or mild HA, M-HA, and severe, moderate or mild HB is indicated as S/M-HB.

Polymorphisms analysis in IL10 (-1082:A/G), TNFA (-308:G/A), and CTLA4 (-318:C/T)

Primer	Primer sequence (5' > 3')	PCR product size (bp)	Analysis type	Allelic variants*	Restriction fragments (pb)	References
CTLA4 C-318F C-318R	 AATGAATTGGACTGGATGG TTACGAGAAAGGAAGCCGTG	247	MseI restriction T/TAA	C/C C/T T/T	226 , 21 226, 130, 96, 21, 21 130, 96, 21	Deichmann et al, 1996
IL10Gf IL10Af IL10r	TACTAAGGCTTCTTTGGGAG CTACTAAGGCTTCTTTGGGAA CAGCCCTTCCATTTTACTTTC	551 552	Allele Specific PCR	A/A A/G G/G	------	Zheng et al, 2001
TNFAF TNFAR	AGGCAATAGGTTTTGAGGGCCAT TCCTCCCTGCTCCGATTCCG	107	NcoI restriction C/CATGG	G/G A/G A/A	87, 20 107, 87, 20 107	Wilson et al, 1992

* Underlined allelic variants indicate hypothetical increased risk association with inhibitor development

Statistical Analysis: Fisher exact test was applied to analyse contingency tables of Inhibitor status (i.e., HR, LR, T and [-]) vs *F8* mutation inhibitor risk groups, and different polymorphisms status (i.e., High, Medium and Low risks). This analysis was achieved by use of GraphPad Prism 5.0 software.

RESULTS

We studied the HA causative mutation in 170 severe patients including 58 cases with HR-INH, 17 LR-INH, 10 with T-INH and 85 [-]. The unbiased population of 119 Argentinean patients with severe HA showed a permanent inhibitor prevalence of 17.6% (21 cases) that was analysed to show the natural distribution of mutation type/location vs inhibitor risk (Figure 1).

To explore other genetic factors we studied DNA polymorphisms in the genes encoding for *IL10* (-1082:A/G), *TNFA* (-308:G/A), and *CTLA4* (-318:C/T). In contrast with previous studies from others populations (Astermark et al, 2006), we found no significant differences in the inhibitor risk of polymorphisms in these genes in Argentina, perhaps associated with differences in ethnical or environmental factors (Figure 2). In contrast, we found significant differences in our unbiased population between three *F8* mutation type/location inhibitor-risk groups: High-risk (8/14=57%) including multi-exon deletions (5/7=71%) and nonsense in the FVIII Light-Chain defects (3/7=43%); Intermediate-risk (13/88=15%) including nonsense in the Heavy-Chain (0/6), frameshifts Ins/Del (3/19=16%), Intron 22 inversions (9/55=16%,), splicing defects (1/4) and single-exon deletions (0/4); and Low-risk (0/17=0%) including in-frame-Ins/Del (0/2), Intron 1 inversions (0/2) and missense mutations (0/13=0%) (Figure 1).

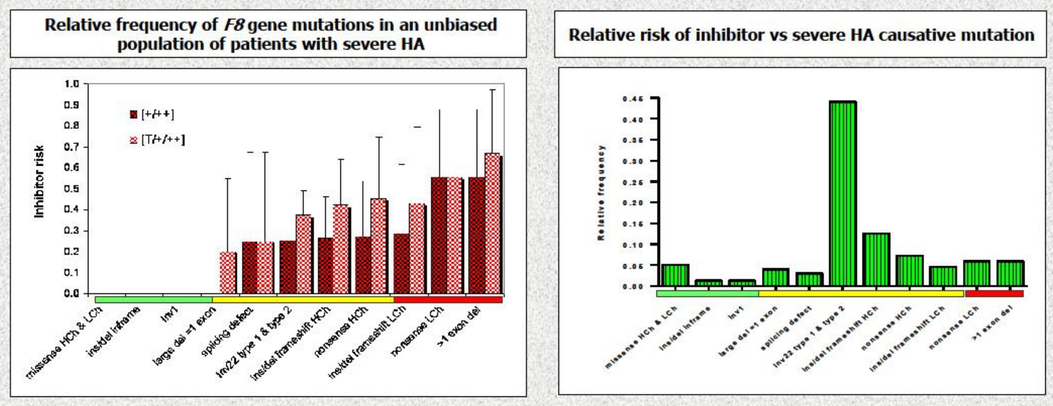


Figure 1. Left: *F8* mutation frequencies of an unbiased Argentinean population of severe HA. Right: Inhibitor relative risks of *F8* mutations in the same population. Three risk groups were classified (i.e., Low, green; Medium, yellow and High, red). Risk groups vs INH status (T/LR/HR or [-]) indicated P = 0.0002. Vs INH status (LR/HR or T/[-]), P = 0.0001.

IL10, *TNFA* and *CTLA4* polymorphisms analysis. Relationship with inhibitor association in Argentina

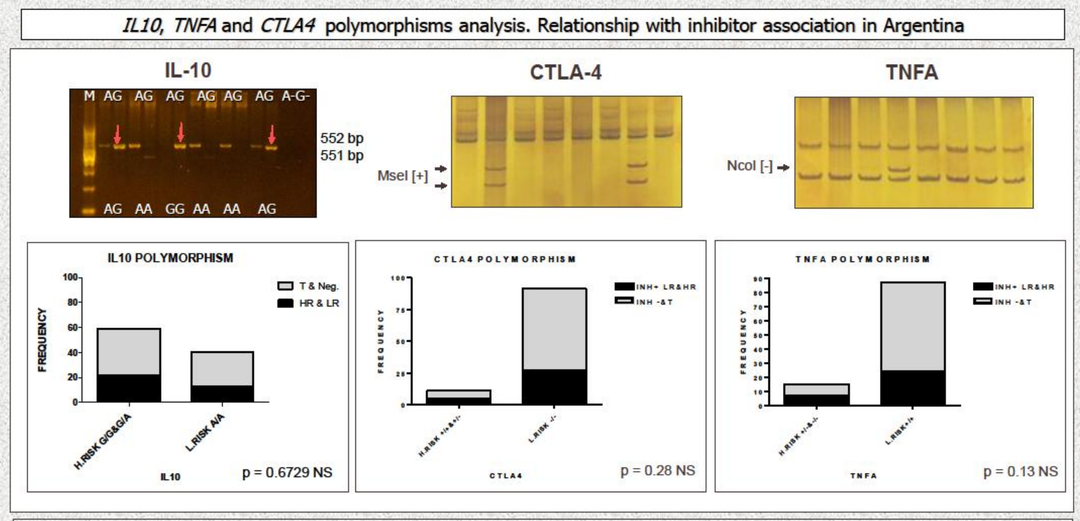


Figure 2. Upper panel. Gel electrophoresis analysis corresponding to DNA polymorphisms in *IL10* (-1082:A/G), *TNFA* (-308:G/A), and *CTLA4* (-318:C/T), in each case the allele associated with high risk of inhibitor development is shown: i.e., *IL10* (-1082: [+]; G), *CTLA4* (MseI [+]; T), *TNFA* (NcoI [-]; A). Lower panel. Results of statistical analysis by Fisher exact Test between the association of each polymorphic allele in genes encoding cytokines and immunoregulatory molecules and development of inhibitory antibody against FVIII. Risk groups vs INH status ([LR/HR] or [T & -]) indicated P = NS.

CONCLUSIONS

In agreement to international series, our population of haemophilia with permanent INH (HR-INH and LR-INH) is mostly composed by gross gene rearrangements (i.e., *F8* Inv22, large-deletions) and secondarily by nonsense mutations and others small defects (e.g., ins/del associated with frameshifts and splicing impairment) particularly affecting the FVIII Light Chain (including a3-A3-C1-C2 domains).

In contrast with previous studies from north European populations, we found no significant association between FVIII inhibitor risk and *IL10*, *TNFA* and *CTLA4* polymorphisms in Argentine patients with severe HA, suggesting ethnical or environmental differences.

REFERENCES

- Astermark J. Haemophilia 2006; 12(3): 52-60.
- Oldenburg J, El-Maarri O, Schwaab R. Haemophilia 2002; 8(2): 23-29.
- Rossetti L – Radic P, Larripa I, De Brasi C. J Thromb Haemost 2008;6(5):830-6.
- Rossetti L, Radic C, Candela M, Pérez Bianco R, de Tezanos Pinto M, Goodeve A, Larripa IB, De Brasi CD. Haematologica 2007;92(6):842-5.
- Deichmann et al. Biochem Biophys Res Comm 1996;225:817-8.
- Zheng C et al. Int J Cancer 2001;95:184-8.
- Wilson AG, di Giovine FS. Hum Mol Genet 1992; 1:353.

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