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A prevalent *CTLA4* missense variant significantly associates with inhibitor development in Argentine patients with severe haemophilia A

V. D. MARCHIONE,* J. R. ZUCCOLI,† M. M. ABELLEYRO,* C. P. RADIC,* D. NEME,‡ M. CANDELA,§ M. DE TEZANOS PINTO,‡§ C. D. DE BRASI*§ and L. C. ROSSETTI*
Instituto de Medicina Experimental (IMEX) CONICET-Academia Nacional de Medicina; †CIPYP (Centro de Investigaciones sobre Porfirinas y Porfirias); ‡Fundación de la Hemofilia Alfredo Pavlovsky; and §Instituto de Investigaciones Hematológicas Mariano R Castex, Academia Nacional de Medicina, Buenos Aires, Argentina

Haemophilia A (HA) (OMIM #306700), an X-linked recessive disorder characterized by reduced activity of coagulation factor VIII (FVIII:C), is caused by deleterious mutations in the *F8*. HA can be treated by administration of the deficient FVIII. However, about 20%–30% of severe HA patients (biochemically defined as FVIII:C < 1 IU/dL) developed FVIII neutralizing antibodies (inhibitors) making replacement therapy ineffective. Inhibitors result in higher therapy costs and decreased quality-of-life and life expectancy of patients with haemophilia. From the Public Health System perspective, Argentina currently compiles 96 HA patients with clinically identified inhibitors out of a total 2220 [1]. This reduced figure (96/2220) is a successful result of undergoing an extended prophylaxis covering more than 65% of patients, in which therapeutic FVIII is not administered under risk conditions, and immune-tolerance induction treatment for inhibitor eradication.

As a typical complex trait (multifactorial) an operative classification of inhibitor development in haemophilia focuses on two main groups of risk factors: modifiable (environmental factors) and non-modifiable (genetic factors, often involving several genes with different relative weight predisposing to the phenotype). Among the former, environmental risk factors include treatment-related factors and immune-system challenges. Among the latter, in HA, the causative *F8* genotype has been established as the main factor conditioning inhibitor development (in Argentina [2], and worldwide, reviewed in [3]), but it also counts a group of secondary risk factors, weaker than the *F8* genotype, such as family history of inhibitors, ethnicity, human lymphocyte antigen haplotype and polymorphisms linked to immune-system genes, such as Interleukin-10 (*IL10*), tumour necrosis factor-α (*TNFA*) and cytotoxic T-lymphocyte associated protein-4 (*CTLA4*) [4,5]. Human genetics evidence indicates that predisposing factors associated with autosomal genes, such as those mentioned above, are ethnically dependent and therefore not consistent among populations worldwide.

In this scenario and encouraged by previous studies of inhibitor concordance in familiarly related patients indicating the possible presence of additional genetic factors (others than the *F8*-genotype) in our population [2], we screened for locally relevant secondary inhibitor risk factors in a relatively large homogeneous population of patients with the Inv22 and a comprehensive severe HA population.

All patients included in the study of inhibitor risk had been extensively exposed to FVIII concentrates, above the number of exposure days (ED) normally regarded as the highest risk period for inhibitor development (i.e. ED > 50 days). A positive inhibitor titre was defined as equal to or more than 0.6 BU per mL.

The screening of additional genetic factors influencing FVIII inhibitor development focused on four single nucleotide polymorphisms (SNPs) in immunomodulatory genes: (a) *IL10* rs1800896, NM_000572.2:c.-1117A>G (IL-10-1082A>G); (b) *TNFA* rs1800629, NM_000594.3:c.-488G>A (TNFA-308G>A); (c) *CTLA4* rs5742909, NM_001037631.2:c.-319C>T (CTLA-4-318C>T); and (d) *CTLA4* rs231775, NM_001037631.2:c.49A>G NP_001032720.1:p.Thr17Ala, (CTLA-4+49A>G) [free notation as recommended by the Human Genome Variation Society (HGVS) and Legacy notation (between brackets) to permit comparisons with other studies].

Genomic DNA was extracted from peripheral-blood leucocytes using standard methods and *F8* mutation characterization was performed as described by Rossetti *et al.*[6]. *IL10* c.-1117A>G was analysed by allele-specific PCR [7]; *TNFA* c.-488G>A by PCR-*Nco*I-artificial-RFLP (restriction fragment length polymorphism) [8]; *CTLA4* c.-319C>T by PCR-*Mse*I-RFLP [9] and *CTLA4* c.49A>G by PCR-*Kpn*I-RFLP [10].

A case–control study of inhibitor risks associated with each type of HA-causative mutation was

Correspondence: Liliana C. Rossetti, Molecular Genetics of Haemophilia Laboratory, IMEX CONICET-Academia Nacional de Medicina. Pacheco de Melo 3081, Buenos Aires 1425, Argentina.
Tel.: 5411 48058803, Ext.: 261; fax: 5411 4803 9475; e-mail: rossetti@hematologia.anm.edu.ar

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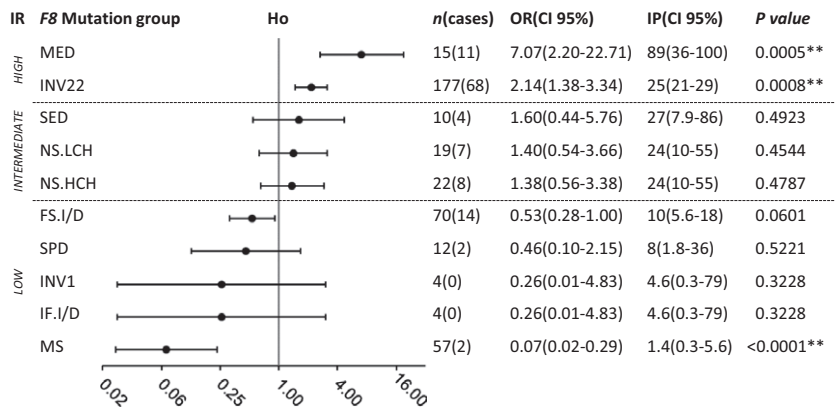

Fig. 1. Relative risk of inhibitor development according to the type/location of the *F8* mutation. The OR and 90% confidence intervals are plotted for each mutation. The vertical line indicates an OR = 1 (Ho (null hypothesis): inhibitor development is not influenced by the type/location of the *F8* mutation). Left panel indicates the *F8* mutation. MED: Multi-Exon Deletion; INV22: intron 22 inversion; SED: Single-Exon Deletion; NS.LCH: Nonsense Light Chain; NS.HCH: Nonsense Heavy Chain; FS.I/D: Frameshift *Indel*; SPD: Splicing Defect; INV1: intron 1 inversion; IF.I/D: In-Frame *Indel*; MS: Missense, grouped by inhibitor risk (IR). Right columns show the total number of patients with each mutation (*n*), (cases) between brackets indicate cases with inhibitor, OR values and absolute inhibitor prevalence (IP) are shown with 95% confidence intervals (CI), and *P* values of the Fisher exact test (** indicates highly significant $P < 0.01$). IP of a mutation group was calculated considering the global absolute inhibitor prevalence of severe HA patients (inhibitor unbiased population) of 17.6% ($Freq_{InhSHA}$), the natural frequency of the mutation group ($Freq_{Mt/SHA}$) in the same (unbiased) severe HA population from Argentine ($n = 107$) [6] and the OR value ($OR_{M+/M-}$) by the formula $[IP_M = (Freq_{InhSHA}*OR_{M+/M-})/[1 + (Freq_{Mt/SHA}*OR_{M+/M-})\text{-}Freq_{Mt/SHA}]]$.

performed in a large cohort of patients with severe HA ($n = 390$) (Fig. 1). This study permits classification of a group of high-risk mutations including multi-exonic deletions with OR (95% CI) of 7.07 (2.20–22.71) ($P = 0.0005$) and the Inv22 with 2.14 (1.38–3.34) ($P = 0.0008$); intermediate-risk mutations including nonsense defects and single-exon deletions, and low-risk mutations properly represented by missense defects with 0.07 (0.02–0.29) ($P < 0.0001$), (Fig. 1). In agreement with those reported in ethnically mismatched populations worldwide, about 44% of severe patients with HA in Argentina showed the Inv22 mutation [3] associated with an augmented absolute inhibitor prevalence, IP (95% CI), of 25% (21–29) (Fig. 1).

The homogeneous strata of Inv22-positive patients ($n = 140$–148) allows investigation of additional genetic factors relatively less conditioners of inhibitor risks than the *F8* genotype by a case/control study. In addition, a comprehensive population of severe HA patients associated with all mutation types ($n = 213$–222) permits reinforcing the statistical analysis.

The choice of Inv22-positive severe HA patients to investigate secondary genetic factors predisposing for inhibitor development was based on two main reasons. First, because the Inv22-positive population provides the biggest number of perfectly homogeneous group of severe HA patients associated with significant chances to develop inhibitors against therapeutic FVIII, fact that was estimated in previous studies in our population [2] and confirmed in this study. Second, and perhaps more importantly, it was proved that the Inv22-mutated *F8* expresses the entire FVIII-amino-acid sequence intracellularly (two non-secreted polypeptides result in a positive intracellular FVIII-cross reacting material); thus, differentiating from genuine null mutations and becoming 'pharmacogenetically relevant' affecting its high-moderate inhibitor risk by the nature of the administered therapeutic FVIII [11].

Once the results of the analysis of inhibitor status concordance among familiarly related HA patients unveiled the possible involvement of secondary genetic factors in our population [2], the choice of a list of candidate genes and polymorphisms came naturally supported by the net of molecules involved in this specific immune response, the significant associations found in other populations and the SNP frequency distribution associated with 26 ethnic groups worldwide showed in the 1000 Genomes Project [12].

All studied SNPs on *IL10*, *TNFA* and *CTLA4* genotype distributions were consistent with those predicted by the Hardy–-Weinberg equilibrium (Table S1b).

IL10 c.-1117A>G, reported in North-European and Brazilian series as an inhibitor risk factor [5], showed a neutral non-significant OR between patients with and without inhibitors in our Inv22-informative population (Fig. 2, Table S1a).

TNFA c.-488G>A analysis showed that the heterozygote genotype [A/G] was slightly higher among patients with inhibitors with non-significant ORs of 1.78 (0.84–3.77) for the comprehensive severe HA population (Table S1a) and 1.67 (0.68–4.08) for the Inv22 strata (Fig. 2). These results are similar in their tendency but not in their statistical significance with those reported in European HA-patient series [5], perhaps due to the fact that the [A/A] genotype was not found in our population and the frequency of the

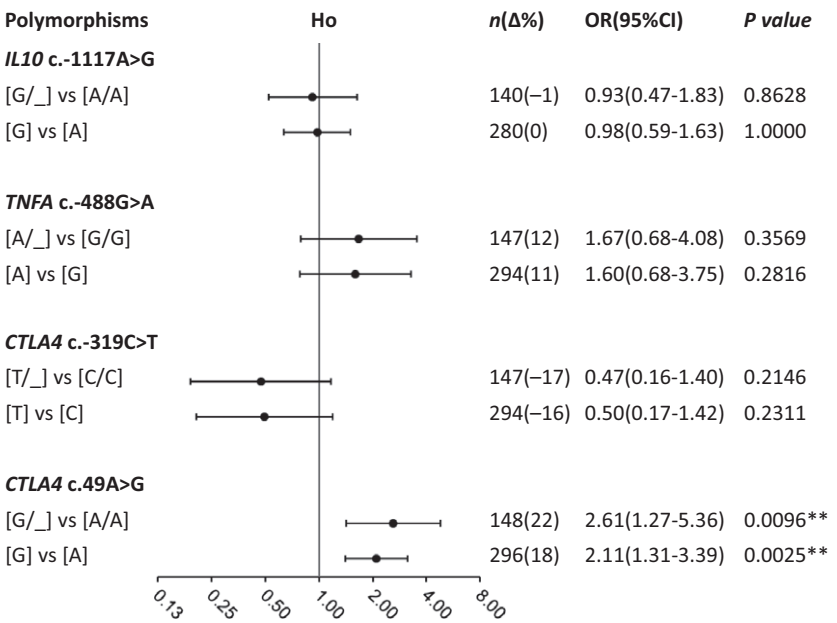

Fig. 2. Risk of inhibitor development associated with SNPs in *IL10*, *TNFA* and *CTLA4* in the Inv22-strata. Analysis of SNP genotypes and alleles were performed. The OR and 90% confidence intervals are plotted for each SNP: *IL10* c.-1117A>G (rs1800896), *TNFA* c.-488G>A (rs1800629), *CTLA4* c.-319C>T (rs5742909) and *CTLA4* c.49A>G (rs231775). Right columns show the total number of genotypes or alleles (*n*), the difference between the percentage of inhibitor positive cases between the exposure groups (e.g. [G] minus [A]) (Δ%), OR values with 95% confidence intervals, and *P* values of the Fisher exact test (** indicates highly significant $P < 0.01$).

[A] allele (q = 0.07) is lower than in other populations [12].

The development of inhibitor antibodies against FVIII is a T-helper (Th) cell-dependent event involving antigen-presenting cells (APC) and B-lymphocytes. The MHC class II molecules present infused FVIII peptides to the T-cell receptor on the Th surface. A co-stimulatory signal, provided by interaction between B7 (CD80/86) and CD28 molecules, is required for promoting anti-FVIII antibody production [4]. CTLA-4 interferes with this co-stimulatory interaction APC-Th cells down-regulating the immune response [4].

CTLA4 c.-319C>T has been associated with higher promoter activity resulting protective for developing inhibitors in HA patients in other populations [4].

Our analysis confirmed this trend in severe HA patients and in Inv22-positive patients, although none of these analyses reach statistical significance, associating the protective allele [T] with ORs of 0.63 (0.26–1.52) ($P = 0.3893$) (Table S1a) and 0.47 (0.16–1.40) ($P = 0.2146$) (Fig. 2) respectively.

CTLA4 c.-319C>T frequencies shown in the 1000 Genomes Project database range from 0% in African populations to about 12% in British populations, while Latin-Americans and Asians show intermediate figures that result similar to those found in our population (6%–6.5%) [12].

CTLA4 c.49A>G associates with a missense variant (p.Thr17Ala) in the leader peptide that causes an incomplete glycosylation in the endoplasmic reticulum ultimately leading to a reduced surface/total expression ratio of CTLA-4 [13].

Interestingly, our studied populations of severe HA patients present a prevalent representation of the three *CTLA4* p.Thr17Ala genotypes (Table S1), with [G] allele frequencies ranging 0.40–0.42 in agreement to the average of those reported in the 1000 Genome Project [12].

A significantly higher inhibitor risk was found associated with the [G] allele with ORs of 1.76 (1.19–2.61) ($P = 0.0051$) for the comprehensive mutation cohort (Table S1a) and 2.11 (1.31–3.39) ($P = 0.0025$) for the Inv22 mutation cohort (Fig. 2). This statistically significant association was consistent considering the combination of genotypes [G/_] (i.e. [G/G] and [A/G]) with a dominant effect indicating ORs of 1.89 (1.04–3.45) ($P = 0.0398$) and 2.61 (1.27–5.36) ($P = 0.0096$), for the large and homogeneous population respectively (Fig. 2, Table S1a).

CTLA4 c.49A>G (p.Thr17Ala) has been associated with susceptibility to a number of autoimmune diseases such as Graves' disease [10], acquired HA and, notably, insulin-dependent Diabetes mellitus in Argentinean population [14].

This is the first series in which *CTLA4* p.Thr17Ala was significantly associated with inhibitor development in haemophilia. The same trend, though non-significant, was observed by others showing an OR of 2.2 (0.6–7.8) associated with the [G] allele in North Europeans HA patients [4].

It became clear that genetic secondary factors predisposing for inhibitor development in HA patients from different regions and ethnics (e.g. immune-gene polymorphisms) show significant differences worldwide. These contrasts were highlighted in massive worldwide studies related to inhibitors in haemophilia as HIGS (Hemophilia Inhibitor Genetics Study) [15] vs. others' non-massive and regional [4,5] such as this study.

Concerning the severe impact on medical practice, and on the economy of national health systems, of inhibitor development against the replacement therapy in patients with haemophilia, and the significant

ethnic-geographical differences associated with multifactorial traits, it is advisable to develop regionally relevant *inhibitor risk scores* including all non-modifiable factors to weigh up the genetic predisposition of each particular patient to develop inhibitors.

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Author contribution

VDM: designed and performed research, analysed data and wrote the manuscript; MMA, CPR: performed research, and approved the final manuscript; JRZ: designed and performed research, and approved the final manuscript; DN, MC: designed research, performed clinical evaluation of patients and approved the final manuscript; MTP: performed clinical evaluation of patients and approved the final manuscript; CDB, LCR: designed and performed research, analysed data and wrote the manuscript.

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Disclosures

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

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Supporting Information

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

Table S1a. FVIII inhibitor development *vs.* *TNFA*, *CTLA4* and *IL10* polymorphisms in Argentine patients with severe HA.

Table S1b. Hardy–Weinberg Equilibrium analysis in the studied set of biallelic markers in *IL10*, *TNFA* and *CTLA4*.