



## A NOVEL GENETIC CLASSIFICATION OF INHIBITOR RISK FOR F8 NONSENSE MUTATIONS BASED ON IMMUNOGENIC PROFILING OF RIBOSOMAL READTHROUGH

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**Background and aims.** Among F8 mutation types, Premature Termination Codon (PTC) lead to the synthesis of truncated endogenous factor VIII (eFVIII) and display a wide variation of inhibitor risk after administration of therapeutic FVIII (tFVIII), lowering the performance of clinical risk prediction models. Translational readthrough (RT), which overrides PTCs and incorporates predictable amino acids subsets, produces traces of eFVIII full-length FVIII including missense and wild-type molecules (WT-RT). In line with data obtained for F8 missense variants, we hypothesized that higher affinity of the administered tFVIII than eFVIII counterpart to MHCII molecules may increase immunological response, and that the RT output may modulate the inhibitor risk.

**Methods.** To provide a new classification of PTC based on RT susceptibility and inhibitor formation, F8 genotypes, inhibitor status and predictable RT output, were investigated in 1048 patients (PTCs, n=335), recorded in the EAHAD database. These data were integrated with expression studies of a wide panel (n=43) of FVIII PTCs fused with Gaussia Luciferase (FVIII-GL). The predicted RT output without WT formation, based on PTCs type (UGA, Trp; UAG, Gln/Tyr, UAA, Tyr/ Gln), were tested using in silico NetMHCII tool, which calculates binding affinity between eFVIII missense peptides arising from RT and molecules MHC class II allowing comparisons across different HLA alleles. Affinity of RT missense-deriving peptides (eFVIII) was compared with that of the corresponding WT counterpart (tFVIII).

**Results.** Wild-type FVIII molecules (WT-RT), which would re-incorporate the WT residue and reduce inhibitor develop-

ment risk, was i) lower in patients with than without inhibitors (p=0.022), ii) not predicted in patients with recurrent PTCs detected in at least three inhibitor-positive cases (n=136, p=0.0001), and iii) much lower (12%) for FVIII light chain PTCs, highly associated with inhibitor development than for the heavy chain (25.2%) and B-domain (27.4%). Comparison of RT output arising from FVIII-GL PTCs in vitro expression, did not show a quantitative difference between WT-RT positive (mixture of WT and missense variants) and negative (missense variants only) groups, further supporting the presence of WT-RT molecules as a determinant of decreased inhibitor risk. Conversely, for F8 PTCs without WT formation (n=297), mean differences in affinity between MHCII molecules and novel missense peptides were higher for missense variants predicted in patients with than without inhibitors (p<0.0001), and increased for PTCs present in more than one patient with inhibitor. A comprehensive in silico analysis of Single Nucleotide Variants in the entire F8 coding sequence, consisting of prediction of WT-RT or differential affinity of FVIII missense peptides (classified as predictable PTCs), revealed that naturally occurring PTCs have a higher inherent potential to produce WT-RT (31%) compared with predictable PTCs (22%, p=0.001), potentially increasing inhibitor risk associated to these latter.

**Conclusions.** Our data provide a new classification of PTCs based on genetic features that, based on predicted RT output and immunogenicity, could be exploited to improve estimate of individual PTC-related inhibitor risk in HLA genotyped patients.