OC 56.2 | Comparative Analysis Of Residual Factor VIII Expression from Recurrent F8 Nonsense Mutations Indicates that Localization in the B-domain Favours Readthrough-mediated Protein Output

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Background: Nonsense mutations, inserting premature termination codons (PTCs), might undergo, with low frequency (<0.01%), spontaneous suppression (readthrough) with production of full-length proteins upon amino acid insertion at the PTC. This process, dictated by nucleotide/protein sequence features, might have implications for hemophilia A (HA) patients.

Aims: To investigate residual factor VIII (FVIII) expression through complementary studies in HA patients' plasma and exploiting a sensitive *in-vitro* expression platform.

Methods: Detection of plasma FVIII levels (ELISA, aPTT), and expression studies (HEK293 cells) with a highly-sensitive naturally-secreted luciferase (Gaussia, GL) fused to FVIII (FVIII-GL).

Results: Plasma samples from HA patients affected by six nonsense mutations (p.R446X, p.R814X, p.K1289X, p.W1726X, p.R1985X, p.R2135X) revealed traces of FVIII. Strikingly, the two B-domain variants (p.R814X, p.K1289X) showed the highest FVIII levels, suggesting a position-dependent effect. Expression studies with the FVIII-GL variants showed that those of the B-domain produced the highest luciferase activity levels, thus supporting in vivo findings. Accordingly, the predicted readthrough-deriving amino acid changes (R446W, R814W, K1289Q/Y, W1726Y, R1985W, p.R2135W) showed a minor impact for those affecting the B-domain.

To verify further our hypothesis, the panel of *F8* mutations was rationally expanded to be representative of the majority of patients with nonsense mutations (60%), including the most frequent (50% of patients) in the B-domain. Through our sensitive platform we observed that all *F8* nonsense variants led to detectable luciferase activity (0.4-6%). Strikingly, when categorized in two groups (B-domain, n = 21; other domains, n = 26), secreted luciferase activity of B-domain variants was significantly higher (p<0.0001) as compared with variants located in the other FVIII domains.

Conclusions: Our findings for the first time indicate that nonsense mutations in the B-domain, known to tolerate missense changes as those potentially arising from readthrough, are favoured in terms of readthrough-mediated protein output, which might have pathophysiological implications for HA patients.

OC 56.3 | Identification and Characterization of *F9* Deep Intronic Variations in Haemophilia B Patients

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Background: With current molecular diagnosis, about 1% of haemophilia B patients remains genetically unresolved. In these cases, deep intronic variation could be causal.

Aims: To identify the causal variation in 6 unrelated mild-to-moderate HB patients in whom no genetic variation was found using conventional genetic exon-focused approaches.

Methods: The whole *F9* was sequenced using Next Generation Sequencing capture method. All candidate variations were confirmed using Sanger sequencing. The putative splicing impact of these deep intronic variations was studied using both *in silico* analysis (Splicing Sequences Finder and MaxEntScan) and minigene assay.

Results: Next generation sequencing data revealed 3 candidate variants in *F9* introns. The c.278-1806A>C was found in three patients with a mild phenotype, while the c.724-2385G>T and the c.723+4297T>A were found in a mild and a moderate haemophilia B patient, respectively. Additionally, a *de novo* 6-kb LINE retrotransposition in *F9* intron 4 was found in the remaining patient with mild phenotype. *In silico* analysis strongly predicted that both c.724-2385G>T and c.723+4297T>A impacted the splicing. Conversely, no impact was predicted for the c.278-1806A>C. For all substitutions, an abnormal mRNA pattern was found using the minigene assay. The c.724-2385G>T led to the insertion of a 84 bp pseudo-exon due to the creation of a *de novo* donor splice site. The c.723+4297T>A led to the exonisation insertion of a 180 bp sequence by enhancing the strength of a pre-existing cryptic donor splice site. The c.278-1806A>C led to the retention of a 199 bp pseudo-exon.

Conclusions: With this comprehensive work combining next generation sequencing and functional assays, we report for the first time deep intronic variants that caused haemophilia B through splicing alteration. This study highlights the usefulness of whole *F9* sequencing for the progressive reduction of genetically unexplained haemophilia B.