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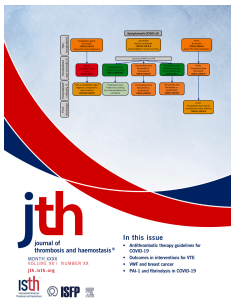

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DNA base editing corrects common Hemophilia A mutations and restores factor VIII expression *in vitro* and *ex-vivo* models

Running title: Rescue of Factor VIII expression by base editing

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Abstract

Background

Replacement and non-replacement therapies effectively control bleedings in Hemophilia A (HA) but imply lifelong interventions. The authorized gene addition therapy could provide a cure but still poses questions on durability. *F8 g*ene correction would definitively restore factor VIII (FVIII) production, as shown in animal models through nucleases mediating homologous recombination (HR). However, low efficiency and potential off-target double-strand break (DSB) still limit HR translatability.

Objectives

To correct common model single point mutations leading to severe HA through the recently developed DSB/HR-independent base (BE) and prime (PE) editing approaches.

Methods

Screening for efficacy of BE/PE systems in HEK293T transiently expressing FVIII variants and validation at DNA (sequencing) and protein (ELISA; aPTT) level in stable clones. Evaluation of rescue in engineered blood outgrowth endothelial cells (BOEC) by lentiviral-mediated delivery of BE.

Results and Conclusions

Transient assays identified the best-performing BE/PE systems for each variant, with the highest rescue of FVIII expression (up to 25% of rFVIIIwt) for the p.R2166* and p.R2228Q mutations. In stable clones we demonstrated that the mutation reversion on DNA (~24%) was consistent with the rescue of FVIII secretion and activity 20-30%). The lentiviral-mediated delivery of the selected BE systems was attempted in engineered BOEC harboring the p.R2166* and p.R2228Q variants, which led to an appreciable and dose-dependent rescue of secreted functional FVIII.

Overall data provide the first proof-of-concept for effective BE/PE-mediated correction of HA-causing mutations, which encourage studies in mouse models to develop a personalized cure for large cohorts of patients though a single intervention.

Keywords: CRISPR, Factor VIII, Gene editing, Hemophilia A, base/prime editors

INTRODUCTION

Hemophilia A (HA; OMIM # 306700) is a rare X-linked recessive disorder caused by mutations in the *F8* gene (MIM 300841), which encodes coagulation factor VIII (FVIII) [1]. FVIII is physiologically produced mainly by Liver Sinusoidal Endothelial Cells (LSECs), and it has also been demonstrated an extra-hepatic production with organ-specific endothelial cells and hematopoietic cells [2–4].

Current treatment of severe HA is based on lifelong prophylactic injection of FVIII (replacement therapy), which however suffers from a short half-life and imposes frequent administrations. This limitation, combined with the development of anti-FVIII neutralizing antibodies in ~30% of treated HA patients [5], boosted research and led to several alternative strategies ranging from the use of extended-half-life FVIII variants, by-passing agents and inhibitors of the physiologic coagulation inhibitors, some of them also into the clinics [6,7]. On the other hand, to provide a definitive cure for HA strong efforts have been pushed on gene therapy [8], and very recently Valoctocogene roxaparvovec [9], an adeno-associate viral vector (AAV5) delivering the B-domain deleted FVIII variant, has received authorization by the EMA and FDA. Clinical trials [9–13] demonstrated an appreciable increase of FVIII activity in plasma over time after a single treatment and a subsequent remarkable reduction of bleeding episodes, allowing most treated HA patients to discontinue prophylaxis. Notwithstanding, the follow-up revealed a decline of the FVIII activity levels over years, thus posing the question on the durability of this therapeutic effect. Moreover, this approach relies on the episomal persistence of the therapeutic transgene in hepatocytes, which inherently restricts its application to adults only [14,15].

In this context, genome editing could provide a definitive cure with a single intervention, as it has been elegantly shown in mouse models for HA via homologous-recombination (HR) triggered by nuclease-driven double-strand break (DSB) [16–20]. Notwithstanding, new DSB/HR-independent DNA editing approaches stemming from the CRISPR-Cas technology have been recently developed (Figure 1) thus opening new therapeutic perspectives for human genetic disorders.

Base Editors (BEs) comprise a catalytically impaired Cas9 nickase delivered on target by its specific guide RNA (gRNA), and a base modification enzyme able to edit a single nucleotide on single-stranded nucleic acid substrate [21–25]. Cytosine base editors (CBE), able to convert C into T, and adenine base editors (ABE), able to convert A into G can collectively mediate all four possible transition mutations (C>T, A>G or, in the opposite strand, T>C and G>A). These BEs have been delivered to a wide range of tissue targets with viral and non-viral modalities to treat genetic disorders [26–30], and some are currently in clinical trials [31,32].

On the other hand, Prime Editors (PE) can mediate targeted insertions, deletions, all 12 possible base-to-base conversions, and combinations thereof [33]. The PE2 architecture is based on a Cas9 nickase fused to an engineered reverse transcriptase (RT) domain that is targeted to the editing site by an engineered prime editing guide RNA (pegRNA), which specifies the target site in its spacer sequence (gRNA). The pegRNA also contains a primer binding sequence (PBS) and an RT template (RTT sequence) encoding the desired edit. Once

landed on the target site, the PE cuts the opposite strand, and the flapping DNA is hybridized with the PBS sequence, thus providing the 3' end exploited by the RT to synthetize new DNA that is then inserted into the genome. To further promote the incorporation of the newly synthesized DNA, a simple single sgRNA can be added to direct PE2 to nick the non-edited strand, thus forming the PE3/PE3b system. Optimization of the PE2 and PE3 architectures, and incorporation of proteins to manipulate DNA repair machinery, led to the development of the recent and more efficient PE4, PE5 and PE6 variants [34,35]. Since its discovery, PE has been applied to a broad type of targets, ranging from cultured cells to human pluripotent stem cells as well as disease mouse models, either to model or to rescue pathogenic mutations [33–36].

Here, for the first time we applied the BE and PE approaches to HA-causing point mutations. Collectively, more than 3000 unique variants have been reported in the European Association for Haemophilia and Allied Disorders (EAHAD) and the Human Gene Mutation (HGMD) databases (EAHAD http://f8-db.eahad.org/; HGMD https://www.hgmd.cf.ac.uk/) [37]. Among them, beside the large IVS1 and IVS22 inversions [38,39] that are not approachable with the proposed BE/PE, point mutations (missense, nonsense, splicing variants) are found in over 30% of severe HA patients and in the vast majority of moderate forms.

Through investigations on a panel of highly recurrent and representative missense/nonsense model mutations associated with severe HA we demonstrated in cellular models that BE and PE systems can be tailored to efficiently correct point mutations at the DNA level, ultimately resulting in a consistent rescue of rFVIII secretion and function.

METHODS

Bioinformatics analysis

The selection and design of gRNA and pegRNA were performed by exploiting the following bioinformatic tools: CRISPR RGEN Tools [40,41], pegIT [42], pegFinder [43], BE-Designer [44], BE-HIVE [45] and CRISPOR [46].

To quantify editing at DNA level, Sanger-sequencing results were analyzed with the EditR tool [47].

Construction of expression plasmids

The generation of expression plasmids for the recombinant FVIII (rFVIII) variants were outsourced to the Twist Bioscience Company (Twist Bioscience, CA, USA). Briefly, the lentiviral pF8 BDD plasmid expressing the B-domain deleted FVIII isoform was edited by inserting into the *XbaI* and *SalI* restrictions sites a synthetized cassette containing the Internal Ribosome Entry Site (IRES)-PuroR fragments. Then, the FVIII portion between the *BsiWI* and *XbaI* restrictions sites was replaced by a synthetized fragment containing the selected FVIII variants. All constructs were validated by NGS sequencing.

The pU6-pegRNA-GG-acceptor and MLM3636 plasmids were purchased from AddGene (plasmids #132777, and # 43860) (AddGene, MA, USA). Cloning in these plasmids was done as described by Anzalone et al [33].

Oligonucleotides used for the construction of pegRNAs and gRNAs were purchased from IDT Inc. (Integrated DNA Technology, NJ, USA) and listed in Supplementary Table 1.

Plasmids are listed in Supplementary Table 2.

Transient transfection assays, generation of rFVIII stable clones and editing

Human embryonic kidney 293T (HEK293T, ATCC CRL-3216) cells were transiently transfected in 24-well plates with pFVIII vectors (800 ng) without or with plasmids for the combination of BE/PE (650 ng) and gRNA/pegRNA (50 ng) with Lipofectamine 2000 (Invitrogen, MA, USA), as per manufacturer's instructions. Culture medium was replaced 4 hours post-transfection with fresh OptiMEM (Sigma-Aldrich, St. Louis, MO, USA). Media were harvested 48 hours after transfection, centrifuged for 5 min at 3000 x g and stored at -20°C. To generate rFVIII expressing cell lines, HEK293T cells were transfected with the *ScaI*-linearized rFVIII expressing plasmids in 24-well plates with Lipofectamine 2000. After 3 days of recovery, cells were selected with 1 μg/mL Puromycin (Sigma-Aldrich, St. Louis, MO, USA). A stable pool of clonal cell line was isolated and plasmid integration was confirmed by PCR (data not shown). To assess editing, stable clones were transfected with the combination of BE/PE (1.2 μg) and gRNA/pegRNA (110 ng) as above reported.

Lentiviral vectors production

The lentiviral pF8 BDD plasmids used to transiently express rFVIII were used to generate rFVIII-LV vectors. The recombinant lentiviral plasmids expressing the ABE8e-NG together with its mutation-specific gRNA were generated by exploiting the golden-gate protocol (NEBridge Golden Gate Assembly). Briefly, fragments containing the lentiviral backbone (region spanning IRES through cPPT/CTS), the ABE8e-NG and gRNA expression cassettes were PCR amplified from plasmids SIN40C.SFFV.MCS.IRES.GFP, NG-ABE8e and MLM3636 (AddGene plasmids #169280, #138491 and #43860, respectively) with the high-fidelity Q5 polymerase and joined by exploiting the *BsmBI*-v2 Type IIS restriction site. Primers and 4-base overhangs were designed by exploiting the NEBridge Golden Gate Assembly Tool.

Lentiviral vectors (LV) were produced by transient transfection in 293T cells using the calcium phosphate method and the pMDLg/pRRE, pVSV-G, pRSV-Rev (Addgene # 12251; # 138479 and # 12253, respectively) as packaging plasmids. LV was harvested at 48 hours post-transfection and filtered through 0.22-μm-pore cellulose acetate filters. LVs were concentrated by ultracentrifugation (2 h at 50,000xg at RT). LV titers were calculated by GFP analysis of HEK293T transduced by serial dilution of LV.

Generation of engineered BOECs and editing

Blood Outgrowth Endothelial Cells (BOECs) from a severe HA patient harboring the *F8* IVS22 inversion [48] were plated at a 10^4 cells/cm^2 density and transduced with LV carrying the FVIIIwt or the FVIII R2166* or R2228Q variants (LV-FVIIIwt, LV-FVIII-R2166*, LV-FVIII-R2228Q) at multiplicity of infection (MOI) of 10.

After 72 hours, cells were selected with 1μg/ml of Puromycin (Sigma-Aldrich) for 3 days. The number of integrated copies of each construct was comparable, and more specifically five, six and four, respectively. HA BOECs were subsequentially transduced with LV expressing the ABE8e-NG and the mutation-specific gRNAs at MOI of 1.25, 2.5, 5, 10 and 20. LV-transduced HA BOECs were cultured for 72 hours, and supernatant collected and stored at -20°C.

Measurement of rFVIII protein levels

Secreted rFVIII levels and cofactor FVIII activity were determined in cell media through a commercial ELISA kit (F8C-EIA, Affinity Biologicals, Ancaster, Canada) and aPTT assay with FVIII depleted plasma (Hyphen Biomed, Neuville sur Oise, France), respectively. Secreted rFVIII antigen and activity levels were expressed as % of the wild-type recombinant FVIII (rFVIII) and specific activity was calculated as the ratio between activity and protein levels. All experiments were run in duplicate and repeated three times.

Evaluation of correction at DNA level

Genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, MA, USA). PCR reactions were carried out with 400 ng of genomic DNA and plasmid-specific primers. The primers used are listed in Supplementary Table 1. PCR products were purified with the QIAquick Gel Extraction kit (QIAGEN, DE) and subjected to Sanger sequencing. Results were analyzed by EditR tool.

Statistical analysis

Data analysis was performed by unpaired t-test with Welch's correction. A value of $p < 0.05$ was considered statistically significant for a 5% confidence interval.

Data Sharing Statement

Relevant data are included in the article and can be found in a data supplement available with the online version of this article. For original data, please contact pnm@unife.it or blsdra@unife.it. Reagents are available under material transfer agreement on request from the corresponding author, Mirko Pinotti (pnm@unife.it).

RESULTS

Among all *F8* point mutations having sequence requirements amenable for the correction through the BE and PE systems (Figure 1), we selected four common *F8* missense and nonsense variants associated with moderate to severe bleeding phenotypes (Figure 2A).

Characterization of rFVIII variants and selection of active BE and PE editors.

To build an experimental model to screen BE/PE for efficiency, we transiently expressed the native B-domain deleted (BDD) FVIII variants in HEK293T cells. As shown in Figure 2B, all mutations led to remarkably reduced FVIII secreted levels as compared with rFVIIIwt (52.9±0.2 ng/mL), albeit to a graded extent (p.R2182H, 11.9±0.8 ng/mL; p.R2016W, 4.9±1.1 ng/mL; p.R2228Q, 2.0±0.1 ng/mL; p.R2166*, <0.1 ng/mL). Conversely, evaluation of the coagulant properties by aPTT assay indicated that the p.R2016W leads to a dysfunctional rFVIII isoform, as witnessed by the 1.2% of activity as compared with rFVIIIwt, likely due to the bystander effect (Figure 2). Differently, the p.R2182H and p.R2228Q variants were associated with coherent reduction of coagulant activity (25.3% and 4.8%, respectively). As expected, we did not detect any appreciable activity in conditioned medium from the p.R2166*-expressing cells as compared with the negative control.

By taking advantage of bioinformatics tools, we designed for each variant a panel of gRNA and pegRNA to be combined with the xABE7.10, ABE8e-NG, ABE8e-SaKKH, ABE7.10, ABEmax-NRTH and Sp-PE2/PE3 editors. To screen them for efficiency, cells were co-transfected with the expression plasmids for the rFVIII variants, editors and gRNA followed by the evaluation of secreted rFVIII levels (Figure 2B).

For the p.R2016W variant, except for the SaKKH-ABE8e/g1b combination, the ABE8e-NG/g1, Sp-PE2/peg1 and Sp-PE3/peg1 produced a significant increase of secreted rFVIII levels (from 4.9±1.1 ng/mL to 14.3±6.3 ng/mL, 13.1±3.5 ng/mL and 12.0±3.0 ng/mL, respectively). Also, the xABE7.10/g1 resulted in increased, albeit not statistically significant, secreted levels (10.8±4.7 ng/mL). For the p.R2166* nonsense variant, all editor systems tested resulted in a significant increase of secreted rFVIII levels in media, with the ABE8e-NG/g4 and ABE8e-NG/g4c displaying the highest correction effect (from <0.1 ng/mL to 13.7±4.2 ng/mL and 9.5±2.1 ng/mL, respectively). The p.R2182H change resulted to be efficiently rescued by the PE2 based system, with secreted rFVIII antigen levels raising from 11.9±0.8 ng/mL to 16.7±0.3 ng/mL. Lastly, the p.R2228Q variant was significantly rescued by all approaches tested (ABE8e-NG with g3, Sp-PE2 with peg3 or peg3c, from 2.0±0.1 ng/mL to 4.3±0.3 ng/mL, to 12.2±2.2 ng/mL or to 13.0±1.3 ng/mL, respectively). Conversely, the ABE8e-NG with g3b was ineffective. For the p.R2182H and p.R2228Q variants, the Sp-PE3 was significantly more effective than Sp-PE2 in rescuing the targeted mutations (from 16.7±0.3 ng/mL to 24.8±4.4 ng/mL and from 12.2±2.2 ng/mL to 18.9±1.4 ng/mL, respectively).

To verify the impact of the rescue on the functional FVIII features, we carried out aPTT-based assays in conditioned medium (Figure 2C).

For the p.R2016W variant, the increase in activity upon Sp-PE2/peg1 treatment was consistent with that in secreted protein levels (22.8±4.1%), while the ABE8e-NG/g1 treatment did not impact FVIII activity (1.3±0.2%). For the p.R2166* variant a remarkable functional rescue was obtained with the ABE8e-NG/g4 (39.7±8.6%), while the impact of the Sp-PE2/peg4 was barely detectable (1.8±1.4%). The p.R2182H, was rescued by the Sp-PE3/peg2/g2 (29.4±2.3%), but not with the ABE8e-NG/g2b. The p.R2228Q variant showed a clear functional

rescue through the either the ABE8e-NG/g3 or the Sp-PE3/peg3/g3 combinations (14.6±0.1% and 48.4±10.5%, respectively).

Overall, this screening phase led to select for each model mutation BE or PE approaches able to efficiently rescue secretion of functional rFVIII.

Correction efficiency of the selected BE and PE systems in stable rFVIII-expressing clones

To better appreciate the rescue, we generated stable clones expressing the rFVIII variants bearing the p.R2166* and p.R2228Q mutations chosen as paradigmatic examples of nonsense and missense mutations associated with the lowest secreted rFVIII levels and with the highest correction efficiency. Secreted rFVIII levels from stable clones were consistent with those observed in transient transfection experiments, with the mutations p.R2166* and p.R2228Q associated with no/barely detectable traces (<1%) and 7.7±1.1% of rFVIII antigen level in cell media, respectively (Supplementary Figure 1). Analysis of rFVIII protein levels in cell lysates revealed reduced (~65% of WT) FVIII protein levels for both mutations. Moreover, proliferation assay revealed that clones expressing rFVIII mutants grew at significantly slower rate (~50%) as compared with those expressing rFVIIIwt (Supplementary Figure 1), which could underlie an endoplasmic reticulum stress triggered by unfolded protein [49].

In this experimental setting the p.R2166* variant was approached with the ABE8e-NG and two different gRNAs and the ABEmax-NRTH with its own specific gRNA. As shown in Figure 3, the p.R2166* was significantly rescued by ABE8e-NG with both gRNAs at both antigen (from 1.4±0.4% to 18.9±1.3% p=<0.0001 or 3.0±0.7% p=0.0093, respectively) and activity (from <1% to 14.4±5.0% p=0.0112 or 2.1±0.3% p=0.010) levels. Differently, the ABEmax-NRTH variant-mediated rescue was poorly detectable.

The p.R2228Q variant was approached with the ABE8e-NG and with the PE2 options (Sp, SpRY and SpG Cas9 variants), each with its own pegRNAs (Figure 3). In co-transfection experiments, ABE8e-NG, Sp-PE2 and SpG-PE2 rescued p.R2228Q at both antigen (from 7.7±1.1% to 21.5±7.0% p=0.0279, 34.8±3.4% p=0.0002 and 17.2±1.2% p=<0.0001) and activity (from 6.3±1.4% to 33.1±10.9% p=0.0499, 36.7±8.4% p=0.0047 and 16.1±6.9% p=0.0643, respectively) levels. Vice versa, SpRY-PE2 variant was poorly effective, albeit associated with an increase in secreted FVIII levels.

To provide direct experimental evidence of the mutation reversion we carried out DNA Sanger sequencing of the targeted gene regions, which revealed the desired A>G conversion for both FVIII variants treated with the BE/PE combinations associated with the most appreciable effect on secreted FVIII levels (Figure 4). Quantification of editing by relative peaks' height from three independent experiments led to estimate a correction efficiency of 29±4% for the p.R2166* change with the ABE8e-NG/g4 and of 27±3% or 21±4% for the p.R2228Q mutation with the ABE8e-NG/g3 or Sp-PE2/peg3c, respectively. Sequencing of the p.R2166* mutant targeted with the ABE8e-NG/g4 also revealed a bystander G to A editing (p.Y2167H) with an efficiency of

38±11%.Altogether these data demonstrate that the selected best-performing BE and PE combination can efficiently revert the target mutations, thus ultimately rescuing the secretion of functional FVIII.

Rescue of FVIII in ex-vivo blood outgrowth endothelial cells (BOEC) by lentiviral delivery of ABE8e-NG

Data from stable clones prompted us to attempt the correction of both FVIII variants in the model of human blood outgrowth endothelial cells (BOECs), lately called endothelial progenitor cells (EPCs), isolated from both peripheral blood and considered a viable *ex-vivo* cellular model for cell and gene therapy for HA [48,50–52]. Since BOECs isolated from HA patients with the mutations under investigation were not available, we generated new models of BOECs consisting of BOECs from a HA patient harboring the *F8* IVS22 mutation, and not producing appreciable secreted FVIII levels, transduced with a lentiviral vector (LV) expressing the FVIII variant under investigation (Figure 5A). As shown in Figure 5B and 5C, BOECs transduced with the LV-FVIII-R2166* or LV-FVIII-R2228Q mimicked what previously detected in stable FVIII clones, with p.R2166* and p.R2228Q associated with undetectable and low levels of functional FVIII levels, respectively. On the other hand, the secreted FVIII from BOECs transduced with LV-FVIIIwt, created as additional control, shortened the FIX-dependent aPTT-based coagulation time from 167.8±6.7 s to 46.9 ± 4.2 s, corresponding to 30 ng/mL.

To trigger editing we selected the ABE8e-NG since the coding cassette of ABE is smaller than the Prime editing (PE) counterpart, and therefore more suitable to be efficiently loaded and delivered via LV.

BOECs were then transduced at different MOI (from 1 to 20) by LV expressing the ABE8e-NG and the two mutation-specific gRNAs. The evaluation of FVIII antigen and activity levels in cell media three-day post-treatment revealed a significant and dose dependent rescue of rFVIII for both mutations. In particular, rFVIII antigen levels increased from 0 to 17.4±0.6 ng/ml (p.R2166*) and 32.9±2 ng/ml (p.R2228Q) at MOI 20 (Figure 5B), which were paralleled by a concurrent decrease in coagulation time (from 167.8±6.7 s to 52.8±0.8 s for p.R2166*; from 151.9±14.7 s to 42.5±2.5 s for p.R2228Q). Analysis of rFVIII specific activity showed that, for both mutations, rescued FVIII possesses a roughly normal specific activity close to that observed for the rFVIIIwt (Figure 5C).

Overall, these data demonstrated that LV-mediated delivery of ABE8e-NG with the mutation-specific gRNA can efficiently rescue secretion of a functional FVIII protein in *ex-vivo* BOEC cells.

DISCUSSION

While the intense research over decades has provided HA patients with a plethora of therapeutic and prophylactic options that remarkably reduced bleeding tendency and ameliorated the quality of life [6], a definitive cure is not available yet. To this purpose, enormous progresses have been made with additive gene therapy based on AAV, with Valoctocogene roxaparvovec that recently received authorization by regulatory agencies, but several open issues have still to be addressed, including the current restriction to adult [9,14,15,53]. Conversely, the correction of the defect detected in the patient's *F8* gene would provide a

definitive cure, particularly in replicative tissues such as liver of young patients. So far, extensive research has been conducted to trigger HR through nuclease-induced DSB[20], which still suffers from low efficiency and poses safety concerns related to potential off-target DSBs.

For these reasons the recently developed DNA editing systems such as BE and PE systems, not relying on DSB and HR, represent attractive approaches to efficiently and permanently revert point mutations, which are the most frequent cause of human disease [54]. Because of the high efficiency of BE, of the extreme versatility of PE, and limited off-target effects, BE and PE have been so far successfully applied in a variety of cell types and organisms, including animal models of human genetic diseases [55,56]. BE is now close to the clinic for heterozygous familial hypercholesterolemia and severe sickle cell disease [57,58]. Despite these promising results, very little attempts have been done with BE or PE in hemorrhagic coagulation factor disorders where even a partial rescue of functional plasma levels would remarkably ameliorate the clinical phenotype. To date only the BE has been attempted in hemophilia B, and proved to revert two *F9* missense mutations [59,60].

In this pioneer project, we explored for the first time the BE and PE approaches on *F8* point mutations, accounting for over 30% of all severe HA patients, the large majority of which theoretically correctable by exploiting the variety of BE and PE systems so far developed (Supplementary table 3).

Through *in vitro* and *ex-vivo* studies on paradigmatic examples of missense and nonsense model mutations associated with severe bleeding phenotypes, we provided the proof of principle of the therapeutic potential of BE/PE approaches for HA. FVIII antigen and activity of the recombinant variants in medium were mostly consistent with the coagulation phenotype in patients, with undetectable levels for p.R2166* nonsense mutation, major (p.R2228Q) or moderate (p.R2182H) impairment of secretion and a combined detrimental effect on both secretion and activity for the p.R2016W, the latter confirming our previous characterization [61]. The in vitro system, which does not to permit the mutation impact on FVIII stability and/or removal from the circulation, could in part explain the not uncommon discrepancy between FVIII levels reported in patients and recombinant data for the p.R2182H variant.

For each mutation, the initial screening phase in transient transfection experimental systems led to the identification of a short panel of BE/gRNA and PE/pegRNA combinations able to efficiently rescue FVIII expression. In particular, the ABE8e-NG and Sp-PE3 confirmed to be the most active in the editing, when compared to the ABE7.10, xABE or Sp-PE2, respectively [33,62]. It is worth noting that some combinations (i.e Sp-PE2/PE3 for the p.R2166* or xABE7.10/g2 for the p.R2182H variant) were ineffective or led to a further reduction of the secreted FVIII levels, which might be attributable to bystander effects of base editors. The same mechanism might lead to rescue secretion but not activity as in the case of the p.R2016W variant targeted by the ABE8e/g1 combination. These data highlight the importance of accurate computational design of the specific correction approaches (BE or PE), followed by extensive experimental screening, to select those promoting effective editing.

The selected BE and PE based combinations were successfully exploited in cells stably expressing the nonsense p.R2166* and the missense p.R2228Q, selected because found to be associated with very low secreted FVIII and displaying the highest correction efficiency. Analysis of the rescued FVIII expression corroborated findings in the transient transfection assays with an overall correction efficiency of 20-30% for both mutations. Noticeably, the consistent increase of the FVIII antigen and activity levels pointed toward a normal specific activity of the rescued FVIII, suggesting that efficient conversion of the point mutation to the wild-type counterpart. This was clearly demonstrated by DNA sequencing of the targeted gene region selected by allele-specific PCR. More specifically, the analysis of sequence trace decomposition showed an editing efficiency of 21-29%, an extent consistent with the rescued FVIII antigen and activity levels. On the other hand, sequencing revealed an undesired adjacent editing for the p.R2166* mutation targeted by ABE8e-NG/g4 combination, which occurred with a comparable efficiency (~38%) than that of the correct one (~30%). This would lead to the introduction of the p.Y2167H change, whose effect is predicted to be detrimental. However, the rescue of the secreted and functional FVIII levels (~20% vs ~30% of mutation reversion) points toward the occurrence, in the majority of cases, of two distinct editing events, i) the candidate mutation reversion only and the rescue impact, or ii) the by-stander effects, with the introduction of the p.Y2167H substitution in the null p.R2166* allele, which does not causes additional harm. Although the safer profile of BE/PE as compared with that of nuclease-mediated editing approaches [63], we recognize the need of extensive genome wide evaluation to assess off-target effects of our F8-tailored combinations, which are hard to be bioinformatically predicted at present.

The successful editing was further demonstrated in the BOECs that better recapitulate the physiological expression site for FVIII. To overcome the unavailability BOEC models harboring the mutations under investigations, and to avoid the potential confounding effect of endogenous FVIII produced by wild-type BOEC [48], we engineered HA patient's BOEC with the *F8* IVS22 inversion, and not expressing appreciable FVIII levels. By LV we integrated the FVIII coding cassette for the two model mutations into the $BOEC^{IVS22inv}$ and demonstrated that both cellular models recapitulated findings from our previous cellular models, with the p.R2166* and p.R2228Q variants associated with undetectable and barely detectable levels of rFVIII, respectively. By recognizing that these ex-vivo HA cellular models may not fully recapitulate conditions (i.e transgene integration position, chromatin context) that might be relevant for the editing process *ex vivo* or *in vivo*, the treatment with the ABE8e-based BE resulted in a remarkable, and dose-dependent, increase of secreted FVIII. This was paralleled by the shortening of the coagulation time in aPTT-based FVIII activity assays that, at the highest dose, mirrored the functional levels expressed by BOECs transduced with the LV-FVIIIwt.

In conclusion, these data provide the experimental proof-of-concept that delivery of tailored BE and PE editors, can effectively revert pathogenic and common HA single nucleotide mutations at the DNA level, thus leading to appreciable rescue of production and secretion of functional FVIII. It is tempting to speculate

that, if translated in patients, the extent of rescue obtained with a single intervention would remarkably ameliorate the coagulation and clinical phenotype with lifelong effects, even in young patients. When considering the editing windows and the PAM constrains, the BE/PE approaches could target up to 90% of point mutations, with therapeutic implication for up to 97% patients bearing these nucleotide changes (Supplementary Table 3).

It is worth noting that these therapeutic approaches could take advantage of non-viral gene delivery systems [59,64,65] preventing the persistence of the editors and thus further minimizing off-target effects. This might be envisaged for *ex-vivo* gene therapy purposes (i.e. Endothelial Cell Progenitor cells)[48] or direct *in-vivo* F8 gene editing in LSECs taking advantage of endothelial specific promoters (i.e Stabilin-2)[66].

Altogether these results lay the foundation for safety and efficacy studies in animal models with specific mutations, not yet available, with base and prime editors to develop a versatile and personalized definitive cure for large cohorts of Hemophilia A patients.

AUTHORSHIP CONTRIBUTIONS

Contribution: T.E performed bioinformatic analyses, research and interpreted data; C.A performed experiments and interpreted data in BOEC cells; P.M and B.D conceived the study, supervised research and coordinated the work; P.M, B.D, F.A and B.F wrote the manuscript.

Conflict-of-interest disclosure: Nothing to declare.

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CONFLICT OF INTEREST DISCLOSURES

Conflict-of-interest disclosure: Nothing to declare

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FIGURE LEGENDS

Figure 1. The Adenine Base and Prime Editing tools.

Schematic representation of the Adenine Base (ABE, left) and Prime Editing (PE, right) tools. ABE is composed by a Cas9 nickase targeting a specific DNA sequence through a specific guide RNA (gRNA). A directly evolved Adenine Deaminase, fused to Cas9 nickase, is responsible for deaminating the target adenine (A) to inosine (I). Cellular DNA repair machinery recognizes the mismatch (I:T) and repairs the opposite nicked strand using the inosine (I) as a template. After DNA replication, the repair process results in an A to G conversion at the target site.

PE is composed by a Reverse Transcriptase (RT) fused to a Cas9 nickase, targeted to a specific DNA sequence through a specific Prime Editing Guide RNA (pegRNA). The pegRNA also provides a Primer Binding Site (PBS) which base pairs with the cut DNA strand and is exploited by the RT to

synthesize new DNA by copying the information by the RT template (RTT), also coded by the pegRNA. The newly synthesized cDNA strand displaces a short segment of the target DNA strand (flap) due to complementarity with the target sequence and the RT Template. Cellular DNA repair machinery recognizes the nick and the displaced flap. The flap is removed, and the edited cDNA strand is integrated into the target DNA using the opposite strand as a template.

Figure 2. BE/PE can rescue rFVIII secretion and coagulant activity impaired by common HA point mutations.

(A) Features of the four-representative *F8* missense/nonsense mutations selected for this study. Data come from Factor VIII Gene (F8) Variant Database (EAHAD; accessed on 01/06/2020). Median of FVIII:C and FVIII:Ag levels are reported.

(B) FVIII antigen levels in media from HEK293T cells transiently transfected with rFVIII expression plasmids alone (UT, white bars) or in combination with BE (light blue bars) or PE (blue bars). Results are expressed as ng/ml. Results are presented as mean ± SD of three independent experiments. *, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

(C) FVIII antigen (light red) and activity (red) levels evaluated in media from HEK293T cells transiently transfected with rFVIII expression plasmids alone (UT) or in combination with BE or PE. FVIII antigen and activity levels are reported as percentage of rFVIIIwt. Results are presented as mean ± SD of three independent experiments.

Figure 3. BE/PE-mediated rescue of rFVIII antigen and activity levels in stable clones.

rFVIII levels in stable HEK293T clones expressing the wild-type or mutant rFVIII cassettes alone (UT) or upon treatment with the selected BE/PE editors. The SpRY and SpG PE tools, made available during the study, were directly tested in the stable clone. FVIII antigen and activity levels are reported as percentage of rFVIIIwt. Results are presented as mean ± SD of three independent experiments. *, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Figure 4. BE/PE-mediated correction of causative HA mutations at DNA level.

Representative electropherograms from DNA sequencing of the targeted region in the stable clones that, upon treatment with BE/PE, showed the highest rescue of rFVIII secretion and function. The red and underlined letters indicate the PAM and gRNA sequences, respectively. The PBS and RTT sequences of pegRNA are indicated by blue and green underlined letters, respectively. The targeted mutation, before and after editing, is reported in green and in the upper and lower part of the electropherograms, respectively. The amino acid change, resulting from the editing, is reported in red.

Figure 5. BE-mediated correction of causative HA mutations in BOECs cellular models.

(A) Schematic representation of the protocol exploited for the generation of mutation-specific BOECs models and evaluation of BE-mediated correction trough LV-mediated delivery of ABE8e editors. See results for experimental details.

(B) rFVIII antigen levels in media from BOEC cells transduced with LV expressing the mutation specific ABE8e editor at different multiplicity of infection (MOI). Results are presented as mean ± SD of two independent experiments. Higher MOIs are reported with increasing saturated violet color.

(C) rFVIII coagulant activity in media from BOEC cells transduced with LV expressing the mutation specific ABE8e editor at different MOI. rFVIII antigen and coagulation time are reported in the x- and y-axes, respectively. A standard curve made with rFVIIIwt (light blue circles) from stable clone is reported. Higher MOIs are reported with increasing saturated violet color. Results are presented as mean ± SD of two independent experiments. UT, untreated

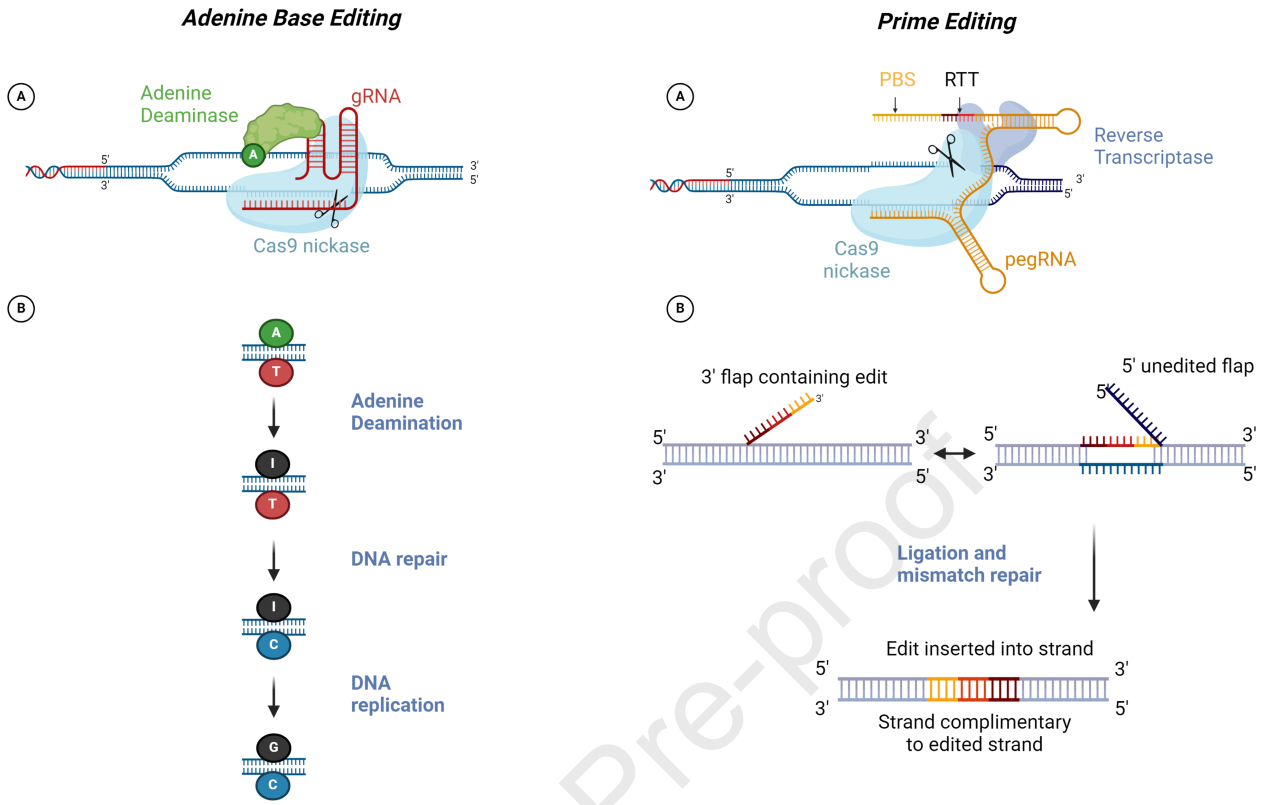
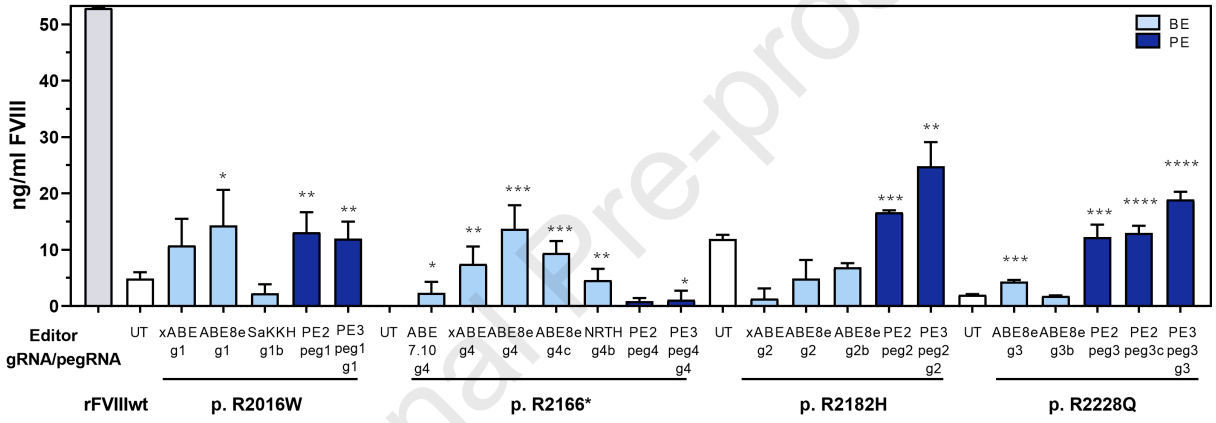


FIGURE 1

A

Mutation type	Location	No of Patients	Nucleotide change	Protein change	FVIII:C%	FVIII:Ag%
Missense	Exon 19	100	c.6046C>T	p.R2016W	1	3,5
Nonsense	Exon 23	54	c.6496C>T	p.R2166*	<1	<1
Missense	Exon 23	67	c.6545G>A	p.R2182H	<1	n.r
Missense	Exon 24	57	c.6683G>A	p.R2228Q	<1	1

B



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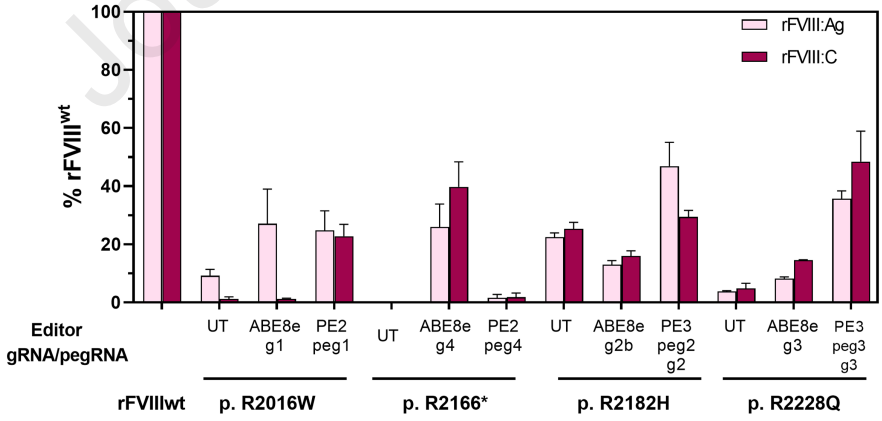
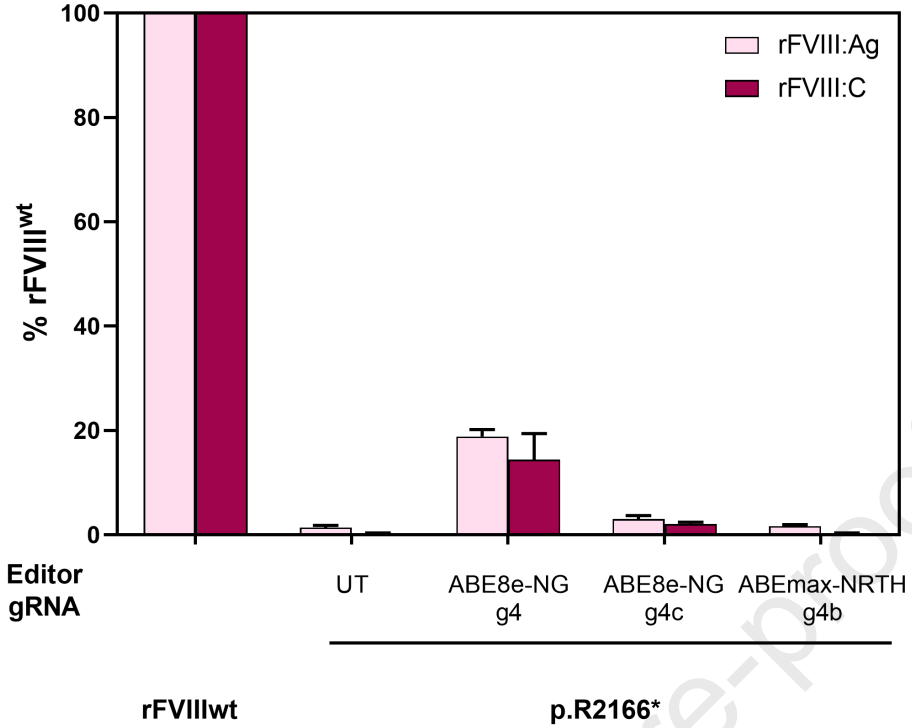


FIGURE 2



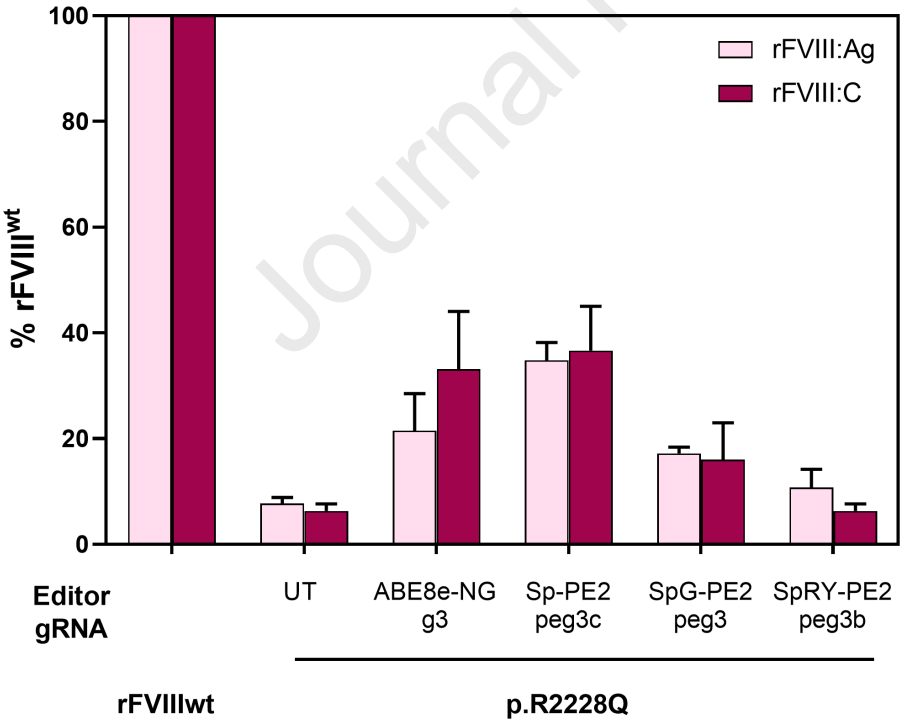
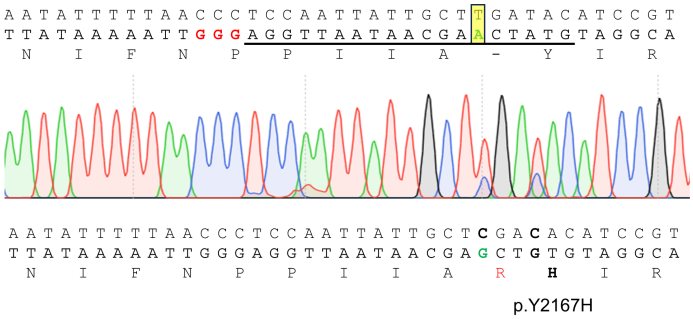


FIGURE 3

c. 6496C>T ***p.R2166**** ABE8e-NG + g4



c. 6683G>A ***p.R2228Q*** ABE8e-NG + g3

Sp-PE2 + peg3c

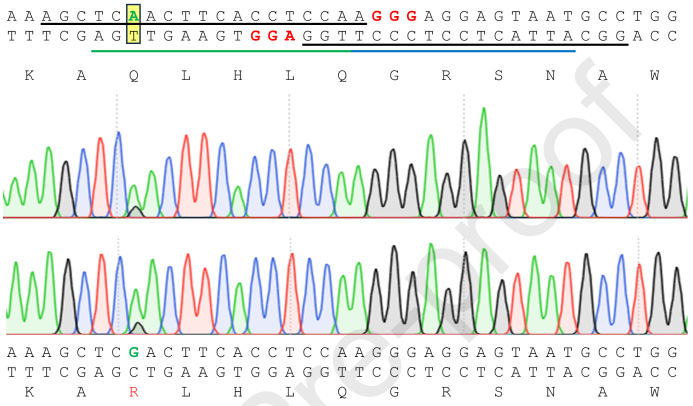
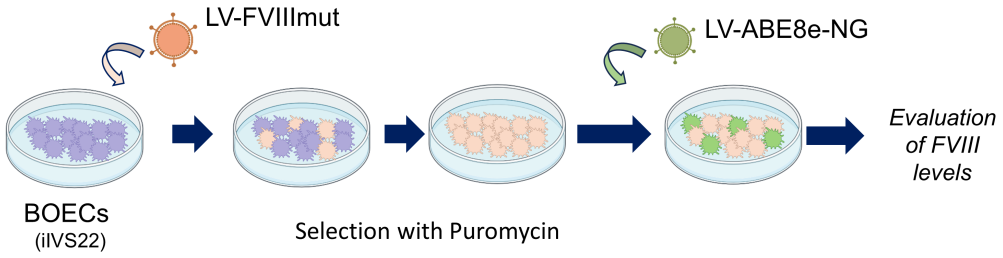
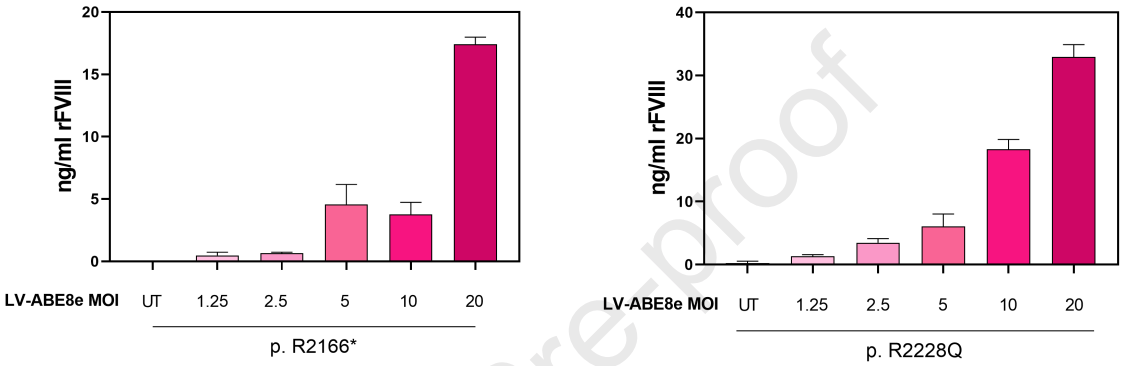


FIGURE 4

A



B



C

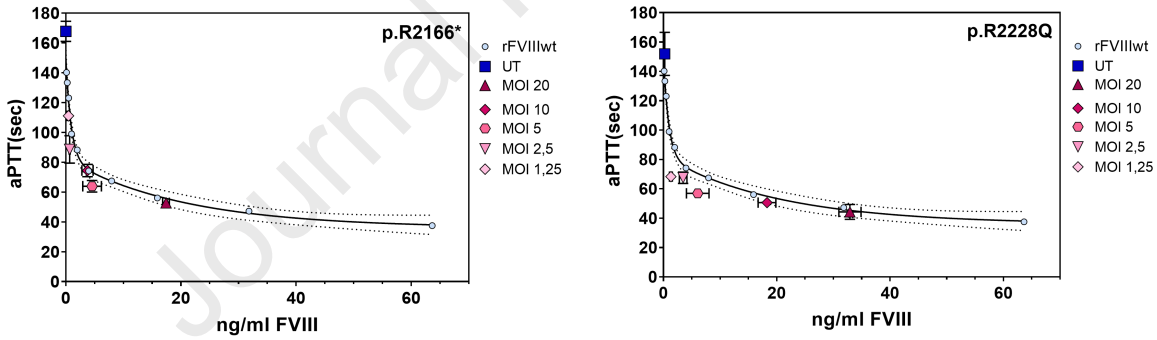


FIGURE 5