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# *F9* MISSENSE MUTATIONS IMPAIRING FACTOR IX ACTIVATION ARE ASSOCIATED WITH PLEIOTROPIC PLASMA PHENOTYPES

**Short title** FIX levels modulation by activation site mutations

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**Essentials**

- The factor IX (FIX) cross-reactive material (CRM) might modulate infused FIX distribution
- The contribution of *F9* genotypes on infused FIX pharmacokinetics was investigated
- FIX activation site (R191/226) variants (HB patients n>300) express medium/high CRM levels
- FIX activation site substitutions may prolong infused FIX Alpha and Beta half-lives and MRT

**Summary**

**Background:** Circulating dysfunctional factor IX (FIX) might modulate distribution of infused FIX in haemophilia B (HB) patients. Recurrent substitutions at FIX activation sites (R191-R226, >300 patients) are associated with variable FIX activity and antigen (FIXag) levels.

**Objectives:** To investigate i) expression of a complete panel of missense mutations at FIX activation sites and ii) contribution of *F9* genotypes on the FIX pharmacokinetics (PK).

**Methods:** FIXag and activity assays in plasma and after recombinant expression of FIX variants. Analysis of infused FIX PK parameters in patients (n=30), mostly enrolled in the *F9* Genotype and PK HB Italian Study (GePKHIS; EudraCT ID2017-003902-42).

**Results:** The variable FIXag amounts and good relation between biosynthesis and activity of multiple R191 variants result in graded moderate-to-mild severity of the R191C>L>P>H substitutions. Recombinant expression may predict the absence in the HB mutation database of the benign R191Q/W/K and R226K substitutions. Equivalent changes at R191/R226 produced higher FIXag levels for R226Q/W/P substitutions, as also observed in p.R226W female carrier plasma.

PK analysis in patients suggested that infused FIX Alpha distribution and Beta elimination phases positively correlated with endogenous FIXag levels. Mean residence time was particularly prolonged (79.4 hrs, 95% CI 44.3-114.5) in patients (n=7) with the R191/R226 substitutions, which in regression analysis were independent predictors (β coefficient 0.699, p=0.004) of Beta half-life, potentially prolonged by the increasing over time ratio between endogenous and infused FIX.

**Conclusions:** FIXag levels and specific features of the dysfunctional R191/R226 variants may exert pleiotropic effects both on HB patients' phenotypes and substitutive treatment.

**Keywords**

Haemophilia B; factor IX activation; recombinant proteins; pharmacokinetics; pharmacogenetics.

## INTRODUCTION

The activation of coagulation factor IX (FIX) has a key role in the coagulation cascade, either via activated factor VII (FVIIa) in complex with tissue factor (TF) or via activated factor XI (FXIa) [1]. Full activation is achieved after cleavage at two highly conserved activation sites [2,3], which results in the release of the activation peptide [4–7]. More than 300 haemophilia B (HB) patients with recurrent *F9* mutations affecting the R191 or R226 residues, encoded by CpG-containing codons [8], have been reported (EAHAD FIX variant Database, https://f9-db.eahad.org/) [9]. Seminal studies have indicated that single cleavage at R191 does not produce catalytic activity but converts FIX zymogen into a factor VIII (FVIII)-binding enzyme [10,11], whereas single cleavage at R226 develops catalytic activity but results in suboptimal binding to the FVIII light chain [11,12]. Accordingly, missense mutations at these positions are associated in HB patients with moderate/mild (R191) [13–21] or severe (R226, $HB_m$ mutation subclass) [14,22–28] FIX deficiency. Failure in the production of activated FIX (FIXa) [14,24,29] has been modelled in HB mice (R226W) [30].

Based on these information, we focused on *F9* mutations affecting the FIX activation sites by systematic recombinant expression of natural and designed variants to interpret HB phenotypes, both residual activity and antigen levels, associated to virtually "all" amino acid substitutions.

Previous results suggested that activation peptide sequences act as plasma retention signals, as observed for human FVII added with the FIX activation peptide, which prolonged 4.3-fold its half-life when tested after infusion in mice [31,32].

Concerning the distribution of FIX infused in HB patients, it has been hypothesized that the presence of cross-reactive material ($CRM^+$), namely the circulating FIX antigen (FIXag) with reduced or null activity, might partially improve the *in vivo* recovery of FIX infused in HB patients [33]. Differently, in HB mice, the presence of dysfunctional endogenous FIX caused by the human R379Q variant [34] may decrease replacement efficacy [35]. Based on these observations, it has been proposed that prophylaxis regimens in HB patients should consider the CRM status [36]. Since observations in patients and animal models may be explained by the dynamic equilibrium between plasma FIX and the extravascular protein bound to the subendothelial basement membrane [37–40], a two-compartment (2CP) pharmacokinetic (PK) model best describes the distribution of FIX infused in HB. We report the PK analysis of FIX infused in severe/moderate HB patients, enrolled in the frame of the *F9* Genotype and PK Haemophilia B Italian Study (GePKHIS), to investigate the relation between distribution of

infused FIX and circulating endogenous FIX variants, among which several substitutions at the FIX activation sites.

## MATERIALS AND METHODS

### Patients and approval

Plasma samples from HB patients (n=30), mostly enrolled in the GePKHIS study, and in addition three related female carriers of the p.R226W variant, were collected and processed at the local haemophilia centres belonging to the Italian Association of Haemophilia Centres (AICE). The study was approved by the coordinator centre (Ferrara, code ACTB02BD04), as well as by each local ethics committee, and was registered in the EudraCT database (ID 2017-003902-42). The study was carried out in accordance with the Declaration of Helsinki principles, and written informed consent was obtained.

### Study design

The protocol was approved for severe/moderate HB patients (Supporting Table 1) undergoing on-demand or prophylactic treatment with Nonacog Alfa (Pfizer, New York, USA).

Inclusion criteria to participate in the study were: severe or moderately severe HB (FIX activity ≤ 3 IU/dL); no bleeds in the last week before assessment of PK; on-demand or on-prophylaxis treatment with Nonacog Alfa for >150 exposure days.

Exclusion criteria were: any kind of bleeding in the last week before the PK; severe hepatic disease; ongoing HIV treatment (HAART); history of anti-FIX inhibitors; previous treatments with extended half-life FIX concentrates. The PK study was not conducted in the HB patient with the p.R191H variant, associated with a mild HB phenotype (FIX coagulant activity >5%; see Supporting Table 1). For this patient, only the basal activity and antigen levels were determined.

### Nomenclature

All amino acids are numbered according to the Human Genome Variation Society (HGVS) nomenclature [41].

### *F9* genotypes of patients

The *F9* genotypes of HB patients undergoing PK (Supporting Table 1) were characterized as described [20]. Causative mutations were classified as missense (n=16, patients n=21), nonsense (n=4), splicing (n=2) and deletion (n=2) variants.

**Expression of recombinant variants**

Recombinant FIX (rFIX) variants were created by site-directed mutagenesis of *F9* cDNA (reference sequences: NM_000133.4, NP_000124.1) cloned in the pCDNA3 vector [42]. Oligonucleotides are listed in Supporting Table 2. All plasmids have been validated by sequencing. Nonsense variants have been produced in previous studies [43,44].

Expression studies were carried out through transient transfection of human embryonic kidney 293 (HEK293) cells in the presence of 5 μg/mL vitamin K (Konakion, 10 mg/mL), essentially as described [42]. Briefly, cells were seeded in 12-well culture plates and transfected in serum-free medium (Opti-MEM, Gibco, Life Technologies, USA) with the Lipofectamine 2000 reagent (Life Technologies) with a ratio DNA (μg):Lipofectamine (μl) of 1:1 (2 μg DNA: 2μl Lipofectamine) and media were collected 48 hours (hrs) post-transfection.

**Evaluation of protein and activity of rFIX variants**

FIXag levels were evaluated by polyclonal anti-human FIX ELISA (FIX-EIA, Affinity Biologicals, Canada) [43,44] to minimize the effects of mutations on detection of FIX epitopes. Known concentrations of purified rFIX were used as reference.

Protein forms derived from FXIa-dependent activation [45] of rFIX variants were evaluated through Western blotting analysis with polyclonal goat anti-human FIX (APGAFIX; Affinity Biologicals) and anti-goat horseradish peroxidase-conjugated (A50-101P; Bethyl Laboratories, Montgomery, TX) antibodies. Blotting images were acquired on the ChemiDoc instrument and analyzed by the Image Laboratory Software version 4.0 (Bio-Rad, USA).

Activity of rFIX variants in media was evaluated through a commercially available chromogenic assay exploiting an optimized FXIa concentration as per manufacturer instructions (Biophen; Aniara Diagnostica, West Chester, USA) [43]. Reaction aliquots were collected for Western blotting analysis. Serial dilutions of wild-type rFIX (rFIX-WT, mean concentration 485±51.5 ng/ml), expressed in the same experimental conditions in parallel to rFIX variants, were used as reference. The activity/antigen ratio was calculated.

**Evaluation of FIX protein and activity in plasma**

Serial dilutions of pooled normal plasma (PNP; Hyphen BioMed, France) were used as reference for analysis through polyclonal anti-human FIX ELISA (Affinity Biologicals) [43,44] of FIXag levels in plasma from HB patients. Activity of infused/baseline FIX was measured by one-stage clotting method [46].

**PK analysis**

Each patient received a single-dose (40±5 IU/kg) of Nonacog Alfa. Plasma samples were collected before infusion and at 1-3-9-24-48-72 hrs post-infusion.

PK parameters were obtained through the 2CP model analysis (WinNonlin 7.0, USA) [46–48], with the best fitting evaluated through the Sum of Squared Residuals (SSR) [49] and the Coefficient of Correlation between observed and predicted FIX concentration/time. The FIX/time concentrations were corrected according to the Björkman formula [50] or by basal level subtraction, as appropriate.

Specific primary (K 1-2, K 2-1 and V1) and secondary (Alpha HL, Beta HL, Clearance, CLD2, Cmax, MRT, V2, Vss and AUC) PK parameters, including *in vivo* recovery (U/dL per IU FIX concentrate/kg body weight infused) were evaluated [46,51,52].

**Statistical analysis**

Statistical analyses were performed using IBM® SPSS® Statistics (version 23.0; IBM Corp., USA). Variables were reported as means with 95% confidence interval (CI). For skewed variables, the analysis was conducted with logarithmically transformed values. Two-tailed Pearson correlations were performed to determine the association between PK parameters and FIXag (%). *F9* mutation type-related differences in PK parameters were analysed by *t*-test. In linear regression analysis, the contribution to PK parameters of FIXag levels and *F9* mutation types was evaluated.

**Results**

***R191/R226 substitutions, plasma and recombinant phenotypes***

To compare expression levels of protein variants with amino acid substitutions at FIX activation sites (R191 and R226), we evaluated FIXag and activity levels obtained from i) analysis of HB

patients' plasma, collected in the frame of the GePKHIS study, ii) inspection of the international database (https://f9-db.eahad.org/), and iii) systematic expression of rFIX variants harbouring natural or designed changes.

In HB patients (Figure 1A), the R191H/C substitutions result in reduced plasma FIX activity (Figure 2A, left panel) and normal (R191H) or moderately reduced (R191C) FIXag (Figure 2A, right panel) levels. The potential effects of the unpaired cysteine in the 191C FIX was explored by Western blotting analysis of plasma and conditioned medium performed in reducing/non-reducing conditions. Data did not support the presence of high molecular weight molecules produced by disulfide bond of the 191C variant, either circulating in plasma or after secretion in medium (Supporting Figure 1). The devoid-of-function R226Q/W variants showed normal or increased antigen levels (Figure 2A, right panel). The increased plasma levels associated to the R226 substitutions is supported by data in HB patients and recombinant expression. Among the 60 patients affected by the R226Q and reported in the database, eight have been characterized for the FIXag, with mean levels >100%. Patients (n=43) affected by the R226W change display (10 patients evaluated) variable FIXag levels (range 130-50%, mean 84%). Among the GePKHIS patients bearing these substitutions, FIXag was >100%.

In expression studies, equivalent substitutions (R191/R226 to Q/W/P/L) (Figure 2B) caused significantly different effects at position R191 and R226, and were compatible with normal or increased secretion in media of the 226 variants as compared with near normal or reduced levels of the 191 variants. As an additional control for catalytically inert serine proteases we also expressed the S411P variant (chymotrypsin numbering S195), which also gave rise to high FIXag values.

Mutations affecting residues adjacent to R191 (A192P) and to R226 (V227F, V228L/F/A/G, Supporting Figure 2) are responsible for noticeable FIXag levels (70-130%), and for residual activity (2-20%) of most variants [17,27,53–55]. Inspection of the exome sequencing database GnomAD (https://gnomad.broadinstitute.org/) revealed the presence of 12 subjects carrying the conservative V227I change (rs137852242), suggesting a rare polymorphism (Supporting Figure 2).

In expression studies we also designed and expressed rFIX control variants bearing a "classic" alanine change (R226A), or amino acid changes conferring negative (R226D/E) or positive (R226K) charge. Comparison between the natural (Q/W/P/L) and the designed (A/D/E, Supporting Figure 3) R226 variants in FXIa-dependent activation experiments (Figure 2C, left panel), failed to detect the FIX form corresponding to the heavy chain upon loss of the activation

peptide. Differently, the designed R226K variant, as expected for the conservative R-to-K amino acid change, displayed an activation pattern similar to that of rFIX-WT (Figure 2C, left panel), and reached an activity and activity/antigen ratio above 50% and 0.5, respectively (Figure 2C, right panel).

To provide insights on the activity features of the R191C/L/P/H variants, the relation between FIXag and activity was investigated by comparing findings in plasma and recombinant systems (Figure 2D), which showed a very good relation only for plasma values ($r^2$=0.987). The normal FIXag levels for the r191L correspond to FIXag reduced to half in plasma, which suggests a FIX molecule with increased clearance *in vivo*. The relation between activity and activity/antigen ratio, which magnifies the functional properties of the multiple natural and designed R191 variants, was very good either excluding ($r^2$=0.69; Figure 2E) or including ($r^2$=0.98; Figure 2E, inset) the R191K variant, characterized by the highest activity and antigen values. The slope clearly indicated that the clinically relevant FIX deficiencies produced by the R191C/L substitutions (Figure 1A) are based on different functional properties. Conversely, the mild phenotype associated with the R191H deficiency is based on high secretion levels but modest catalytic properties.

Overall, comparison of *in vivo* and recombinant results of substitutions at activation sites provided a gradient of residual activity (R191) and antigen (both R191 and R226) values. The recombinant expression permits to extrapolate information that are not available in patients but help interpreting findings in patients.

***Plasma FIX activity and antigen levels in female carriers of the p.R226W variant***

We investigated plasma FIX activity and antigen levels in related female carriers [56,57] (n=3) of the p.R226W variant (Figure 3), to explore its potential influence on the proportion of the WT FIX and the inactive FIX variant circulating in plasma. The mean FIXag levels were higher (153.4±18.6%), than the mean FIX activity (78.0±30.3%), which resulted in an activity/antigen ratio decreased to half (0.50±0.15, Figure 3), suggesting a similarly increased amounts of both FIX molecules.

***Correlation between FIX antigen levels and FIX PK parameters in HB patients***

In the frame of the GePKHIS study, the decay of infused FIX was determined in 29 HB patients, characterized for *F9* genotypes. FIX variants corresponding to *F9* mutations in HB patients (Figure 1B and Supporting Table 1) were recombinantly expressed, which confirmed their causative role and supported the PK analysis. The characteristic biphasic decay curves and the SSR values are reported in Supporting Figure 4, and the distribution of 2CP PK parameter values in Table 1.

The FIXag levels were directly correlated (Table 1) with Alpha HL and Beta HL, and inversely with CLD2, Cmax, and with K 1-2 and K 2-1, the latters two primary parameters modelling the flow between plasma and extravascular compartments (Supporting Figure 4). The correlation between FIXag levels and *in vivo* recovery, expressed as the ratio between maximum FIX activity and the FIX dose, was not significant (r=-0.289, p=0.128). On the other hand, the *in vivo* recovery has been reported to be quite variable even in the same patient on different occasions [52].

***FIX antigen levels and PK parameters in HB patients grouped by mutation type***

The significant correlation between FIXag and Alpha HL (p=0.004; Table 1) prompted us to analyse the Alpha HL distribution in relation to missense variants. Individual FIXag and Alpha HL values and the overall relation ($r^2$=0.386; Pearson r=0.622) are reported in Figure 4A.

FIXag levels (Table 2, first line) in patients (n=21) with missense mutations were higher than in patients (n=8) with null (nonsense, splicing and deletion) mutations (p=0.049). Patients with mutations at the activation sites (n=7) showed FIXag levels higher than those with null mutations (p=0.002) and than those patients (n=12) with other missense mutations ("no activation sites", n=14; p=0.001).

The distribution of PK parameters according to the 2CP model was compared in patients with different mutation types (Table 2). Whereas differences between missense and null mutations were not detectable, missense changes at the activation site were associated with significantly lower K 1-2, K 2-1 and CLD2 values, and longer Alpha HL, Beta HL and MRT than those observed in patients with null mutations, as well as in patients with other mutations (n=22, Table 2, last column).

PK parameter values for Alpha HL, Beta HL and MRT in HB patients classified by mutation types are reported in Figure 4B. The distribution of PK values indicated high variability, either within patients with identical *F9* mutations (p.R191C, p.R226Q, p.G236D; Figure 4B) or within a single group of mutations. This observation is exemplified by the Alpha HL values of the p.R379X

among null mutations, and by the p.N138H and p.A37D among missense changes not located at activation sites (Figure 4B).

***Predictors of Alpha HL, Beta HL and MRT: FIX antigen levels and F9 genotypes***

The regression analysis of predictors of Alpha HL, Beta HL and MRT was conducted including the FIXag levels and the *F9* mutation types, grouped either in missense and null, or activation site variants and other mutations (Table 3). In the model including missense *vs* null mutations, FIXag values were independent predictors of Alpha HL (β coefficient 0.492, p=0.011), and as a trend of Beta HL (β coefficient 0.374, p=0.064). In the model including activation site variants *vs* other mutations, *F9* genotypes were independent predictors of Alpha HL and MRT, and particularly of Beta HL (β coefficient 0.699, p=0.004).

## Discussion

Our data provide experimental evidence, based on *in vivo* and recombinant studies, to interpret the HB mutational patterns and phenotypes associated to the recurrent substitutions at the FIX activation sites.

Substitutions at R191 and R226 differentially affected residual antigen levels, with normal or increased protein levels associated to the R226 variants. High antigen values may be also associated to other catalytically inert variants as well as to missense mutations affecting the V227 and V228 positions (Supporting Figure 2). Whereas the natural R226 substitutions do not permit the formation of a catalytically competent FIXa and cause severe HB, the benign R226K substitution, designed to mimic the positively charged WT arginine residue, permitted both efficient secretion and activation/activity, and accordingly is not comprised among HB mutations. Differently, most substitutions (L/A/G) affecting the R228 position cause moderate/mild HB (Supporting Figure 2).

Multiple R191 variants are characterized by normal or modestly reduced antigen levels, and by a positive relation between activity and activity/antigen ratio. These observations suggest that the detrimental effects of substitutions at R191 on folding/secretion/scavenging processes [38,40,58], which all contribute to decrease antigen levels, decrease also activation and/or catalytic activity of the FIX R191 variants in a roughly proportional manner. In turn, these features shape the plasma and bleeding phenotypes of patients, and produce the graded moderate to mild HB severity of the

R191C>L>P>H substitutions. The R191H change results in the mildest and most frequent form (>100 HB patients), based on its normal FIXag levels and modest activity/antigen ratio. On the other hand, the benign R191Q/W/K changes, which predict FIX activity above the levels defining mild HB [59], are not reported neither in the HB nor in exome sequencing databases.

Overall, the recombinant experiments i) assist the interpretation of the wide and graded differences in plasma FIX activity and antigen levels observed in the numerous patients affected by mutations at the R191 position, and of the degree of the associated disease severity, and ii) may predict the absence in the HB mutation database of several benign substitutions at the R191/R226 positions. A limitation of the recombinant approach is that antigen levels *in vitro* might not parallel those *in vivo*, caused by variable transfection efficiency or limited cellular capacity for post-translational modifications. However, each protein variant was expressed in six independent replicates in a cellular system widely used for its efficiency. Noticeably, the recombinant approach permits to detect FIX variants that are secreted, and thus could bind vascular and extravascular receptors, but might have increased clearance and thus show lower FIX antigen levels in plasma (i.e R191L). Further, the recombinant expression of FIX variants corresponding to HB causative mutations permitted us to refine the 2CP model PK analysis.

Previous observations in HB patients led to hypothesize that the FIX $CRM^+$ status may favourably influence [33,46] the PK of infused FIX or that dysfunctional endogenous FIX would impair prophylaxis in HB mouse models [35]. With potentially discrepant information as background, we investigated in HB patients the hypothesis that dysfunctional endogenous FIX, which circulates in variable amounts, might positively or negatively modulate the distribution of infused FIX. Prompted by the presence in our HB cohort of several patients affected by mutations at the activation sites causing retention of the activation peptide, previously reported to act as a plasma retention signal [31,32], we also explored whether the variably abundant antigen levels in these patients could further modulate the persistence of infused FIX in plasma. Worth noting, our study is the first report on the relation between FIX PK parameters and specific *F9* mutations and well-defined mutation groups.

The consistent correlation of primary and secondary PK parameters with dysfunctional endogenous FIX levels in HB patients supported the study of patients grouped by mutation types, which in turn dictate the concentration and features of dysfunctional molecules. Although FIXag levels were higher in patients with missense mutations than in those with null ones, their mean PK parameter values did not significantly differ, which may be explained by very low or null antigen

levels associated to several missense changes (Figure 4A). The wide variability in antigen levels for the non-activation site missense changes could contribute to obscure mutation group differences in PK parameters. Coherently, the grouping of missense and null mutations in linear regression analysis indicated FIXag levels, but not *F9* genotypes, as significant predictors of PK parameters, particularly of Alpha HL. Differently, the activation site variants were associated with longer Alpha HL, Beta HL and MRT than null mutations. The MRT, a parameter that may influence patients' treatment, was 80% longer (79.39 hrs, 95% CI 44.3-114.5) than in patients with null mutations (44.2 hrs, 95% CI, 30.8-57.5, p=0.031) or with other missense changes (45.9 hrs, 95% CI 34.9-56.9, p=0.015). The regression analysis suggested activation site variants as independent predictors of several PK parameters, and particularly of Beta HL (β coefficient 0.699, p=0.004), which may be prolonged by the increasing proportion over time of the abundant endogenous FIX variants as compared to infused FIX. The very high endogenous/infused FIX ratio reached in the Beta elimination phase could magnify the effects of activation site variants, and the increasing competition for receptors may decrease both the forward and backward flow between central plasma and extravascular compartments [38–40] of infused FIX (Supporting Figure 5), thus prolonging its Alpha and Beta half-lives. The contribution to FIX PK of missense mutations, potentially produced also by other mutation types [60], may consist of quantitative components such as the residual FIXag levels, and in addition, for the R226 and R191 substitutions, of qualitative components such as the retention of the activation peptide cleaved at only one site. It has been demonstrated that removal of the activation peptide from zymogen FIX results in exposure of a binding site for low-density lipoprotein receptor-related protein, which favors FIXa catabolism [58]. Whereas mutations at position R191 may modulate PK outcomes in the presence of intermediate FIXag levels, those at position R226 may influence PK parameters in the presence of high levels of the secreted FIX variants. It is tentative to speculate that in female carriers both levels of the FIX R226W variant and FIX WT would be increased, as suggested by the increased FIXag levels and the half-reduced activity/antigen ratio.

Although based on a limited number of patients and a small number of related female carriers, data suggest that mutations at the activation sites have pleiotropic effects in plasma by dictating in patients i) the residual FIX activity (only variants at R191), ii) the variably abundant FIXag, and by potentially modulating iii) the PK features of the infused FIX and, in female carriers, iv) the variant and wild-type FIX levels. These *F9* genetic components, together with others producing

large differences among patients affected by identical *F9* mutations, are expected to contribute the wide and elusive variability of PK outcomes in HB patients.

Our data support, in patients characterized for *F9* mutations, the hypothesis [33] that the FIX $CRM^+$ status might partially improve PK of FIX infused in HB patients, and that the activation peptide, retained in the activation site variants, may contribute to prolong the Beta phase of FIX distribution in plasma, as inferred in mouse models [31,32]. The relative impacts of FIXag levels versus retained activation peptide on FIX PK were indirectly disentangled by linear regression analysis that suggested activation site mutations as independent predictors, which warrants further studies. These *F9*-related features would permit to better estimate the improved pharmacokinetic profile of currently used and novel long-acting FIX molecules [61,62].

## Conclusions

*In vivo* and recombinant results suggest that combination of FIXag levels and type of dysfunctional molecules predicted in plasma by *F9* mutations, particularly those at the activation sites, may exert pleiotropic effects on i) the endogenous FIX and thus HB patients' phenotypes, as well as ii) infused FIX half-lives and thus MRT in substitutive treatment.

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## Authorship contributions

A.B. created recombinant vectors and performed expression studies on FIX recombinant variants; M.M. performed PK analysis; B.L. designed and performed statistical analysis of PK analysis data; A.B. and B.L. performed statistical analysis of recombinant expression and of *F9* genotype-PK parameters association; D.B., P.R., L.B., M.L.S., P.G., D.C., A.C.M., M.S.N. and G.C. characterized and recruited patients, collected plasma samples according to the PK study design and performed clotting assays; E.B., provided assistance on the pharmacovigilance study; M.P. and G.C. critically revised the manuscript; A.B., M.M. and F.B. conceived the study and designed

research; A.B., M.M., B.L. and F.B. analysed and interpreted data and wrote the manuscript. All authors revised and approved the final version of the manuscript.

**Disclosure of Conflicts of Interest**

A.B. reports grants and personal fees from Pfizer, grants from Bayer, grants and non-financial support from Grifols, outside the submitted work. M.M. reports personal fees from Kedrion, grants from Pfizer, personal fees from Novo Nordisk, personal fees from SOBI, personal fees from Bayer, personal fees from Bioverativ, personal fees from Octapharma, personal fees from CSL Behring, outside the submitted work. A.C.M. has acted as a paid consultant to Bayer, CSL, Kedrion, Novo Nordisk, Pfizer, Roche, Shire, and Sobi, and as a paid invited speaker for Bayer, CSL, Novo Nordisk, Shire, and Sobi, outside the submitted work. G.C. reports personal fees from Roche, personal fees from Bayer, personal fees from Shire, grants and personal fees from CSL Behring, grants and personal fees from Sobi, personal fees from Uniqure, grants from Pfizer, personal fees from Kedrion, personal fees from Novo Nordisk, personal fees from Werfen, outside the submitted work. M.P. received grants from Novo Nordisk and Pfizer and personal fees from Pfizer, outside the submitted work. F.B. reports grants from Bayer, outside the submitted work, and the financial support of GePKHIS by an unconditioned Investigator Initiated Research grant from Pfizer. The remaining authors state that they have no conflict of interest to declare.

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**Table 1.** Correlation between FIX antigen and PK parameters of rFIX infused in HB patients.

| | **Mean (95% CI)** | | |
|---|---|---|---|
| *FIX antigen (%)* | *43.70 (15.91-71.49)* | | |
| | | **Pearson's correlation** | |
| **Primary PK parameters** | | **r** | **p** |
| K 1-2 (1/hrs) | 0.14 (0.06-0.23) | -0.461 | **0.012** |
| K 2-1 (1/hrs) | 0.26 (0.13-0.40) | -0.469 | **0.010** |
| V1 (dL/kg) | 0.93 (0.78-1.08) | 0.327 | 0.084 |
| **Secondary PK parameters** | | | |
| Alpha HL* (hrs) | 5.11 (3.61-6.61) | 0.516 | **0.004** |
| AUC (U.h/dL) | 1813 (1539-2086) | -0.107 | 0.580 |
| Beta HL (hrs) | 43.24 (32.93-53.55) | 0.397 | **0.033** |
| CL (dL/hrs/kg) | 0.031 (0.026-0.037) | 0.024 | 0.900 |
| CLD2 (dL/hrs/kg) | 0.10 (0.04-0.16) | -0.397 | **0.033** |
| Cmax (IU/dL) | 58.71 (48.73-68.69) | -0.407 | **0.029** |
| MRT (hrs) | 53.50 (42.99-64.01) | 0.311 | 0.101 |
| V2 (dL/kg) | 0.59 (0.39-0.79) | 0.273 | 0.151 |
| Vss (dL/kg) | 1.52 (1.22-1.82) | 0.340 | 0.072 |

r, Pearson's coefficient. p, Pearson analysis.

K 1-2, transfer rate from central (1) to peripheral (2) compartment; K 2-1, transfer rate from peripheral (2) to central (1) compartment; V1, Volume of central compartment. Alpha HL,

alfa distribution half-life; AUC, area under the curve; Beta HL, beta elimination half-life; CL, clearance; CLD2, inter-compartment clearance; Cmax, at zero time extrapolated FVIII concentration; MRT, mean residence time; V2, volume of peripheral compartment; Vss, volume of distribution at steady state. *, normally distributed variable.

**Table 2.** FIX antigen and 2CP PK parameters in HB patients grouped for F9 mutation types.

| **Mutation types** | **Missense (all)** | | **Null** | | **Missense (activation sites)** | | **Missense (no activation sites)** | |
|---|---|---|---|---|---|---|---|---|
| **Patient number** | 21 | | 8 | | 7 | | 14 | |
| | **Mean (95% CI)** | p | **Mean (95% CI)** | p | **Mean (95% CI)** | p | **Mean (95% CI)** | p# |
| ***FIX antigen (%)*** | *60.05 (23.48-96.62)* | ***0.049*** | *0.79 (-0.94-2.52)* | ***0.002*** | *163.3 (43.97-228.5)* | ***0.001*** | *21.95 (7.30-36.60)* | ***<0.0001*** |
| **PK parameters** | | | | | | | | |
| K 1-2 (1/hrs) | 0.17 (0.05-0.29) | 0.825 | 0.09 (0.01-0.16) | **0.004** | 0.03 (0.02-0.04) | **0.028** | 0.23 (0.06-0.41) | **0.016** |
| K 2-1 (1/hrs) | 0.30 (0.11-0.48) | 0.664 | 0.17 (0.09-0.26) | **0.001** | 0.04 (0.01-0.07) | **0.002** | 0.42 (0.16-0.68) | **0.001** |
| V1 (dL/kg) | 0.91 (0.78-1.04) | 0.981 | 0.99 (0.46-1.52) | 0.320 | 1.11 (0.85-1.38) | **0.018** | 0.81 (0.68-0.94) | 0.071 |
| Alpha HL* (hrs) | 5.70 (3.70-7.70) | 0.196 | 3.56 (2.07-5.06) | **0.001** | 9.53 (6.32-12.74) | **0.002** | 3.79 (1.76-5.81) | **<0.0001** |
| AUC (U.h/dL) | 1857 (1526-2189) | 0.633 | 1696 (1089-2303) | 0.966 | 1757 (988-2527) | 0.576 | 1907 (1503-2311) | 0.717 |
| Beta HL (hrs) | 47.04 (33.18-60.90) | 0.299 | 33.28 (23.73-42.82) | **0.009** | 72.97 (38.15-107.8) | **0.002** | 34.07 (25.33-42.82) | **<0.0001** |
| CL (dL/hrs/kg) | 0.030 (0.024-0.035) | 0.438 | 0.036 (0.019-0.053) | 0.701 | 0.032 (0.017-0.047) | 0.778 | 0.029 (0.023-0.035) | 0.978 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CLD2 (dL/hrs/kg) | 0.12 (0.04-0.20) | 0.836 | 0.07 (0.04-0.10) | **0.008** | 0.03 (0.02-0.04) | 0.068 | 0.16 (0.04-0.28) | **0.042** |
| Cmax (IU/dL) | 58.35 (45.84-70.85) | 0.866 | 59.66 (39.29-80.05) | 0.092 | 42.60 (30.41-54.73) | **0.023** | 66.22 (49.09-83.35) | **0.021** |
| MRT (hrs) | 57.06 (43.17-70.95) | 0.297 | 44.16 (30.84-57.49) | **0.031** | 79.39 (44.26-114.51) | **0.015** | 45.90 (34.87-56.93) | **0.005** |
| V2 (dL/kg) | 0.64 (0.37-0.92) | 0.674 | 0.45 (0.31-0.59) | **0.050** | 1.14 (0.35-1.92) | **0.006** | 0.39 (0.28-0.50) | **0.003** |
| Vss (dL/kg) | 1.55 (1.17-1.93) | 0.756 | 1.44 (0.81-2.07) | 0.087 | 2.25 (1.25-3.25) | **0.004** | 1.20 (1.00-1.40) | **0.004** |

PK parameters in *F9* mutation type groups were compared by *t*-test; the table includes four columns reporting *p* values, three of them flanked by the parameter values subjected to statistical evaluation. For p#, the comparison is made between missense mutations in the activation sites (n=7) and other mutations (the sum of null, missense no activation site, n=22, parameter values not reported in the table). *, normally distributed variable. PK parameters definition as in Table 1.

**Table 3. Linear regression models for predictors of the PK parameter variability**

| | ß-coefficient | p | Predictors |
|---|---|---|---|
| Alpha HL (hrs) | 0.492 | **0.011** | FIX antigen |
| | -0.066 | 0.719 | *F9* genotypes - Missense *vs* null mutations |
| | 0.102 | 0.642 | FIX antigen |
| | 0.569 | **0.015** | *F9* genotypes - Activation sites *vs* other mutations |
| Beta HL (hrs) | 0.374 | 0.064 | FIX antigen |
| | -0.062 | 0.751 | *F9* genotypes - Missense *vs* null mutations |
| | -0.111 | 0.623 | FIX antigen |
| | 0.699 | **0.004** | *F9* genotypes - Activation sites *vs* other mutations |
| MRT (hrs) | 0.274 | 0.182 | FIX antigen |
| | -0.099 | 0.623 | *F9* genotypes - Missense *vs* null mutations |
| | -0.121 | 0.625 | FIX antigen |
| | 0.594 | **0.023** | *F9* genotypes - Activation sites *vs* other mutations |

Definition of PK parameters and mutation types as in Tables 1 and 2, respectively. The FIX antigen and PK parameter values in *F9* mutation groups are reported in Table 2. Missense mutations (patients n= 21) *vs* null mutations (patients n= 8); mutations in the activation sites (patients n= 7) *vs* other mutations (patients n= 22). ß-coefficients, standardized regression coefficients.

**FIGURE LEGENDS**

**Figure 1. Natural and designed amino acid substitutions at FIX activation sites.**

A) Schematic representation of human coagulation FIX showing the pre- and pro-peptides (PP), and light and heavy chains (LC and HC, respectively). Haemophilia B (natural) variants affecting the R191 and R226 sites are reported above the FIX scheme (n, patient number in the EAHAD FIX variant Database https://f9-db.eahad.org/) [9], with the prevalent HB phenotypes associated with mutations on top. Blue and red variants, patients also available in the GePKHIS study. Designed recombinant variants are indicated below the FIX scheme. Colour code: blue, red and black, natural variants; orange, designed variants. The alignment and conservation of residues in the two activation sites (red arrows) is also shown.

B) FIX variants available in the GePKHIS study. Missense (with amino acid changes not involving activation sites) and null (nonsense, splicing and deletion) variants are reported above and below the FIX scheme, respectively.

**Figure 2. Characterization of FIX variants at the activation sites.**

A) Activity (left panel) and antigen (right panel) levels of the 191 and 226 variants estimated in GePKHIS patients, reported in the international EAHAD FIX variant database (https://f9-db.eahad.org/) [9] or upon recombinant expression. Reference for plasma or recombinant values were PNP or rFIX-WT, as appropriate.

B) Comparative expression of equivalent amino acid substitutions at 191 and 226 positions, after transient expression in HEK293 cells. The S411P variant (chymotrypsin numbering S195) was expressed as an additional control for inactive FIX with high antigen values.

C) Left panel. Western blotting analysis of the activation profile of natural (red) and designed (orange) recombinant 226 variants in the presence (+) or absence (-) of FXIa. Protein forms, resulting from FIX activation, as well as relative molecular weights (kDa), are indicated on left and right of the blot, respectively. Serial dilutions of rFIX-WT were loaded as control and reference. rFIX variants with similar FIX antigen levels (226P/L/A/D/E/K, range 70%-100%) were tested after equal dilution (1:10) in PBS buffer, while those with antigen levels exceeding wild-type rFIX (226Q/W), prior to be diluted in PBS buffer, were first normalized to ~100% by dilution in medium from untransfected cells.

Zym, zymogen FIX; FXIa, activated factor XI.

Right panel. FIX antigen and activity levels, as well as activity/antigen ratio, of the designed 226K variant.

D) Comparative analysis of activity and antigen levels of FIX variants bearing natural missense changes found in HB patients (p, red squares; left panel) and after recombinant expression (r, blue circles; right panel).

E) Relation between mean activity levels and activity/antigen ratio of FIX variants bearing natural (blue circles) or designed (orange circles) amino acid substitutions.

Inset, Analysis of the relation with the 191K variant included.

Results, indicated as % of reference, are reported as mean±SEM (panels A, patients bar, and E) or mean±SD of n=6 replicates (panels A, recombinant bar, B and C).

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ ****, $p<0.0001$.

**Figure 3. Analysis of female carriers of the p.R226W variant.**

Plasma FIX antigen and activity levels, as well as activity/antigen ratio, estimated in female carriers (n=3) of the p.R226W variant. Results are reported as mean±SEM. *, $p<0.05$.

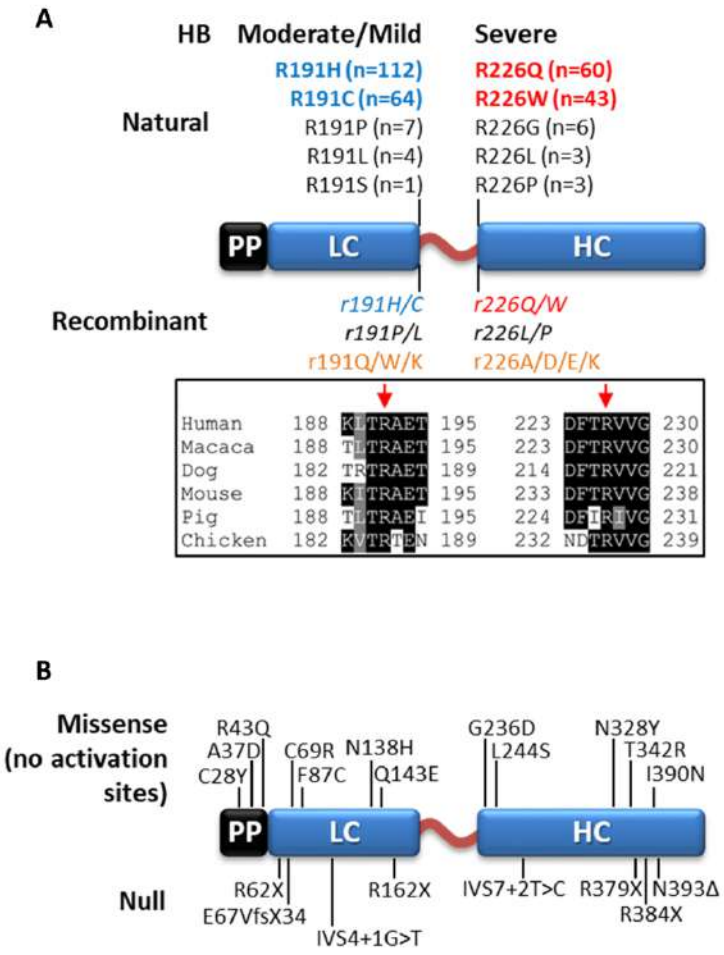
**Figure 4. FIX PK parameters, FIX antigen and *F9* genotypes.**

A) Alpha HL parameter, *F9* genotypes and FIX antigen levels. Relation between mean antigen levels of expressed FIX missense variants of the HB cohort and Alpha HL parameter. Alpha HL of the R191C (n=3 patients), R226Q (n=3) and G236D (n=2) variants is reported as mean of PK values from each patient.

B) Alpha HL, Beta HL and MRT and *F9* genotypes. Comparative plot of selected PK parameters (Alpha HL, upper panel; Beta HL, middle panel; MRT, lower panel) among FIX variants categorized by three mutation groups: null (nonsense, splicing, deletion), missense variants affecting (act. sites) or not (no act. sites) the FIX R191 or R226 activation sites.

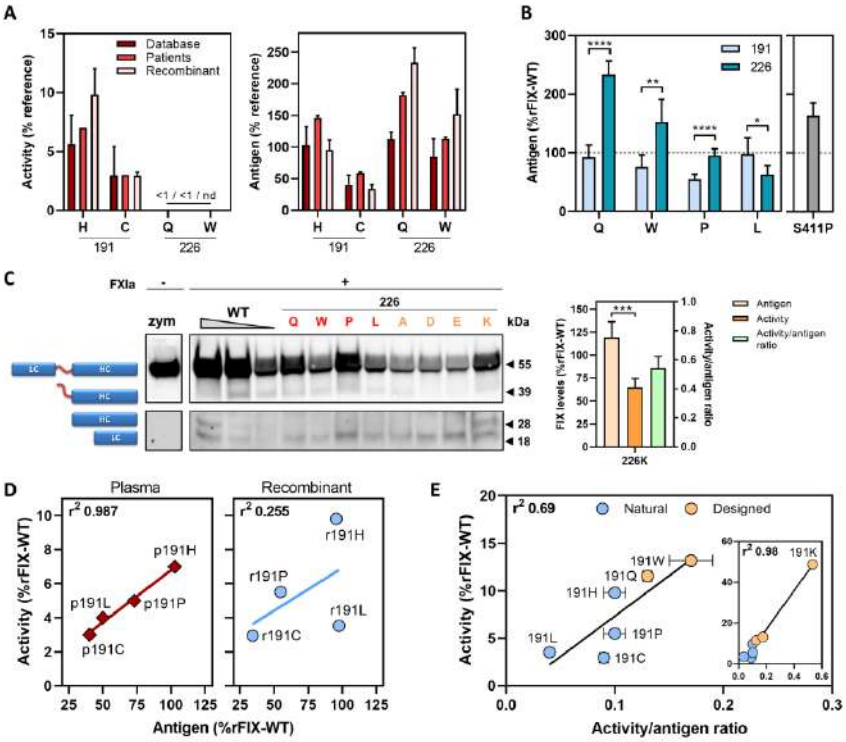
Circles, activation site variants (blue, R191C; red, R226Q/W); grey squares, nonsense variants; grey triangles, no activation site variants.

**Figure 1**




jth_15552_f1.tif

Figure 2




jth_15552_f2.tif

**Figure 3**

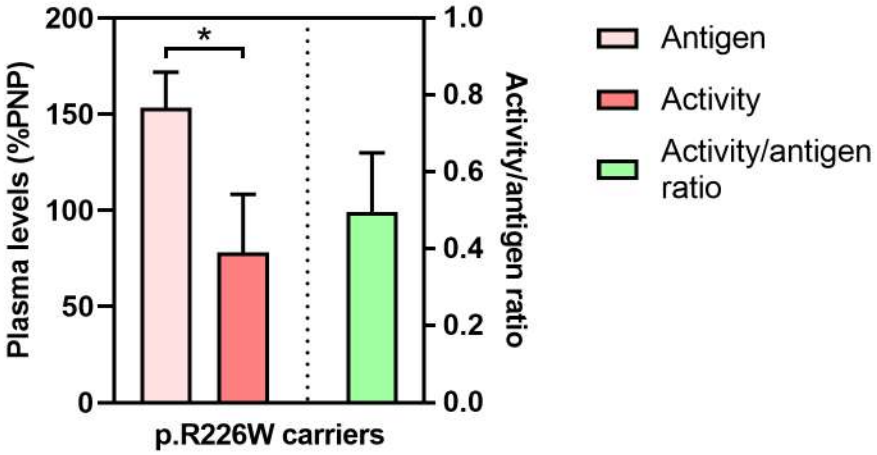


**Figure 4**