Pharmacokinetics of extended half-life albumin-fused factor IX and heterogeneous *F9* variants in hemophilia B: a retrospective cohort study

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Ethical approval and informed consent: patients with severe HB were enrolled in a global phase 3 study (www.clinicaltrials.gov, #NCT0101496274). A small group of patients switching from SHL to EHL concentrate, and thus under-going PK analysis in the frame of Italian Association of Hemophilia Centres (AICE) initiatives (Genotype-Phenotype PK Study, GePKHIS, EudraCT ID2017–003902–42) were also investigated. The study was performed in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained from all subjects involved in the study.

Availability of data and material: all relevant data are included in the manuscript. Further original data will be made available by contacting the corresponding author within the regulations of the ethical approval.

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ABSTRACT

No information is available about the influence exerted by *F9* variants on extended half-life (EHL)-recombinant factor IX (rFIX) pharmacokinetics (PK). Adult patients with severe HB (n=41), infused with EHL albumin-fused-rIX-FP, were investigated for key parameters, obtained by Non-Compartmental Analysis (NCA), and for *F9* variants, grouped by non-null (n=27) and null (n=14) lesions. Distribution of PK parameters did not differ between genotype groups. However, clearance in the first tertile, including favorable PK profiles, was slower (p=0.003) in patients with null (mean 0.52 mL/h/kg) than in non-null (mean 0.62 mL/h/kg) variants, and non-null genotypes were more frequent in the third tertile (p=0.046). Missense variant bioinformatics analyses predicted more severe disease features in the third than in the first tertile. These exploratory results suggest i) a moderate role of *F9* variant type in inter-individual EHL-rFIX clearance variability and ii) differences in *F9* genotype-PK NCA parameter association between EHL- and standard half-life-rFIX products.

Key words: hemophilia B; *F9* variants; extended half-life rFIX; pharmacokinetics; clearance.

Introduction

Pharmacokinetics (PK) studies of replacement therapy may be instrumental to improve and personalize management of hemophilias, enabling healthcare providers to tailor prophylactic treatments able to decrease the risk of bleeding or prevent unnecessarily frequent infusions.[1]

In Hemophilia B (HB), PK of standard half-life (SHL) and extended half-life (EHL) factor IX (FIX) concentrates, mostly recombinant proteins (rFIX), indicated high inter-individual variability,[2] which supports the presence of both genetic and acquired components. Whereas the influence of age and body weight have been clearly established,[3] the presence of cross-reactive material (CRM+), namely the circulating FIX antigen (FIX:Ag) with reduced or null activity, has been hypothesized to modulate the distribution of FIX products infused in HB patients, with FIX CRM+ status favorably influencing the PK of infused FIX.[4,5] Differently from HB patients, in HB mouse CRM+ model expressing the missense variant Arg333Gln, the presence of endogenous dysfunctional FIX may deleteriously affect the hemostatic response to prophylactic therapy.[6] More recently, the decay of infused SHL recombinant FIX (SHL-rFIX) concentrates was investigated in HB patients character-

ized for *F9* genotypes.[7] The distribution of two compartment analysis (2CP) PK parameters indicated high inter-individual variability, even in patients with identical *F9* variants. Beta elimination HL was positively correlated with endogenous FIX:Ag levels, and with missense changes at the FIX activation sites (p.Arg191, p.Arg226), which predict the presence of endogenous FIX CRM+ molecules retaining the activation peptide. Conversely, several PK parameters suggested that null mutations predicted worse PK profile.

No information is available about the influence exerted by *F9* variants on PK features of EHL FIX concentrates, which benefit from binding of the albumin-fused FIX to the recycling neonatal Fc receptor (FcRn).[2,8-10]

We investigated the association of *F9* genotypes with PK profile of rFIX-human albumin fusion protein (rIX-FP).

Patients and Methods

Inclusion criteria

For this retrospective study the inclusion characteristics were as follows: i) male patients previously treated with a stable dose of FIX concentrate, ii) with severe HB (FIX activity, FIX:C <2%), iii) age ≥18 years, iv) undergoing PK analysis in a global phase 3 study (n=35) evaluating the pharmacokinetics, efficacy, and safety of the EHL-rFIX product,[2] and v) a small group of patients (n=12) switching to the same EHL-rFIX product, and thus undergoing PK analysis; vi) all the patients were in a steady state bleeding free condition for at least 1 month.

A different cohort of HB adult patients (GePKHIS study n=37) with similar inclusion characteristics[7] but infused with SHL recombinant Nonacog alfa FIX, were also investigated.

Exclusion criteria

Patients with i) inhibitor history or detectable inhibitor to FIX, titrated by the Bethesda method according to the Nijmegen modification, titer ≥0.6 Bethesda units; ii) known hypersensitivity to FIX or hamster protein, iii) on immunomodulating treatment; iv) having CD4 cell count <200/mm^3; v) levels of serum aspartate/alanine aminotransferase >5 times the upper limit of normal; vi) absence of bleeding in the last two weeks, as inferred from lack of pain or swelling of the joints, or absence of clinically evident hemarthrosis.

PK analysis

Patients were infused with 50 IU/kg of rIX-FP and ≥6 data points were analyzed measuring rIX-FP activity with a validated 1-stage clotting method.[2] Three main PK parameters, Area Under the Curve (AUC) from the time of dosing extrapolated to infinity, based on the last observed FIX concentration (AUC 0-inf), total body clearance normalized to body weight (CL) and terminal half-life (t 1/2), were obtained by standard Non Compartmental Analysis (NCA) using Phoenix WinNonlin software version 7.0 (Pharsight).[5] NCA parameters, after infusion with SHL recombinant Nonacog alfa FIX (40±5 IU/kg), were also evaluated in the GePKHIS study.[7]

Genetic and bioinformatics studies

F9 mutations determined by Sanger gene sequencing for genetic diagnosis were collected. Patients with genetic reports not permitting unequivocal *F9* variant classification and mapping were excluded. For the NCA PK phenotype-*F9* genotype association studies, overall 41 patients were eligible for the rIX-FP and 37 for the Nonacog alfa FIX, respectively. *F9* variants were classified as null (premature termination codons=PTC, splicing at +1 or +2 positions, deletions and frame-shift) and non-null (missense, splicing at -3 or +4 positions, and synonymous).

Missense mutations were analyzed by several bioinformatics tools, including supervised machine learning-based classifier,[11-14] built to prioritize missense variants with likely involvement in human disease.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0. Correlations between PK parameters were assessed using Pearson correlation test. Derived *post-hoc* tertiles were defined for further analysis. Observed and expected patients' number in CL tertiles were compared by Fisher's Exact Test. *F9* mutation type-related differences in PK parameters were analyzed by *t*-test or Mann-Whitney test for small number of samples (n≤10). In addition, the Benjamini-Hochberg procedure was used in tertile analysis to minimize the influence of type II errors and adjust significant p-values for false discovery rate (FDR). Data are reported as mean and 95% Confidence Interval (CI).

Results and Discussion

Binding of FIX to collagen IV increases the concentration of endogenous or infused FIX in the extravascular space (at the steady state after an infusion, FIX 40%),[15–17] in turn modulating FIX PK. Differently from Hemophilia A patients, in whom FVIII PK studies failed to detect *F8* variation type as a significant contributor, PK studies in HB patients suggested that *F9* variants type may influence SHL-rFIX PK.[7]

The main aim of the study was to interpret by *F9* genetic components the inter-patient variability in EHL-rFIX NCA PK.

PK parameter correlation

We analyzed three key parameters, AUC 0-inf, CL and t 1/2, which may help to tailor substitutive treatment in HB patients after infusion with the EHL-rIX-FP (Table 1). We first investigated in all patients (n=41) the correlation between parameters. Whereas AUC 0-inf and CL were highly correlated (Pearson r -0.88, p<0.0001), correlation between t 1/2 and AUC 0-inf (r 0.52, p=0.0005), and that between t 1/2 and CL (r -0.59, p<0.0001), were moderate.

F9 genotype-PK parameter association

To investigate association between PK parameters and *F9* genotypes (Table 1, Figure 1), the variants were classified as null and non-null. Missense variants composed the non-null group (25/27, 93%) and were the most represented gene alterations in the whole population (25/41, 61%). The p.Gln237Gln synonymous change (Figure 1, orange dot) was classified in the

non-null group, based on the presence in patients plasma of residual FIX:Ag levels (2-3%), association with moderate phenotype and minigene expression studies.[18]

NCA analysis of CL, AUC 0-inf and t 1/2 did not reveal significant differences for the EHL-rFIX concentrates in relation to the genotype groups (Table 1, Figure 1).

Table 1. Non-Compartmental Analysis of pharmacokinetics parameters in hemophilia B patients grouped by null and non-null *F9* variants.

Pharmacokinetics parameters	All patients Mean (95% CI)	*F9* variants Null n	Null Mean (95% CI)	Non-null n	Non-null Mean (95% CI)	*p*
CL (mL/h/kg)	0.83 (0.75-0.91)	14	0.75 (0.62-0.87)	27	0.87 (0.77-0.98)	0.124
First tertile	0.58 (0.54-0.62)	*5*	0.52 (0.46-0.58)	*9*	0.62 (0.58-0.65)	**0.003 (0.027)**
Second tertile	0.80 (0.74-0.85)	*7*	0.80 (0.72-0.87)	***6***	0.80 (0.70-0.89)	0.974
Third tertile	1.11 (1.01-1.22)	***2***	1.14 (0.44-1.83)	***12***	1.11 (0.98-1.23)	0.440
AUC 0-∞ (h*IU/dL)/10^3	6.53 (5.83-7.23)	14	7.01 (5.58-8.45)	*27*	6.28 (5.46-7.10)	0.321
First tertile	4.27 (3.75-4.80)	*3*	3.80 (0.06-7.55)	*11*	4.40 (3.91-4.90)	0.676
Second tertile	6.24 (5.81-6.67)	*6*	6.37 (5.65-7.08)	*7*	6.13 (5.41-6.86)	0.713
Third tertile	9.06 (8.37-9.74)	*5*	9.72 (8.54-10.9)	*9*	8.69 (7.77-9.60)	0.083
t ½ (h)	106 (98-114)	14	109 (95-123)	27	104 (94-115)	0.567
First tertile	78 (72-85)	*4*	79 (65-93)	*10*	78 (69-87)	0.767
Second tertile	106 (102-111)	*4*	107 (89-126)	*9*	106 (101-111)	0.822
Third tertile	134 (124-143)	*6*	131 (123-140)	*8*	135 (118-153)	0.741

AUC 0-∞, area under curve from time 0 to infinity; CL, clearance; t ½, terminal half-life; Null, premature termination codons, frameshift mutations and deletions; non-null, missense changes, -3 splice site nucleotide change and synonymous codon; numbers of patients in tertiles are indicated in *italics;* number of patients in ***bold italics***: chi-square (2X2) for *F9* variant type distribution between CL tertiles (p=0.046); the CL in the first tertile was slower in patients carrying *F9* null variants than in non-null sub-group (p=0.003/adjusted for FDR p=0.027, **bold**); *F9* mutation type-related differences in pharmacokinetics parameters were analyzed by *t*-test or Mann-Whitney test for small number of samples (n≤10).

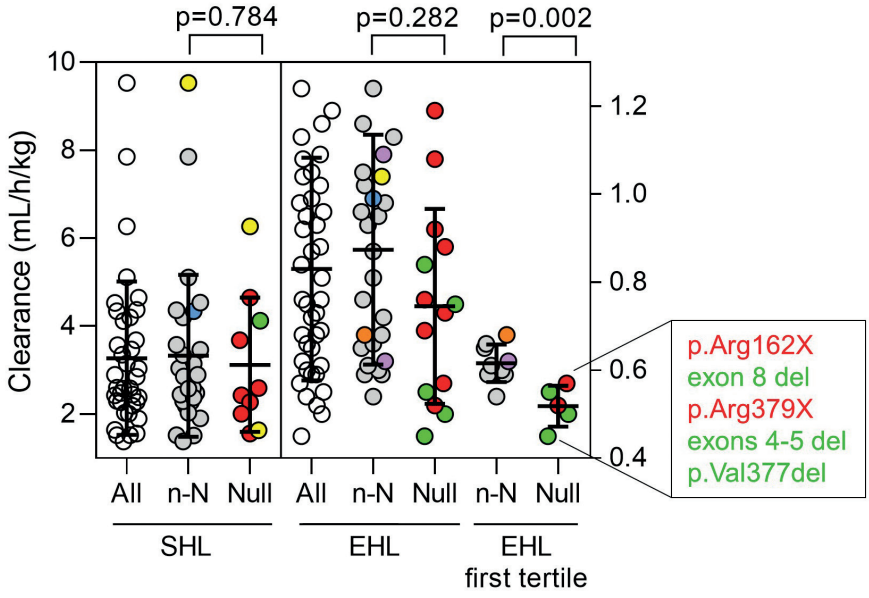

Figure 1. Scatter dot plot of CL values in all HB patients (All), or grouped in non-null (n-N) and null (Null) *F9* variants. SHL, standard half-life FIX concentrate; EHL, elongated half-life FIX concentrate. Mean values and standard deviation are indicated for each *F9* mutation group. SHL, null variants (n=10, 27%; deletion, n=1; PTC, n=7; splicing site changes at +1 and +2 position, n=2); non-null variants (n=27, 73%; missense, n=26; splicing site changes at +4 position, n=1. EHL, null variants (n=14, 34%; deletion, n=5; PTC, n=9); non-null variants (n=27, 66%; missense, n=25; splicing site changes, at -3 position, n=1; synonymous change, n=1). See main text for the Gln237Gln synonymous change (orange dot) classification in the non-null group. Gray dots, missense variants; pink dots, p.Phe87Cys; blue dots, p.Arg226Trp. Green dots, deletion; red dots, PTC; yellow dots, splice site. Deletions and PTC in the first EHL tertile are detailed on the right. The highest CL value (1.67 mL/h/kg), corresponding to the p.Cys108Trp missense mutation, is not shown in the EHL plots. *F9* mutation type-related differences in CL were analyzed by *t*-test or Mann-Whitney test, as appropriate.

Tertile analysis of *F9* genotype- EHL-rFIX CL association

As exploratory investigation aimed at further evaluating the most favorable/unfavorable PK profiles in patients treated with EHL-rFIX concentrates, we performed post-hoc tertile analysis of variables and investigated association with genotype groups (Table 1). The CL in the first tertile, with similar mean and median distributions (n=14, mean 0.58 mL/h/kg, 95% CI 0.54-0.62; median 0.59 mL/h/kg, 95% CI 0.52-0.65) was slower in patients carrying *F9* null variants than in non-null sub-group (p=0.003, adjusted for FDR p=0.027), which was reflected as a trend in higher AUC (p=0.083). These observations highlighted (Figure 1) a small group (n=5) of patients with very similar and favorable PK values, and with *F9* null genotypes (3 deletions and 2 premature termination codons, detailed in Figure 1). Although our observations need to be confirmed in an independent HB cohort, the CL value in the genetically null patients in the first tertile was 1/3 slower than in the whole HB cohort (Table 1), and thus candidate to improve hemostatic efficacy. The explanation of better EHL-rFIX NCA PK profiles in patients that do not present endogenous FIX in their plasma or extravascular compartment, and of hypothetically better EHL-rFIX recycling magnified by binding between albumin and FcRn, requires further investigation. Interestingly, non-null genotypes were more frequently represented in the third CL tertile (n=12, 44%; Table 1). Differences in genotype distribution between the second and third tertiles reached statistical significance (null *vs* non-null, Fisher's Exact Test, p=0.046; Table 1). Accordingly, a substantially biphasic distribution of CL values was observed in the non-null group (Figure 1), and particularly among missense variants.

Bioinformatics analysis of *F9* missense variants

Missense variants in the first CL tertile (n=9) were compared by several bioinformatics tools with those (n=10) in the third CL tertile, including the most unfavorable PK profiles. Missense variants in the third tertile displayed mean scores close to 1 with several tools, which indicates a confident prediction of a disease mutation, and thus severely deficient/dysfunctional endogenous FIX. Differently, the first tertile was characterized by slightly lower scores with wider distribution, leading to trend for difference between tertiles (Provean, p=0.078; Mutation Assessor, p=0.070; Variant Effect Scoring Tool 4 (VEST4), p=0.10). Intra-cluster similarity obtained by combination of scores was represented by the Silhouette score (Figure 2, group A, first tertile; group B, third tertile). An appreciable proportion of missense variants (6/9, 7/10) was found to be clustered by this analysis. Although the low number of variants prevents further classification by FIX domains, it is worth noting that in the group A, the best clustering was found for missense changes in the catalytic domain (p.Glu291Lys, p.Thr332Lys, p.Gly351Val, p.Arg379Pro). The p.Arg226Trp, which abolishes a key FIX activation site, was correctly included among missense changes within cluster B (Figure 2). This missense variant, present in both SHL and EHL rFIX concentrate cohorts (Figure 1, blue dots), was similarly ranked in the respective CL distributions (SHL, 4.20 mL/h/kg; EHL, 0.98 mL/h/kg). The p.Phe87Cys

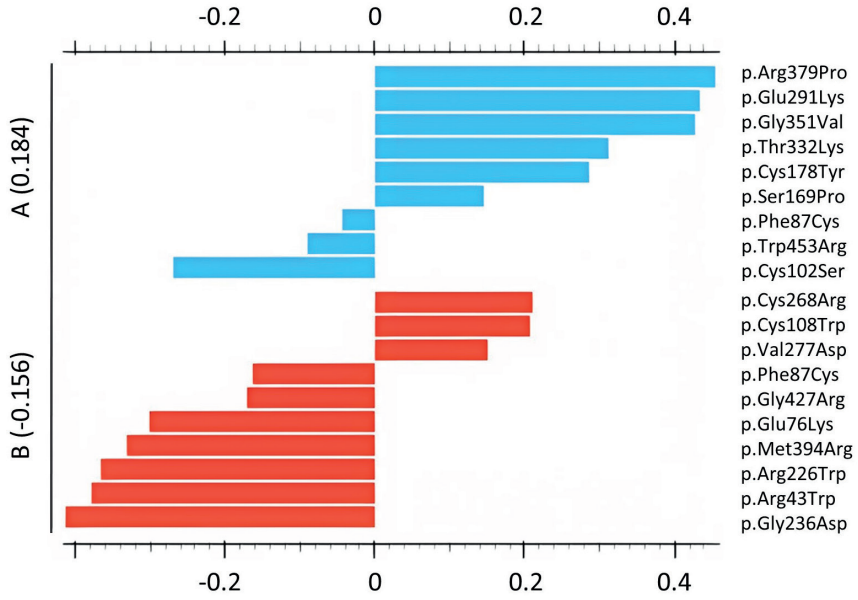

Figure 2. Bioinformatics analysis of *F9* missense variants grouped in the first (A) and third (B) CL tertiles. Blue bars, first tertile; red bars, third tertile. *F9* variants are listed on the right. The p.Phe87Cys was present both in the first and third tertiles. Individual Silhouette scores (range -0.40 to 0.44) are reported together with values of intra-cluster similarity for each group (A and B, left axis). Based on the prediction of several bioinformatics tools, silhouette shows which variants lie well within their cluster (A, p.Arg379Pro, p.Glu219Lys, p.Gly351Val, p.Thr332Lys, p.Cys178Tyr and p.Ser169Pro; B, p.Gly236Asp, p.Arg43Trp, p.Arg226Trp, p.Met394Arg, p.Glu76Lys, p.Gly427Arg and p.Phe87Cys), and which one are clearly misplaced (i.e., p.Cys268Arg, p.Cys108Trp and p.Cys102Ser). The entire clustering is displayed by combining the silhouettes into a single plot, allowing an appreciation of the relative quality of the clusters.

variant, present in two patients with different EHL CL values (0.60 and 1.09 mL/h/kg; Figure 1, pink dots), was well clustered in group B and poorly in group A (Figure 2).

Overall, genetic observations associated with tertile analysis can be considered only as hypothesis-generating.

Comparison of association between *F9* genotype and EHL-/SHL-rFIX PK

For comparison, we report the CL distribution, obtained by NCA, for the EHL-rFIX-FP and a SHL-rFIX product in 37 HB patients[7] (Figure 1, All). Although direct comparison is limited by cohort differences, CL was fourfold faster with the SHL (mean 3.27 mL/h/kg; 95% CI 2.7-3.9) than EHL (mean 0.83 mL/h/kg; 95% CI 0.75-0.91) FIX concentrate, in accordance with previous results.[8,9] The CL distribution in the *F9* genotype groups (Figure 1, Null, n-N) in the two HB cohorts did not reveal significant differences, neither for the EHL- nor for the SHL-rFIX concentrates (Figure 1). To note, genotype differences in tertile distribution were detectable in the EHL- but not in the SHL-PK profiles (Figure 1), which suggests that the *F9* genotype-PK association may differ between SHL- and EHL-rFIX concentrates, favored by differences in key FIX receptors. Whereas the collagen IV receptor would contribute to biodistribution of both concentrates, the neonatal Fc receptor would recognize and extend only the albumin-fused EHL-rFIX. Interestingly, we observed differences in Clearance, a parameter less affected by NCA that does not permit to properly distinguish distribution and terminal phases. The ability of NCA analysis to support the investigation of *F9* genetic components in FIX-PK still represent an open issue.

Main observations and open issues about the *F9* genetic components in EHL-rFIX PK NCA, and their comparison with SHL-rFIX PK, are summarized in the *Supplementary Table 1*.

Study limitations

The proposed investigation is retrospective, includes patients from several countries, and was not designed for genotype-phenotype association. Further, the comparison between *F9* variant class and NCA PK parameters between SHL- and EHL-rFIX concentrates was performed in different HB cohorts, and exploratorily extended to the *post-hoc* and less robust tertile analysis, more prone to chance findings. Although clearance was normalized to body weight and young HB patients were excluded from the analysis, an extended multivariable adjustment was not performed. Another limitation is the classification of variants as null *vs* non-null, which may group mutations with different biological mechanisms, particularly in the heterogeneous non-null group. The limited number of genotypes does not allow further and statistically reliable differentiation by genotype variant. Furthermore, the hypothesis of the presence of low-titer inhibitors, which may influence the observed PK outcome, could not be investigated in our retrospective study.

Conclusions

Although several study limitations prevent generalizability of our findings, and render this study essentially exploratory in nature, a small number of *F9* gene deletions/premature termination codons was found associated with slower CL, and thus favorable EHL-rFIX NCA PK profiles in patients benefitting from the SHL- to EHL-rFIX switch. Conversely, a higher frequency of *F9* non-null genotypes was found in the third CL tertile, characterized by unfavorable PK outcomes. Intriguingly, bioinformatics comparison between *F9* missense variants in the first and third CL tertiles suggested that those predicted to cause the most severe FIX damage were partially clustered with unfavorable CL values. Whereas these observations may contribute to interpret the wide variability of PK outcomes in HB patients treated with EHL-rFIX products, their clinical utility definition requires further studies in independent and larger cohorts of patients.

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Online supplementary material:

Supplementary Table 1. F9 *genetic components in SHL-/EHL-rFIX by NCA PK.*