





# Fusion of engineered albumin with factor IX Padua extends half-life and improves coagulant activity


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## Introduction

Haemophilia B (HB) is associated with bleeding symptoms whose severity is related to the degree of coagulation factor IX (FIX) deficiency.<sup>1</sup> Prophylaxis, effectively reducing bleeding episodes and preventing joint damage, represents the standard-of-care for HB.<sup>2</sup> However, it requires frequent

## Summary

The short half-life of coagulation factor IX (FIX) for haemophilia B (HB) therapy has been prolonged through fusion with human serum albumin (HSA), which drives the neonatal Fc receptor (FcRn)-mediated recycling of the chimera. However, patients would greatly benefit from further FIX-HSA half-life extension. In the present study, we designed a FIX-HSA variant through the engineering of both fusion partners. First, we developed a novel cleavable linker combining the two FIX activation sites, which resulted in improved HSA release. Second, insertion of the FIX R338L (Padua) substitution conferred hyperactive features (sevenfold higher specific activity) as for FIX Padua alone. Furthermore, we exploited an engineered HSA (QMP), which conferred enhanced human (h)FcRn binding [dissociation constant ( $K_D$ ) 0.5 nM] over wild-type FIX-HSA ( $K_D$  164.4 nM). In hFcRn transgenic mice, Padua-QMP displayed a significantly prolonged half-life (2.7 days,  $P < 0.0001$ ) versus FIX-HSA (1 day). Overall, we developed a novel FIX-HSA protein with improved activity and extended half-life. These combined properties may result in a prolonged functional profile above the therapeutic threshold, and thus in a potentially widened therapeutic window able to improve HB therapy. This rational engineering of both partners may pave the way for new fusion strategies for the design of engineered biotherapeutics.

**Keywords:** albumin fusion proteins, factor IX Padua, FcRn receptor, human serum albumin, protein engineering.

administrations, which create vein access problems and interfere with patients' life, resulting in poor adherence to prophylactic regimens, particularly in young patients.<sup>3</sup> Different strategies have been employed to extend the short half-life of FIX (18–22 h) and to reduce the frequency of intravenous injections. Three extended half-life products are currently on the market,<sup>4</sup> namely Fc-fused Alprolix® (Bioverativ

Therapeutics, Waltham, MA, USA),<sup>5,6</sup> pegylated Refixia® (Novo Nordisk, Bagsvaerd, Denmark),<sup>7,8</sup> albeit not widely available and not authorised for prophylaxis in children in many countries, and the albumin-fused Idelvion® (CSL Behring, Marburg, Germany).<sup>9,10</sup> Idelvion is designed with a cleavable linker connecting the two fusion partners that, upon FIX activation, drives the release of albumin and activated FIX.<sup>11,12</sup> Hence, this fusion strategy improves FIX half-life without compromising its functional properties.<sup>9,10</sup>

The fact that albumin is biodegradable, non-immunogenic and effector negative, which minimises the risk of side effects, makes it attractive in this context. Moreover, as albumin consists of a single polypeptide, it can easily be fused to a protein partner and expressed as a monovalent albumin-fused product. Indeed, fusion to albumin is being explored to improve the pharmacokinetics of various therapeutic proteins.<sup>13,14</sup> The albumin and immunoglobulin G (IgG)-Fc fusion strategies relies on the acquired capacity of these proteins to undergo the neonatal Fc receptor (FcRn)-mediated recycling pathway.<sup>15-17</sup> Indeed, FcRn is responsible for the average 3-week long albumin and IgG half-life.<sup>15,16,18,19</sup> FcRn primarily resides in the endosomal compartment of a broad range of cells, including haematopoietic and non-haematopoietic cells,<sup>20,21</sup> where it can bind albumin and IgG with high affinity. The interactions between FcRn and its ligands are pH-dependent, and are initiated at slightly acidic (5.5–6.0) but not at neutral (7.4) pH.<sup>15,22-25</sup> In this way, the receptor can bind fluid-phase pinocytosed albumin and IgG in low-pH sorting endosomes, and transport them back via recycling endosomes to the plasma membrane, where they are ultimately released due to the neutral pH.<sup>26-30</sup> Thus, FcRn rescues albumin and IgG from intracellular degradation, which extends their plasma half-life.

The dissection of the molecular bases underlying albumin-FcRn interactions have prompted the design of novel albumin variants with improved FcRn binding and extended half-life.<sup>13,31,32</sup> Among the three homologous albumin domains,<sup>33,34</sup> the C-terminal domain III (DIII) holds the principal binding site for FcRn.<sup>24,35</sup> We recently demonstrated that three amino acid substitutions within DIII (E505Q/T527M/K573P, QMP) improve binding of human serum albumin (HSA) to human (h)FcRn by >180-fold.<sup>13</sup> Notably, QMP fusion to recombinant activated coagulation factor VII (rFVIIa) led to a 3.6-fold extended half-life (2.9 days) compared to the wild-type fusion (0.8 days) in hFcRn transgenic mice, without affecting the therapeutic potential of rFVIIa.<sup>13</sup>

In addition to half-life extension, the biological properties of coagulation factors may also be improved by increasing their activity. In this scenario, the gain-of-function FIX Padua variant (R338L substitution), which has an approximately sevenfold higher specific activity compared to wild-type FIX,<sup>36</sup> may represent an attractive candidate for fusion purposes. Notably, FIX Padua has been explored in HB gene therapy, where it was shown to sustain coagulant levels and

decrease the annualised bleeding rate in 10 patients with HB. Importantly, no serious adverse events or development of inhibitory antibodies were reported.<sup>37</sup>

In the present study, we combined FIX Padua with the QMP albumin variant via a novel linker design, which resulted in a functional product with hyperactive features and extended half-life in hFcRn transgenic mice.

## Materials and methods

Detailed methods are available as Supporting Information.

### *Plasmids design and protein expression*

Chimeric constructs consisting of *F9* complementary DNA (cDNA; reference sequences: NM\_000133-4, NP\_000124-1), including 1.4 kb of intron 1,<sup>38</sup> designed cleavable linkers and the cDNA of mature (amino acids 25-609) HSA (reference sequences: NM\_000477-7, NP\_000468-1), either wild-type or QMP, were cloned in the pCDNA3 expression vector. The FIX Padua variant (R338L) was inserted through site-directed mutagenesis. All constructs were validated by sequencing (primer details in Table SI).

Expression studies in HEK293 cells and stable clone selection were performed as described previously.<sup>12,39</sup>

### *Protein purification and characterisation*

Fusion proteins were purified with a HSA affinity matrix followed by size exclusion chromatography and concentrated by centrifugation units.<sup>13</sup> The activation profile of FIX and FIX-HSA proteins was evaluated by incubation with plasma-derived activated factor XI (pdFXIa) followed by Western blotting as described previously.<sup>40</sup>

Characterisation of secreted protein levels through polyclonal enzyme-linked immunosorbent assay (ELISA),<sup>40,41</sup> as well as FIX activity by chromogenic<sup>41</sup> or activated partial thromboplastin time (aPTT)-based<sup>42</sup> assays, was as described previously. Specific activity was calculated as the ratio between activity and protein levels, with the value of 1 corresponding to normal specific activity of the reference molecule (FIX or FIX-HSA).<sup>39</sup>

### *FcRn binding studies*

The ELISA-based assays on FIX-HSA fusions for hFcRn binding were performed as described previously.<sup>13,43</sup>

Binding kinetics to hFcRn were measured through surface plasmon resonance (SPR), with FIX-HSA fusion proteins immobilised on sensor chips and of serial dilutions of soluble monomeric hFcRn-His injected.<sup>13</sup> Binding curves were zero adjusted and the reference flow-cell value was subtracted. Binding kinetics were determined using the 1:1 Langmuir binding model provided by the Biacore T200 Evaluation Software (version 3.0).

### Mouse studies

Studies in mice (male, aged 6–8 weeks, weighing 20–30 g, three to five mice/group) were performed at The Jackson Laboratory, in accordance with guidelines and regulations approved by the local Animal Care and Use Committee. Hemizygous hFcRn transgenic Tg32, homozygous hFcRn transgenic Tg32 albumin knock-out (KO) and FcRn KO mice received 2 mg/kg fusion proteins, and blood was collected from retro-orbital sinus at the scheduled time points. The concentration of fusion proteins in plasma was quantified by ELISA.<sup>13</sup> The  $\beta$ -phase half-life was calculated using the formula:  $t_{1/2} = \log 0.5 / (\log Ae/A0) \times t$ , where  $t_{1/2}$  = the half-life of the variant evaluated, Ae = the concentration remaining, A0 = concentration on day 1 and t = the elapsed time.

### Statistical analysis

Statistical differences were analysed by unpaired *t*-test, with  $P < 0.05$  considered as statistically significant.

## Results

### Construction of a FIX-HSA fusion protein with optimised linker

The efficient release of albumin from the FIX-HSA chimera is crucial to ensure proper coagulant activity.<sup>9,43</sup> First, FIX was recombinantly fused to the N-terminal end of mature HSA via a cleavable amino acid linker resembling one of the FIX activation sites, R145-A146 (linker a; FIX-a-HSA), which is used in the clinically approved Idelvion.<sup>9</sup> To investigate whether proper activation of the fusion could be further enhanced, two alternative linkers, which included the second FIX activation site, R180-V181 (linker b; FIX-b-HSA), or a head-to-tail combination of the two activation sequences (linker ab; FIX-ab-HSA), were designed (Fig 1A). Upon transient expression in HEK293 cells, albeit showing protein levels significantly lower ( $P < 0.0001$ ) than those of wild-type FIX, the three fusion proteins were efficiently secreted (Fig S1A, left panel), with the FIX-b-HSA and FIX-ab-HSA being produced at approximately half the levels of FIX-a-HSA ( $P < 0.0001$ ). Importantly, incubation with FXIa, a physiological FIX activator, generated protein fragments of the expected molecular weights (Fig S1B). To assess the functional integrity of fusion proteins, their ability to restore coagulation in FIX-deficient plasma was evaluated by aPTT-based assays. The results supported that the three variants were functional, as demonstrated by the shortening of coagulation times upon addition of medium containing any of the three fusion proteins (Fig S1A, right panel). In order to compare the three fusions, specific activity was calculated as the ratio between activity and protein concentration (Fig 1B). The FIX-ab-HSA fusion, containing the combination of the two FIX activation sites (linker ab), showed the highest

specific activity [mean (SD) 1.20 (0.13)]. This was a significant improvement compared to the Idelvion-like FIX-a-HSA [mean (SD) 1.00 (0.1),  $P = 0.0046$ ] and the FIX-b-HSA [mean (SD) 0.86 (0.12),  $P = 0.0001$ ] fusions.

These results demonstrated the favourable features of the designed linker ab, and prompted the selection of the FIX-ab-HSA chimera (henceforth referred to as FIX-HSA) as the scaffold for subsequent engineering steps.

### Design of a double-engineered FIX-HSA fusion protein

To enhance the therapeutic potential of the FIX-HSA chimera, we engineered both fusion partners.

First, to improve the procoagulant features, we exploited the gain-of-function FIX Padua variant by introducing the R338L substitution in FIX alone (Padua) and in the FIX-HSA fusion (Padua-HSA). Upon transient expression, their activity profiles (Fig S1C) demonstrated an approximate eightfold enhanced procoagulant activity of both Padua variants as compared to the corresponding wild-type versions (Fig 1C). Importantly, no significant difference in activity of the Padua variant was measured as a result of fusion to albumin.

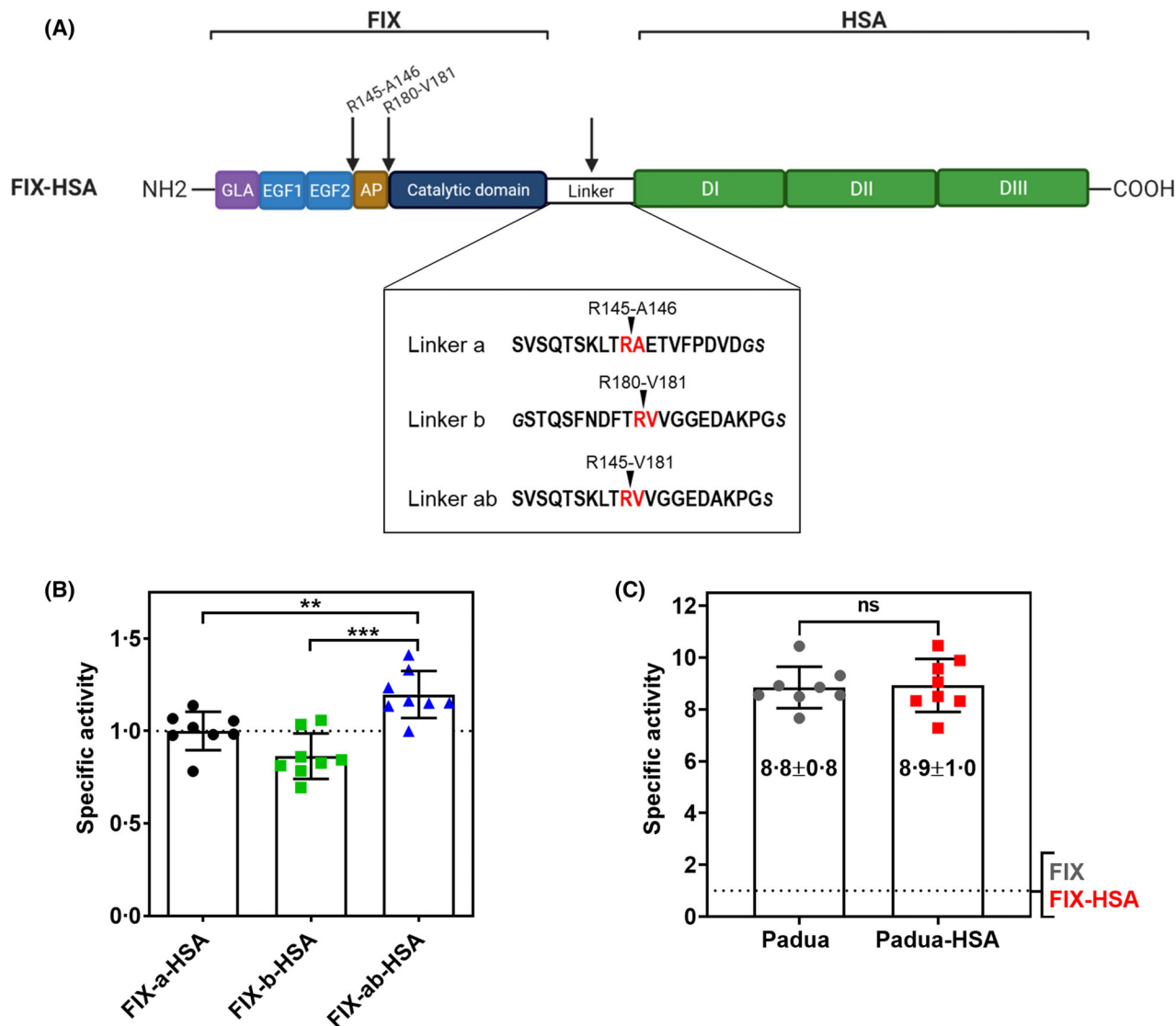
Second, to enhance the pharmacokinetic properties, we fused the engineered QMP albumin<sup>13</sup> to the FIX Padua variant (Padua-QMP) through the optimised linker ab (Fig 2A). The resulting chimera, as well as the wild-type counterpart, were stably expressed in HEK293 cells and purified. A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed pure fractions of FIX-HSA and of the double engineered fusion, which migrated with the expected molecular weights (~115 kDa) (Fig S2).

### Padua-QMP shows enhanced procoagulant and hFcRn binding properties

The functional features of the purified FIX-HSA and Padua-QMP fusions (Fig 2A) were evaluated in FIX-deficient human plasma through aPTT-based assays. Both variants restored coagulation times in a concentration-dependent manner (Fig 2B), with the specific activity of the Padua-QMP being sevenfold higher ( $P < 0.0001$ ) than that of the wild-type fusion (Fig 2B, inset).

Binding of fusion proteins to hFcRn was evaluated using ELISA, which showed that Padua-QMP bound more strongly to the receptor than the wild-type fusion at acidic pH (Fig 2C). As a control, we included the commercial fusion protein Idelvion, which bound hFcRn similarly to the designed wild-type FIX-HSA fusion.

Furthermore, to determine the binding kinetics, we performed SPR analysis with the fusion variants immobilised on the sensor chip and monomeric fractions of the receptor injected at pH 5.5. Whereas FIX-HSA showed a dissociation constant ( $K_D$  164.4 nM) comparable to that of Idelvion ( $K_D$  150.0 nM), the QMP amino acid substitutions greatly



**Fig 1.** Fusion of FIX to HSA with three different linker sequences. (A) Schematic representation of the FIX-HSA fusion protein (upper panel) with the amino acid sequences of the three linkers tested (lower panel). The cleavage sites in FIX and the linker sequences are indicated by arrows (upper panel) and highlighted in red (lower panel). According to the Human Gene Variation Society (HGVS) nomenclature,<sup>60</sup> whose numbering begins from the first translation initiation methionine-coding AUG, the corresponding positions for FIX activation sites are R191-A192 and R226-R227. Created with Biorender.com. (B) Specific activity of transiently expressed fusion proteins with different cleavable linkers. The dotted line indicates the specific activity (=1) of the reference construct (FIX-a-HSA). (C) Specific activity of transiently expressed Padua variants. The dotted line represents the specific activity (=1) of the wild-type constructs. All results are reported as mean  $\pm$  standard deviation. Specific activity was calculated as the activity/antigen ratio. The corresponding *P* values are indicated as not significant (ns,  $P > 0.05$ ), \*\* ( $P \leq 0.01$ ), \*\*\* ( $P \leq 0.001$ ), by unpaired *t*-test. AP, activation peptide; DI, domain I; DII, domain II; DIII, domain III; EGF1 epidermal growth factor-like domain 1, EGF2, epidermal growth factor-like domain 2; FIX, factor IX; GLA, gamma-carboxyglutamic acid domain; HSA, human serum albumin. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

improved the binding affinity, resulting in a  $K_D$  of 0.5 nM (Table I). Importantly, Padua-QMP was shown to bind pH dependently, as bound hFcRn was released from the immobilised fusion protein upon injection of a pH 7.4 buffer (Fig 2D–F; Fig S3).

Thus, the designed Padua-QMP displays improved procoagulant activity and favourable pH-dependent hFcRn binding properties.

#### *Padua-QMP shows extended half-life in hFcRn transgenic mice*

We have previously demonstrated that HSA, as well as fusions built on HSA, poorly bind to mouse FcRn, which hampers evaluation of such molecules in conventional mouse models.<sup>13,24,45,46,47</sup> Therefore, to study the effect of FIX-HSA engineering on plasma half-life we took advantage of

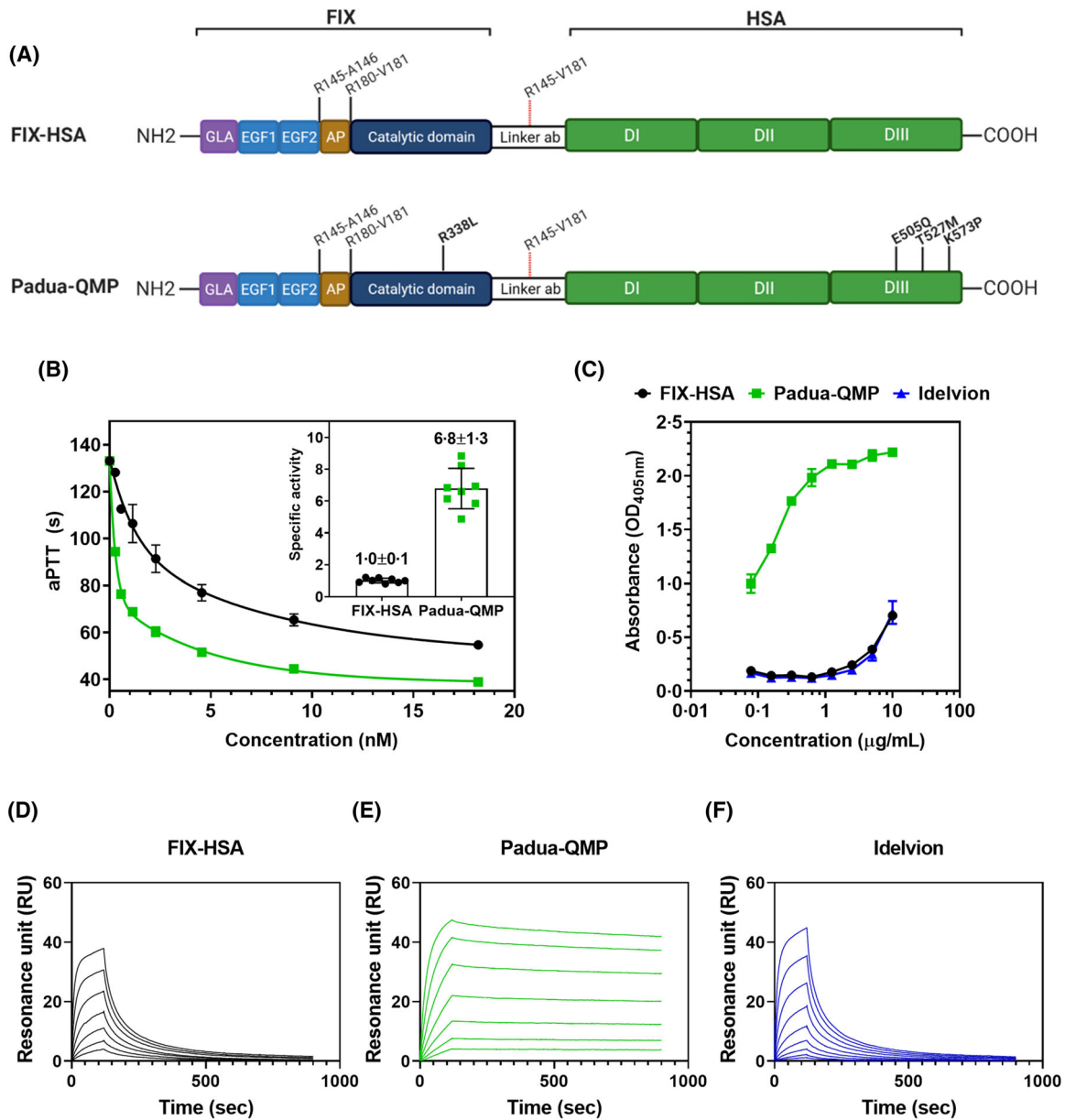


Fig 2. Engineering of the FIX-HSA fusion improves procoagulant activity and hFcRn binding. (A) Schematic representation of the designed fusion proteins of FIX and HSA connected via the cleavable linker ab. FIX is activated by proteolytic cleavage at the two cleavage sites indicated (R145-A146 and R180-V181). Simultaneous detachment of HSA is accomplished as the linker sequence (linker ab) contains an upstream–downstream combination of the two cleavage sites in FIX (R145-V181). FIX-HSA consists of wild-type FIX and wild-type HSA (upper panel). Padua-QMP contains point mutations in FIX (R338L, Padua) and HSA (E505Q/T527M/K573P) as indicated in bold. According to Human Gene Variation Society (HGVS) nomenclature,<sup>60</sup> the corresponding positions/substitutions are R191-A192/R226-R227 (activation sites) and R384L (Padua variant) for FIX, and E529Q/T551M/K597P for the QMP variant. Created with Biorender.com. (B) Representative procoagulant activity of serial dilutions of purified fusion proteins, evaluated by aPTT-based assays. The resulting specific activity is reported (inset). (C) Binding of titrated amounts of purified fusion proteins to immobilised hFcRn at pH 5.5. Results are reported as mean ± standard deviation of duplicates from one representative experiment. (D–F) Representative sensorgrams showing binding of serial dilutions (0–4 µM) of monomeric hFcRn injected over immobilised (~200 RU) fusion proteins at pH 5.5. AP, activation peptide; aPTT, activated partial thromboplastin time; DI, domain I; DII, domain II; DIII, domain III; EGF1 epidermal growth factor-like domain 1, EGF2, epidermal growth factor-like domain 2; FIX, factor IX; GLA, gamma-carboxyglutamic acid domain; HSA, human serum albumin; hFcRn, human neonatal Fc receptor. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

transgenic mice genetically modified to express the hFcRn heavy chain and not the mouse counterpart.<sup>16,48,49</sup> Importantly, these transgenic mice express a chimeric version of

the receptor, as the hFcRn heavy chain pairs with the mouse  $\beta_2$  microglobulin, which has been shown to bind mouse and human albumin with comparable affinity.<sup>50</sup>

**Table I.** Binding kinetics of fusion protein variants to hFcRn at pH 5.5.

Protein variant	Mean (SD):		
	$K_a, \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$K_d, \times 10^{-3} \text{ s}^{-1}$	$K_D, \text{ nM}$
FIX-HSA	4.5 (0.4)	7.4 (0.2)	164.4
Padua-QMP	18.2 (0.4)	0.1 (0.0)	0.5
Idelvion	4.2 (0.1)	6.3 (0.4)	150.0

FIX, factor IX; HSA, human serum albumin;  $K_a$ , association constant;  $K_d$ , dissociation constant.

The kinetic rate constants were determined by the 1:1 Langmuir bimolecular interaction model. The kinetic values reflect the mean (SD) of three runs for each fusion protein.

First, we determined the half-life of Idelvion and of our designed fusion proteins in Tg32 hFcRn transgenic mice that express mouse albumin (29 mg/ml).<sup>16,51</sup> Blood samples were collected up to 10 days after injection of fusion proteins. ELISA was performed to quantify plasma levels, which revealed that FIX-HSA and Idelvion showed a similar half-life of ~1 day, whereas that of Padua-QMP was 2.7 days (vs. FIX-HSA,  $P = 0.0006$ ; vs. Idelvion,  $P < 0.0001$ ) (Fig 3A). Thus, the QMP albumin variant extended the half-life by >2.5-fold even in the presence of competing endogenous mouse albumin.

The experiment was then performed in hFcRn transgenic mice lacking mouse albumin (Tg32 albumin KO mice).<sup>48</sup> From these mice, blood was collected for up to 30 days after injection, as longer half-lives were expected in the absence of mouse albumin competing for hFcRn binding. Indeed, fusion proteins were detectable in plasma 30 days after administration, with half-lives of 7.8 days and 11.1 days for FIX-HSA and Padua-QMP, respectively (Fig 3B). Similar to the designed wild-type fusion, a half-life of 7.2 days was determined for Idelvion. Thus, in the absence of mouse albumin as competitor, a 1.4-fold longer half-life was measured for Padua-QMP in comparison with FIX-HSA ( $P < 0.0091$ ) and Idelvion ( $P < 0.0005$ ) as a result of albumin engineering.

To confirm that improved hFcRn binding was responsible for the extended half-life, we also injected the fusions in mice lacking expression of FcRn (FcRn KO mice).<sup>16</sup> As expected, all three fusion proteins showed equally rapid clearance from the circulation, as their half-lives dropped to only 17–19 h (Fig 3C, left panel). Prompted by the overlapping clearance curves, we also assessed FIX-dependent coagulant activity in plasma from FcRn KO mice (Fig 3C, right panel). A slightly significant shortening of coagulation times for reference fusions over the baseline was detectable only for Idelvion until day 1. Noticeably, Padua-QMP continued to confer a significant increase in FIX activity until day 3 (Fig 3C, right panel).

Altogether, these data demonstrate that the engineered albumin variant extends the half-life of the Padua-QMP chimera in an FcRn-dependent manner and provide *in vivo*

evidence for the hyperactive features of the Padua-QMP molecule.

## Discussion

Fusion to HSA represents an attractive strategy to improve plasma half-life of therapeutic proteins, and has been applied to increase FIX half-life (Idelvion) from 22 to 102 h in humans.<sup>9,10,52</sup> However, the half-life extension achieved by fusion to wild-type albumin is limited, and far from that of endogenous albumin, whose high concentration exerts a considerable competitive pressure for hFcRn binding.<sup>13</sup> In this view, even a minor increase in receptor binding may favour and improve the ability of hFcRn to rescue a fusion product from intracellular degradation. As such, to provide a more effective product for HB replacement therapy, we designed an engineered FIX-HSA fusion protein with further extended half-life and improved functional features.

This was first achieved by optimising the linker design, which represents a pivotal element to ensure proper biological properties.<sup>44</sup> To this purpose, two novel cleavable linker sequences were compared with that present in Idelvion.<sup>9,53</sup> We found that a combination of the two FIX activation sequences conferred higher specific activity to the resulting FIX-HSA chimera, probably due to more efficient release of albumin.

Once the best-performing fusion strategy was identified, we combined this design with the hyperactive FIX Padua variant. While FIX Padua has been safely explored for gene therapy purposes,<sup>37</sup> it has not yet been explored in the context of enhanced half-life proteins for replacement therapy purposes. When combined with albumin, FIX Padua conferred the expected sevenfold higher specific activity in clotting assays, thus supporting its suitability as a fusion partner to generate a more active product. Moreover, given the high specific activity of FIX Padua, its potential for thrombogenicity has to be carefully addressed. It is worth noting that this variant has been found to be associated with thrombosis in a patient with FIX activity levels of ~770%, but neither in his brother nor his mother displaying FIX levels of ~550% and ~330%, respectively.<sup>36</sup> Consistently, in non-human primates treated with adeno-associated viral vector serotype 5 (AAV5)-delivered FIX Padua, increased FIX activity up to 500% was not associated with prothrombotic states, which, together with previous studies, support the FIX Padua safety profile.<sup>54-56</sup> The potential risk for immunogenicity could also be an issue. It is worth noting that, in the above reported studies in mouse and canine HB models, as well as in patients, the viral-mediated delivery of FIX Padua was not associated with inhibitor development.<sup>37,54,55</sup> In our present study, no thrombotic events occurred after injecting Padua-QMP into mice expressing FIX, and the monophasic log-linear decay up to 30 days of FIX-HSA plasma levels did not point toward immunogenicity.

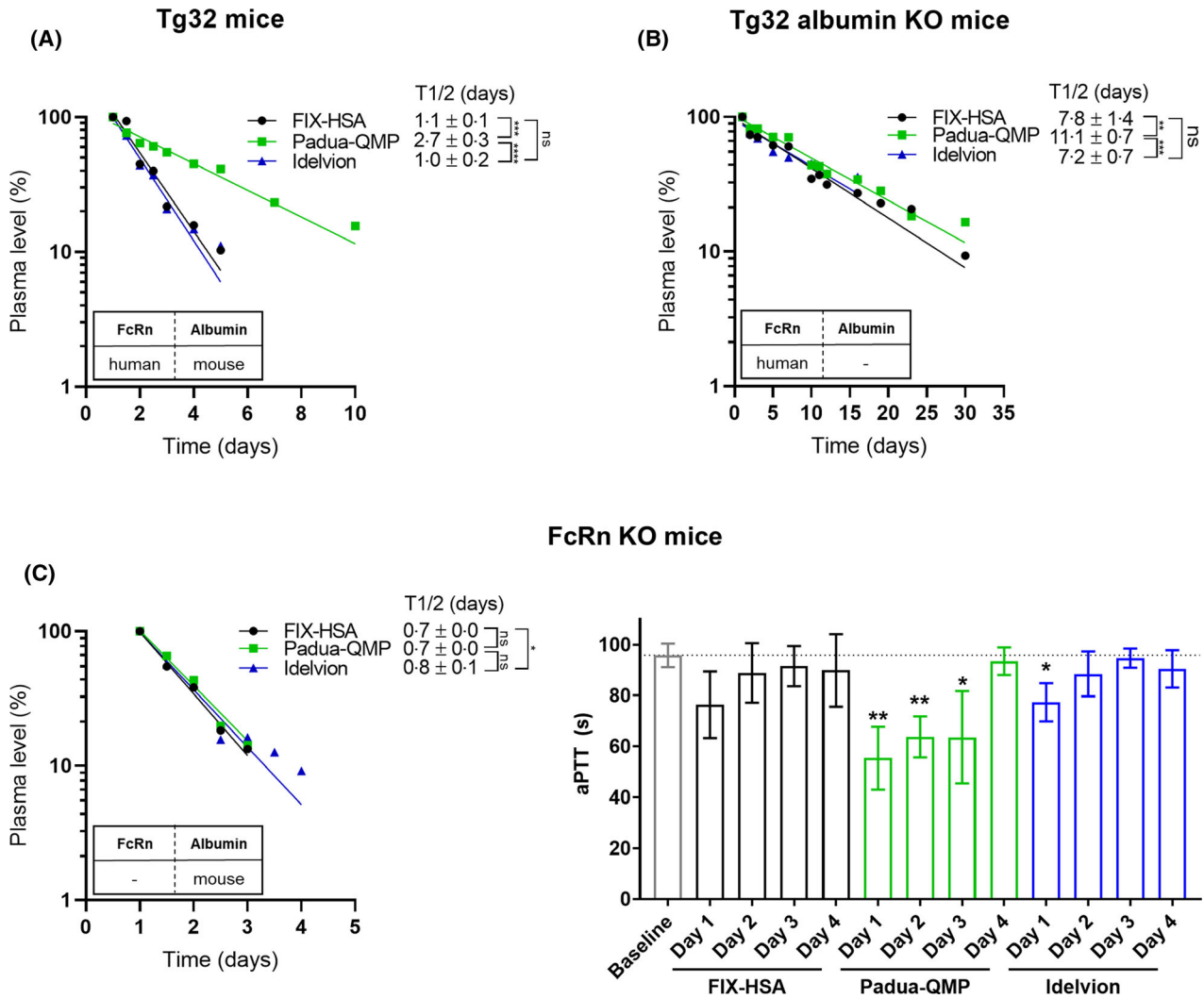


Fig 3. FcRn extends the plasma half-life of the engineered Padua-QMP fusion. Clearance curves of FIX-HSA (black circles), Padua-QMP (green squares) and Idelvion (blue triangles) in hFcRn transgenic mice (Tg32) (A), hFcRn transgenic mice deficient for mouse albumin (Tg32 albumin KO) (B), and FcRn deficient mice (FcRn KO) (C, left panel). Plasma samples from FcRn KO mice were also evaluated for FIX-dependent shortening of coagulation times (C, right panel). The mice received 2 mg/kg fusion protein by intravenous injection. Plasma levels are reported as the percentage remaining in the circulation compared to the level measured 24 h after injection (day 1, 100%). The values represent the mean  $\pm$  standard deviation (SD) of 3–5 mice. Mean  $\beta$ -phase half-life ( $t_{1/2}$ )  $\pm$  SD is reported in days in the upper right corner of each figure. The corresponding  $P$  values are indicated as not significant (ns,  $P > 0.05$ ), \* ( $P \leq 0.05$ ), \*\* ( $P \leq 0.01$ ), \*\*\* ( $P \leq 0.001$ ), \*\*\*\* ( $P < 0.0001$ ), by unpaired  $t$ -test.  $P$  values from unpaired  $t$ -test on *in vivo* half-life data are reported in Table SII. FIX, factor IX; HSA, human serum albumin; hFcRn, human neonatal Fc receptor; KO, knock-out. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Noticeably, the extended plasma half-life of FIX Padua was achieved by combining it with the recently developed QMP albumin variant,<sup>13</sup> which should increase its chance of outcompeting endogenous albumin and thus favour its rescue from intracellular degradation. Due to large cross-species differences in FcRn binding, transgenic mice expressing the hFcRn heavy chain, instead of the mouse receptor, were used to evaluate FIX-HSA fusions. Importantly, while mouse albumin binds with higher affinity than HSA to the fully human receptor,<sup>32,45,46,47</sup> it binds the chimeric FcRn version expressed by the transgenic mice with an affinity similar to that of HSA.<sup>50</sup> This supports that the circulating endogenous

mouse albumin represents a suitable competitor for HSA-based molecules assessed in the Tg32 hFcRn transgenic mice, where the Padua-QMP gained a  $>2.5$ -fold longer half-life compared with FIX-HSA and Idelvion.

It is worth noting that our data on protein levels in Tg32 mice do not take into account the functional contribution of the hyperactive FIX Padua, as these mice express normal FIX levels, not allowing the comparison of FIX-HSA *in vivo* efficacy. However, the available HB mice, expressing mouse FcRn, do not either represent a good model to assess the contribution of HSA engineering to the improved HB phenotype correction due to the cross-species differences.

Indeed, HSA binds poorly to mouse FcRn, and although QMP improves binding to the mouse receptor, the kinetic values are far from that measured towards hFcRn.<sup>13</sup> Notwithstanding, in FcRn KO mouse plasma, although levels of the three fusion proteins were comparable, only Padua-QMP demonstrated a significant shortening of FIX-dependent coagulation times over the baseline until day 3, which highlights its improved functional properties.

In summary, we produced a novel FIX-HSA fusion protein with enhanced coagulant activity and hFcRn binding properties translating into significantly extended plasma half-life in hFcRn transgenic mice. These features may allow for a prolonged functional profile above the therapeutic threshold and, consequently, reduce the frequency of injections required in a prophylactic treatment regimen for patients with HB. This, in turn, could allow the widening of the therapeutic window with a consequent lowering of treatment burden.<sup>58</sup> As treatment is typically provided in amounts that are just sufficient to limit joint bleeds, a hyper-functional product, requiring lower doses to achieve the same or even superior functional levels, may favour achieving an optimal functional trough level.<sup>57-59</sup>

Overall, our present study demonstrates that combining a natural gain-of-function FIX variant with a rationally engineered HSA may result in novel fusion proteins with improved biological properties and bioavailability, which in turn may pave the way for new strategies for the design of engineered biotherapeutics.

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## Author contributions

Silvia Lombardi designed fusion proteins, performed expression as well as functional studies, analyzed data and wrote the manuscript; Kristin H. Aaen and Jeannette Nilsen purified fusion proteins, performed hFcRn binding studies and ELISA assays on mouse samples, analysed data and wrote the manuscript; Mattia Ferrarese established and validated stable clones and analysed data; Torleif T. Gjølborg performed ELISA on Tg32 albumin KO mouse samples and analysed data; Francesco Bernardi and Mirko

Pinotti analysed data and revised the manuscript; Jan T. Andersen and Alessio Branchini conceived the study, designed and performed research, analysed data and wrote the manuscript. All authors approved the final version of the manuscript.

## Conflicts of interest

Jan T. Andersen is a co-inventor of patents, which are entitled 'Albumin Variants and uses thereof' and relate to the data described in this paper, for example, EP3063171B1, US10208102 and US10781245. Alessio Branchini received grants and non-financial support from Grifols. Mirko Pinotti received grants from Novo Nordisk. Alessio Branchini and Mirko Pinotti received grants and personal fees from Pfizer. Francesco Bernardi, received a research grant from Pfizer. The remaining authors have no competing financial interests to declare.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig S1.** Transient expression of the FIX-HSA fusion proteins in HEK293 cells. (A) Secreted levels of the fusion proteins and FIX measured by polyclonal anti-FIX ELISA (left panel), and representative activity profiles of serial dilutions of media in FIX-deficient plasma evaluated by aPTT-based assay (right panel). (B) Western blotting analysis of the activation profile of FIX and FIX-HSA fusions (left panel), in the absence (zym) or in the presence (act) of the physiological activator FXIa. A schematic representation of the protein fragments deriving from FXIa-mediated cleavage is provided (right panel). (C) Representative activity profile of serial dilutions of transiently expressed fusion proteins, either wild-type or containing the R384L (Padua) substitution, evaluated by chromogenic assays. The corresponding *P* values are indicated as not significant (ns,  $P > 0.05$ ), \*\*\*\* ( $P < 0.0001$ ), by unpaired *t*-test. aPTT, activated partial thromboplastin time; FIX, factor IX; HSA, human serum albumin.

**Fig S2.** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the FIX-HSA fusion proteins. Purified fractions of Idelvion (lane 2), FIX-HSA (lane 3) and Padua-QMP (well 4) were analysed by SDS-PAGE and Coomassie staining. FIX, factor IX; HSA, human serum albumin.

**Fig S3.** Complete SPR sensorgrams showing binding of FIX-HSA fusion proteins to hFcRn. Serial dilutions (0–4  $\mu$ M) of monomeric hFcRn were injected over immobilised (~200 RU) fusion proteins at pH 5.5. Regeneration was performed by injecting a pH 7.4 buffer at the time point indicated by the arrow. hFcRn, human neonatal Fc receptor; SPR, surface plasmon resonance.

**Table SI.** Oligonucleotides used to create expression vectors for designed fusion proteins.



**Table SII.** *P* values from unpaired *t*-test on *in vivo* half-life data.

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