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## BRIEF REPORT

# In vivo intranasal delivery of coagulation factor IX: a proof-of-concept study

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## Abstract

**Background:** Hemophilia B (HB) is a bleeding disorder characterized by coagulation factor (F) IX (FIX) deficiency. The current standard-of-care for severe HB is prophylaxis with long-term repetitive intravenous (i.v.) infusions of recombinant FIX (rFIX) with standard half-life or extended half-life. Unmet needs remain regarding the development of non-invasive administration routes for coagulation factors. The aim of this study was to evaluate the effectiveness of intranasal delivery (IND) of rFIX and rFIX fused to Fc fragment (rFIX-Fc) in mice.

**Methods:** Drops of rFIX and rFIX-Fc were deposited in the nostrils of wild-type, FcRn knock-out, FcRn humanized, and FIX knock-out mice. rFIX mucosal uptake was evaluated by measuring plasma FIX antigen and FIX activity (FIX:C) levels, and by performing histologic analysis of the nasal mucosa following IND.

**Results:** After IND, both rFIX and rFIX-Fc were equally delivered to the blood compartment, irrespective of the mouse strain studied, mostly through a passive mechanism of transportation across the mucosal barrier, independent of FcRn receptor. Both plasma FIX antigen and FIX:C activity levels increased following IND in FIX knock-out mice.

**Conclusion:** This proof-of-concept study describes evidence supporting the nasal route as an alternative to FIX i.v. infusion for the treatment of HB.

## KEYWORDS

administration, factor IX, Fc receptor, hemophilia B, intranasal, mice, neonatal

## 1 | INTRODUCTION

Hemophilia B (HB) is a rare inherited X-linked recessive bleeding disorder characterized by coagulation factor IX (FIX) deficiency [1]. The bleeding phenotype of HB is correlated with FIX clotting activity (FIX:C) in plasma. Severe HB (FIX:C<1IU/dL) is characterized by spontaneous and prolonged bleeding episodes, mostly in the joints, but which can be life-threatening such as when intracranial hemorrhage (ICH) occurs [2]. The current standard-of-care for severe HB is prophylaxis, which consists of long-term regular (several times a month) intravenous (i.v.) infusions of recombinant FIX (rFIX) with standard half-life or extended half-life (EHL-rFIX) [3,4]. These rFIX can also be administered when bleedings occur (on-demand treatment) to stop them [3,5]. Although effective in preventing or treating bleeds, long-term regular i.v. infusions greatly impair patients' and caregivers' quality of life [4,6]. Recently, the first gene therapy product for the treatment of HB was approved in US by the Food and Drug administration and in Europe by the European Commission, showing stable FIX:C levels over 18 months after a single i.v. infusion in adult patients with HB [7]. However, not all patients are eligible or may benefit from gene therapy. There is thus an unmet need regarding the development of non-invasive routes for the administration of coagulation factors, especially for newborns who are at risk of ICH, and for pediatric patients who have poor venous access [8].

Intranasal delivery (IND) is receiving increased attention as a potential non-invasive method capable of delivering therapeutically relevant concentrations of many different substances, including large biologics, in preclinical and clinical settings [9–11]. In addition, IND might be the preferred choice over oral administration of biologics as it provides advantages: easy self-administration and dose adjustment, rapid onset of effects, avoidance of hepatic first pass elimination, avoidance of the extremely low pH and digestive enzymes in the gastrointestinal tract [12], and potential direct pathways to the central nervous system that bypass the blood brain barrier [13].

Among the elements involved in mucosal bioavailability, a ubiquitous receptor seems to play a major role in the capture of drugs: the neonatal receptor for the fragment crystallizable (Fc) region of immunoglobulin (FcRn) [14]. This receptor acts as transporter, allowing the active passage of substances through the epithelial barrier [15–17]. Two EHL-rFIX products, genetically fused to the Fc fragment of immunoglobulin (rFIX-Fc) or to mature albumin [18,19], demonstrated an EHL. This is due to a delay in protein degradation following FcRn binding, which allows the recycling of the products back into the blood circulation [20,21]. Therefore, we hypothesized that rFIX, and particularly EHL-FIX such as rFIX-Fc, could be delivered through IND for the treatment of HB.

The aim of this proof-of-concept study was to evaluate the effectiveness of rFIX (standard half-life-rFIX and EHL-rFIX) IND in mice.

### Essentials

- Lifelong prophylactic intravenous injections of coagulation factors are safe and effective in preventing bleeding in severe hemophilia but impacts patients' and caregivers' quality of life.
- This proof-of-concept study evaluated the feasibility of intranasal delivery of recombinant factor IX (rFIX) proteins for the treatment of hemophilia B in mice.
- Both rFIX and rFIX-Fc reached the blood compartment and increased FIX activity levels in mice following intranasal delivery.
- Although further development and characterization are needed, this study paves the way for an alternative administration route for coagulation factors that may improve patients' quality of life.

## 2 | MATERIALS AND METHODS

### 2.1 | Intranasal delivery

The animal study protocols were approved by the *local animal ethics evaluation committee* (CECCAPP: C2EA-15) and authorized by the *French Ministry of Education and Research* (protocol code APAFIS #36194-2022010421473996 v7, approved in 2022). Male and female wild-type (WT) C57BL/6J mice and transgenic mice with C57BL/6J background that were either FcRn knock-out (FcRn KO), FcRn KO and humanized for FcRn (hFcRn), or FIX KO were purchased from the *Charles River Laboratory* (Les Oncins, Saint Germain Nuelles, France). All mice were either 8 to 12 weeks or 76 weeks to evaluate age differences. Recombinant rFIX (Nonacog alfa, BENEFIX, Pfizer), and rFIX-Fc (Eftrenonacog alfa, ALPROLIX, SOBI) were purchased. For IND studies, the identity of the factors was blinded to the investigator who performed the experiments. Mice were weighed (verification of sufficient weight 20–30g), and then anesthetized by intraperitoneal injection (mixture of ketamine and xylazine). A maximum volume of 10  $\mu$ L was administered as a drop into each nostril (20  $\mu$ L of one biologic corresponding to 10 International Units (IU) for a 20–30g mouse). Sham treatment consisted of a saline solution. Three IUs of rFIX and rFIX-Fc were also administered intravenously in the retro-orbital vein of mice that served as controls.

### 2.2 | FIX detection and measurement

Blood samples were collected at different time points after i.v. administration (15 min, 4 h, and 24 h) and IND (15 min) from exsanguination following decapitation on anesthetized mice. Blood samples were centrifuged (15 min, 3500 rpm) at room temperature

(RT) and frozen ( $-20^{\circ}\text{C}$ ) for further analysis. Plasma FIX antigen levels (FIX:Ag) were quantified using the Asserachrom IX:Ag (Stago) enzyme-linked immunosorbent assay kit, as described previously [22,23]. FIX:C was measured using a validated chromogenic assay [24] (Biophen Hyphen Biomed). Murine plasma samples were loaded onto a Mini-Protean TGX 4% to 15% Gel (Biorad) for electrophoresis under reducing conditions (Tris 25 mmol/L, glycine 192 mmol/L, SDS 0.1%, pH 8.3 running buffer) and then transferred onto a nitrocellulose membrane (Trans-Blot Turbo Mini 0.2  $\mu\text{m}$  NC Transfer pack, Biorad) according to the manufacturer's instructions. The nitrocellulose membrane was then blocked with 6% skimmed milk-PBS and exposed to horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-human FIX (1 mg/mL) IgG (1:2000 dilution; Affinity Biologicals).

### 2.3 | Histologic study

The nasal cavities from each animal were taken to perform histologic studies on turbinate tissue sections. Under gentle agitation, samples were first fixed in 4% paraformaldehyde for 3 days at RT, then decalcified at  $4^{\circ}\text{C}$  in 10% EDTA (Sigma) at pH 7.4 for 7 days, and cryopreserved at  $4^{\circ}\text{C}$  in sucrose 30% (Sigma) for 3 days. After immersion in optimum cutting temperature compound (OCT, TissueTek, Sakura Finetek), the samples were quickly frozen and stored at  $-80^{\circ}\text{C}$  until processing. Sagittal sections, 8- $\mu\text{m}$  thick, were performed using a cryostat microtome (CM1860, Leica). Sections were successively incubated at RT with acetone, 3% hydrogen peroxide (Sigma), PBS containing 3% bovine serum albumin and 5% normal goat serum, and for 1 hour with HRP-conjugated anti-FIX antibody (1:100, goat polyclonal, Affinity Biologicals). HRP was revealed using 3-amino-9-ethylcarbazole (AEC) substrate (Vector Laboratories). Sections were

counterstained with Hematoxylin QS (Vector Laboratories), mounted with Glycergel (Agilent Technologies). Images of nasal sections were captured using an inverted microscope (40 $\times$  oil lens and 10- $\mu\text{m}$  scale bar, Nikon Ti-E microscope).

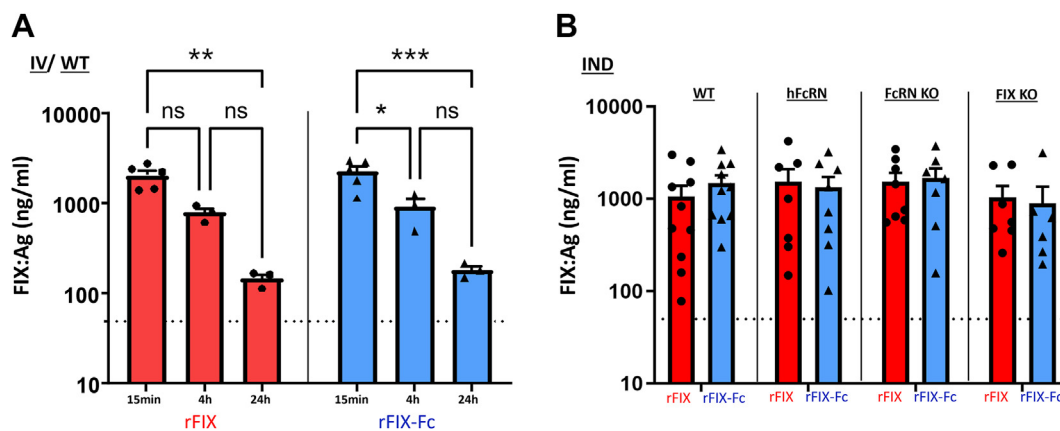
### 2.4 | Statistical analysis

All experiments were carried out at least in duplicate. Data are presented with a logarithm scale for FIX:Ag plasma levels (ng/mL) and FIX:C (IU/dL) and expressed using means  $\pm$  SEM. Statistical analyses were done using GraphPad Prism 8 software by unpaired *t*-test or two-ways analysis of variance test (Dunnett's), with *P* value  $<.05$  considered statistically significant.

## 3 | RESULTS AND DISCUSSION

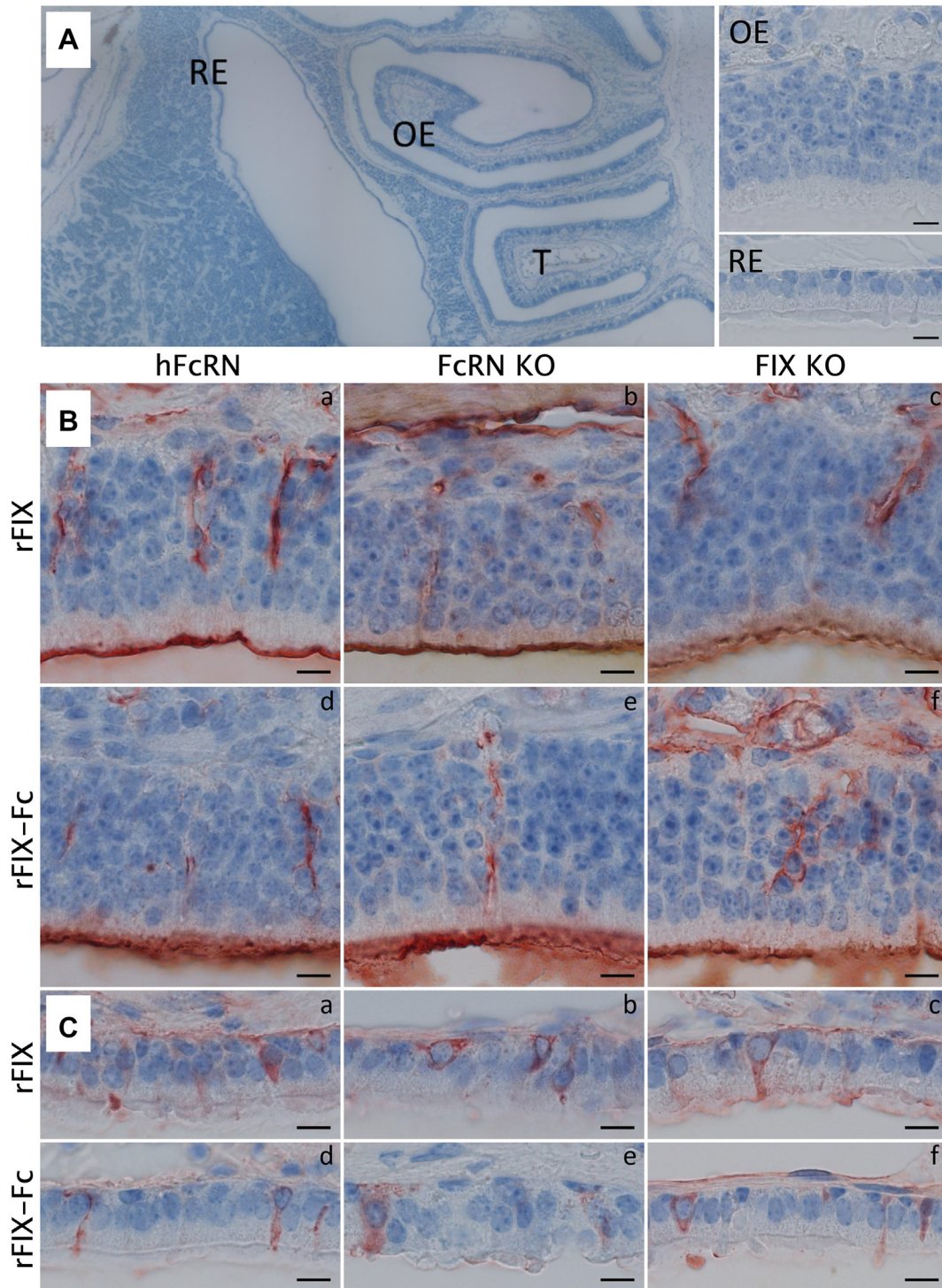
As control, 20- to 30-g WT mice first received i.v. administration of 3IU ( $\sim 100$ -150IU/kg) of rFIX or rFIX-Fc ( $n = 22$ ). This resulted in plasma FIX:Ag levels of  $2066 \pm 275$  ng/mL and  $2259 \pm 345$  ng/mL, respectively, at 15 minutes after i.v. administration (Figure 1A). There was no statistical difference between rFIX and rFIX-Fc regarding FIX:Ag levels ( $P = .591$ ,  $P = .292$ , and  $P = .083$ , at 15 minutes, 4 hours, and 24 hours after administration, respectively), and pharmacokinetics up to 24 hours after i.v. administration were comparable, as previously described [20].

Although advantages of IND have been demonstrated for small molecules (eg, sumatriptan, oxytocin, insulin) [10,11,25], it is not yet clear whether molecules with higher molecular weight could also be administered effectively via the nasal route, through a passive or receptor-dependent pathway [17]. Therefore, a total of 95 (85 were 8-

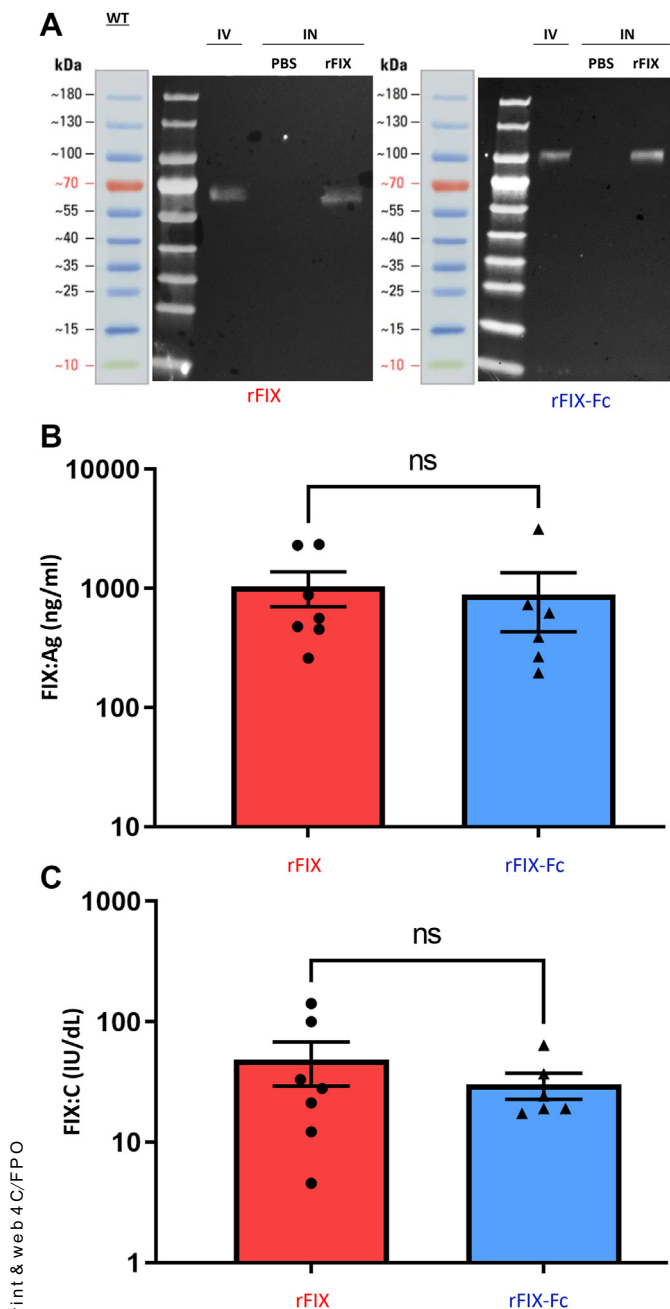


**FIGURE 1** Plasma FIX:Ag levels of rFIX and rFIX-Fc after IV and IND. (A) Kinetics of plasma rFIX and rFIX-Fc concentration 15 min, 4h, and 24h after IV administration in WT mice. Each symbol corresponds to an individual.  $N = 3$ -5. (B) rFIX and rFIX-Fc concentrations 15 min after IND in several mouse strains. Each symbol corresponds to an individual.  $N = 7$ -10. One-way ANOVA statistical analysis was applied and completed by a Dunnett's multiple comparison test. Data are presented as mean  $\pm$  SEM. FcRn, Neonatal Receptor of Fc fragment of Immunoglobulin; hFcRn, humanised strains for FcRn; KO, knock-out; ns, non-significant ( $P > .05$ ); rFIX, recombinant factor IX (SHL-rFIX); rFIX-Fc, recombinant factor IX fused with the Fc fragment of an immunoglobulin (EHL-rFIX); WT, wild type.





**FIGURE 2** rFIX and rFIX-Fc histological analysis of the mucosal uptake after IND. (A) Section of the nasal cavity with respiratory epithelium (RE), olfactory epithelium (OE), and turbinates (T) visible. (B) Section of the olfactory epithelium: results with a humanised FcRn (hFcRn) mouse showed a strong labelling on the outer side of the epithelium within the mucus covering the ciliated cell layer, as well as between the sustentatory cells and neurons up to the lamina propria, and in the vasculature, showing evidence of passive passage with no clear differences between rFIX and rFIX-Fc (a vs d). Similar results were observed with a FcRn KO mouse (b vs e) and with a FIX KO mouse (c vs f). (C) Section of the respiratory epithelium: results with a hFcRn mouse showed both paracellular and transcellular passive passage with a strong labelling between the ciliated cells and inside the ciliated cells with no clear differences between rFIX and rFIX-Fc (a vs d). Similar results were observed with a FcRn KO mouse (b vs e) and with a FIX KO mouse (c vs f). Experiments were performed on 3 mice for each strain to confirm reproducibility but results from only one mouse are shown.



**FIGURE 3** rFIX and rFIX-Fc protein expression and FIX:Ag and FIX:C levels after IND. Both rFIX and rFIX-Fc proteins were found with a single band at their expected molecular weight at 55kDa and 98kDa in wild type mice, respectively (A). FIX:Ag plasma levels (B) and FIX clotting activity (FIX:C) in IU/dL (C) of rFIX and rFIX-Fc of the FIX KO mice ( $n = 7$  and  $n = 6$  for rFIX and rFIX-Fc, respectively). Unpaired *t*-test was performed. Data are presented as mean  $\pm$  SEM. FcRn, Neonatal Receptor of Fc fragment of Immunoglobulin; hFcRn, humanised strains for FcRn; IN, intranasal; KO, knock-out; ns, non-significant ( $P > .05$ ); rFIX, recombinant factor IX (SHL-rFIX); rFIX-Fc, recombinant factor IX fused with the Fc fragment of an immunoglobulin (EHL-rFIX); WT, wild type.

12-weeks old and 10 were older) mice of 4 different strains (WT, hFcRn, FcRn KO, and FIX KO) were used to study the IND of 2 high-molecular weight proteins, rFIX (55 kDa) and rFIX-Fc (98 kDa) [1,20].

Fifteen minutes after IND of 10IU per mice ( $\sim 330$ -500 IU/kg), plasma FIX:Ag levels in WT mice were  $1063 \pm 323$  ng/mL for rFIX and  $1476 \pm 316$  ng/mL for rFIX-Fc ( $P = .823$ ; Figure 1B). The bioavailability after IND was approximately 5- to 6-times lower than that after i.v. administration.

FcRn has been described as playing a key role in the mucosal uptake of biologics [26]. However, the absence of significant differences between rFIX and rFIX-Fc regarding FIX:Ag levels 15 minutes after IND in WT mice suggests that the uptake is FcRn-independent. The nasal mucosal uptake of rFIX and rFIX-Fc was thus tested in 2 FcRn-transgenic mouse models, one that lacks the expression of murine FcRn (FcRn KO) and one that expresses human FcRn (hFcRn) instead. No significant difference in FIX:Ag levels was observed between hFcRn and FcRn KO mice compared with WT mice for both rFIX and rFIX-Fc; FIX:Ag levels after rFIX-Fc IND were  $1328 \pm 398$  ng/mL in hFcRn and  $1673 \pm 463$  ng/mL in FcRn KO mice ( $P = .279$  and  $P = .354$ , respectively; Figure 1B). This confirms that rFIX and rFIX-Fc mucosal uptakes were independent of FcRn.

Histologic analyses of nasal mucosa after rFIX and rFIX-Fc IND were performed (Figure 2). There were no significant differences between strains (WT, hFcRn, FcRn KO, FIX KO) nor biologics tested (rFIX vs rFIX-Fc), supporting the hypothesis that mucosal uptake of rFIX and rFIX-Fc is mostly passive and FcRn-independent. Both paracellular and transcellular mechanism were observed in the olfactory epithelium and respiratory epithelium (RE) (Figure 2). A significant amount of biologics were still visible at the mucosal surface of the olfactory epithelium explaining the lower uptake after IND than that after i.v. administration.

Then, to ensure that the low pH of the nasal mucosa (6.5) does not alter rFIX and rFIX-Fc proteins, we performed Western blot analysis on the plasma of treated mice. Both rFIX and rFIX-Fc proteins were found with a single band at their expected molecular weight at 55 kDa and 98 kDa, respectively (Figure 3A and 3B), confirming that mucosal uptake of rFIX or rFIX-Fc did not alter FIX proteins, irrespective of the mouse strain tested, including FIX KO mice (FIX:Ag levels were  $891 \pm 458$  ng/mL after rFIX-Fc IND, and  $1039 \pm 339$  ng/mL after rFIX administration,  $P = .237$ ). In addition, FIX:C levels were measured at 15 minutes after IND in FIX KO mice. FIX:C was  $48.7 \pm 19.5$  IU/dL for rFIX and  $30.8 \pm 7.4$  IU/dL for rFIX-Fc ( $P = .421$ ; Figure 3D), which was consistent with the FIX:Ag levels measured in these same FIX KO mice (Figure 3C). Results were similar in 8- to 12-week-old and 76-week-old mice (WT ( $n = 5$ ) and hFcRn ( $n = 5$ ), data not presented in Figure 1).

Overall, these results showed that IND of rFIX and rFIX-Fc is effective in increasing plasma FIX:Ag and FIX:C levels in mice and could be considered as an alternative to i.v. administration for the treatment of HB, although higher doses are required, and potentially more frequent administrations would be needed for an efficient prophylaxis use. The variability of plasma FIX:Ag levels after IND was high in all mouse strains (Figure 1B), suggesting that the variability of response depends on the methodology used for IND. Both the duration of exposure and the position of the material in the nasal cavity may determine the success of the administration method [27].

Optimization and standardization of the IND method is therefore needed to lower the variability of response and ensure optimal and repeatable effectiveness for further development in larger animal models and humans. Recently, the use of a polymeric biodegradable film has been described in mice, and was reported as reproducible [27]. It was used with the desire of a nose-to-brain administration, as IND is known to be a valuable tool for administering drugs targeting the brain [25,28]. Enhancing brain uptake of rFIX proteins may be of particular interest in the context of HB where the incidence of ICH is higher than that in the population without hemophilia [8]. Especially in severe HB, neonates or patients with difficult venous access, IND of rFIX could be advised as prophylaxis. Further studies in neonatal mice will be required to evaluate the impact of an immature nasal mucosa on mucosal uptake, safety, and biodistribution of rFIX and rFIX-Fc following IND. Here, we showed that molecules with molecular weight up to 98 kDa can reach the blood compartment following IND. Therefore, it is likely that IND could also be applied to other rFIX molecules such as rFIX fused to albumin, or to rFVIIa for the treatment of FVII deficiency or Glanzmann thrombasthenia [29].

This study showed the proof-of-concept that IND is a non-invasive route of administration that may be developed as an alternative for delivering coagulation factors for the treatment of bleeding disorders such as HB. Additional preclinical studies are required to optimize the methodology of IND and bioavailability and further characterize the efficacy and safety of such an approach in the context of HB. If proven safe and effective for preventing and treating bleeds, we strongly believe that using IND may considerably improve patients' and caregivers' quality of life.

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#### AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design as well as the drafting of the article. M.F. was responsible for statistical analysis of data. S.L.Q. and C.M. revised it critically for important intellectual content, and all authors gave final approval of the version to be submitted.

#### RELATIONSHIP DISCLOSURES

S.L.Q. has received grant/research support from CSL Behring, honoraria from SOBI, Roche, LFB Biomedicaments, Octapharma,

NovoNordisk, and is currently a full-time employee at CSL Behring. All other authors have no competing interests in relation to this work.

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