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Regular article

Targeting Protease Nexin-1, a natural anticoagulant serpin, to control bleeding and improve hemostasis in hemophilia

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Short title: Targeting Protease Nexin-1 to treat hemophilia

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- Protease nexin-1 is an underestimated endogenous inhibitor of thrombin
- Targeting protease nexin-1 improves coagulation in hemophilia

ABSTRACT

Hemophilia A and B, diseases caused by the lack of factor VIII (FVIII) and factor IX (FIX) respectively, lead to insufficient thrombin production, and therefore to bleeding. New therapeutic strategies for hemophilia treatment that do not rely on clotting factor replacement, but imply the neutralization of natural anticoagulant proteins, have recently emerged. We propose an innovative approach consisting of targeting a natural and potent thrombin inhibitor, expressed by platelets, called protease nexin-1 (PN-1). By using the calibrated automated thrombin generation assay, we showed that a PN-1-neutralizing antibody could significantly shorten the thrombin burst in response to tissue factor, in platelet rich plasma (PRP) from patients with mild or moderate hemophilia. In contrast, in PRP from severe hemophilic patients, PN-1 neutralization did not improve thrombin generation. However, after collagen-induced platelet activation, PN-1-deficiency in $F8^{-/-}$ mice or PN-1 blocking in severe patients led to a significantly improved thrombin production in PRP, underlining the regulatory role of PN-1 released from platelet granules. In various bleeding models, $F8^{-/-}$/$PN\text{-}1^{-/-}$ mice displayed significantly reduced blood loss and bleeding time compared to $F8^{-/-}$ mice. Moreover, platelet recruitment and fibrin(ogen) accumulation were significantly higher in $F8^{-/-}$/$PN\text{-}1^{-/-}$ mice than in $F8^{-/-}$ mice in the ferric chloride-induced mesenteric vessel injury model. Thromboelastometry studies showed enhanced clot stability and lengthened clot lysis time in blood from $F8^{-/-}$/$PN\text{-}1^{-/-}$ and from hemophilia A patients incubated with a PN-1-neutralizing antibody, compared to their respective controls. Our study thus provides proof-of-concept that PN-1 neutralization can be a novel approach for future clinical care in hemophilia.

INTRODUCTION

Hemostasis is an essential process allowing minimal blood leakage after an injury, by inducing clotting at the site of vascular damage while maintaining an acceptable level of blood fluidity. This process is characterized by a finely tuned balance between procoagulant, anticoagulant and fibrinolytic systems. Rupture of this balance may result in bleeding disorders like hemophilia. Hemophilia A and B are monogenic X-linked recessive bleeding disorders characterized by a disrupted clotting cascade. Deficiency of procoagulant factor VIII (FVIII) in hemophilia A, or of factor IX (FIX) in hemophilia B, leads to impaired thrombin generation, giving rise to defects in clot formation and therefore spontaneous bleeding. Recently, there has been a surge in innovation in hemophilia treatments. A new considered therapeutic strategy that does not rely on FVIII or FIX replacement, implies the neutralization of natural anticoagulant proteins such as antithrombin (AT) and tissue factor pathway inhibitor (TFPI) to rebalance hemostasis. A small interfering RNA targeting AT[1], and a humanized monoclonal antibody inhibiting TFPI[2] are under study to promote hemostasis in hemophilia. In the same line of thought, an activated protein C (APC)-specific serpin able to rescue thrombin generation in vitro has been proposed to restore hemostasis in hemophilia mouse models[3]. Recently, targeting protein S (PS), the cofactor facilitating the action of APC, has also been proposed to improve hemostasis in hemophilia[4]. Importantly, in contrast to conventional replacement therapy, such blockade of endogenous anticoagulant proteins will not lead to the development of inhibitory alloantibodies against coagulation factors. Moreover, this could represent a treatment approach for patients both without, and most importantly, with inhibitors to clotting factor concentrates.

We hypothesized that another natural anticoagulant protein, protease nexin-1 (PN-1) or serpinE2, could also be an interesting target to promote hemostasis in hemophilia. Indeed, the serpin PN-1 is a highly efficient thrombin inhibitor [5] but is also known to be an effective inhibitor of FXIa[6], a key factor in the amplification of the coagulation cascade. In vitro studies demonstrated that PN-1 inhibits thrombin more effectively than AT[7], even in the presence of heparin[5]. Heparin also strongly accelerates FXIa inhibition by PN-1, making PN-1 a far better inhibitor of FXIa than C1 inhibitor[6,8]. In contrast to AT, PN-1 is barely detectable in plasma but is present in blood cells, and in particular in platelets[9]. Most of platelet PN-1 is stored in the α-granules and is secreted during platelet activation. PN-1 also displays anti-fibrinolytic activity[10], but this activity is relatively minor compared to its anti-thrombin-directed activity.

We have previously shown that PN-1 present in platelets can significantly inhibit both thrombin activity and thrombin generation[11]. Moreover, in vivo studies showed that thrombus formation induced after a vascular lesion was accelerated and facilitated in PN-1-deficient mice compared to wild-type mice. Thus, PN-1 accumulates at the site of the platelet thrombus where it displays its antithrombotic activity via its ability to block both the activity and the generation of thrombin.

Here we examined, for the first time, the effects of PN-1 inhibition on thrombin generation in murine and human hemophilia platelet-rich plasma, and on hemostasis and thrombosis in mouse hemophilia models.

MATERIALS & METHODS

Human hemophilia blood. Blood from patients with hemophilia A and B was provided by the Hemophilia Center (Hospital André Mignot, Le Chesnay, France). Approval was obtained from the Ethic Evaluation Committee and the Institutional Review Board of the French Institute of Medical Research and Health; and all subjects gave written informed consent for blood sampling.
Hemophilia patients without inhibitors were classified depending on the deficit severity level. Mild, moderate and severe hemophilia refer to circulating FVIII or FIX levels between 5% and 40%, between 1% and 5% and less than 1%, respectively. None of the patients with hemophilia B, or with mild and moderate hemophilia A were on prophylactic treatment at the time of blood sampling. Four patients with severe hemophilia A were on prophylactic treatment but had a circulating FVIII level strictly inferior to 1% at the time of blood sampling.

Preparation of human plasma. Blood was drawn by venipuncture using a vacutainer system (Becton Dickinson) containing 109 mM trisodium citrate as anticoagulant in the ratio of 1-part anticoagulant to 9-parts of blood. Platelet-rich plasma (PRP) was obtained by centrifugation at 120 *g* for 15 min at room temperature (RT). Platelet-poor plasma (PPP) was obtained by a double centrifugation: after a first centrifugation of blood at 1200g for 12 min, plasma was subsequently centrifuged at 10 000 g for 5 min to remove residual platelets and cell fragments. PRP was adjusted to 160 x 10^3 platelets/μL with autologous PPP.

Mice. PN-1-deficient mice (PN-$1^{-/-}$) were generated by heterozygous mating and bred in-house. PN-1 heterozygous mice (PN-$1^{+/-}$) and F8 deficient mice were crossed to obtain double knock-out mice for F8 and PN-1 ($F8^{-/-}$/PN-$1^{-/-}$). $F8^{+/+}$/PN-$1^{-/-}$ (PN-1-deficient), $F8^{+/+}$/PN-$1^{+/+}$ (Wild-Type) and $F8^{-/-}$/PN-$1^{+/+}$ (hemophilia) mice were generated by heterozygous mating and bred in-house. All mice had a C57Bl/6 genetic background. PN-1 and F8 genotypes were determined by PCR. All experiments were performed on littermates in accordance with French ethical laws (agreement number, APAFIS n° 4342).

Preparation of murine plasma. Mouse blood was sampled from the inferior vena cava under 5% isoflurane anesthesia. The abdominal cavity was opened, and blood was drawn using a 23-gauge needle and 1 mL plastic syringe previously charged with 100 μL of 3.2 % sodium citrate. PRP was prepared immediately after blood drawing by centrifugation at 200 g for 3 min at RT. PPP was

obtained by a double centrifugation: the first at 2500 g for 15 min at RT, the second at 10 000 g for 5 min to remove residual platelets and cell fragments. PRP was adjusted to 450 $x10^3$ platelets/µL with autologous PPP.

Thrombin generation assay (TGA). Assays were performed in a 96-well micro-titer plate in a final volume of 120 μL/well. Samples spiked with thrombin calibrator (Diagnostica Stago) were run in parallel with each cycle of test sample. Thrombin generation was triggered by adding $CaCl_2$ (16.7 nM final concentration) and fluorogenic substrate Z-Gly-Gly-Arg-AMC (417 µM final concentration) in Fluo Buffer pH 7.5 (20 mM HEPES, 60 g/L BSA). Fluorescence was measured during 60 min and analyzed by the Thrombinoscope software (Thrombinoscope® BV). All experiments were carried out in triplicate.

Human Assay: Adjusted hemophilic PRP was preincubated with 100 µg/mL blocking in-house polyclonal rabbit anti-PN-1 antibody [11] that was produced after injection of recombinant human PN-1 in rabbits, or 100 µg/ml Irrelevant IgG (Immunoresearch) for 15 minutes at 37°C before transfer to microtitration plates containing 0.5 pM tissue factor (TF; Diagnostica Stago) in the presence or not of collagen at 5 μg/mL (Horm, Nicomed Pharma).

Mouse Assay: Adjusted hemophilic PRP from $F8^{+/+}$/PN-$1^{+/+}$, $F8^{+/+}$/PN-$1^{-/-}$, $F8^{-/-}$/PN-$1^{-/-}$mice or $F8^{-/-}$/PN-$1^{+/+}$ preincubated or not with 100 µg/mL blocking in-house polyclonal rabbit anti-PN-1 antibody[11] or 100 µg/ml Irrelevant IgG (Immunoresearch), were transferred to microtitration plates containing 1 pM TF, in the presence or not of collagen at 5 µg/mL final (Horm, Nicomed Pharma).

Tail clip bleeding model. Mice were anesthetized with 2% isoflurane. The tail was immersed in a pre-warmed saline solution (37°C) in a 15 mL tube. Ten minutes later, 3-mm of the tail tip was amputated using a scalpel and the tail immediately re-immersed in the saline. Bleeding time was measured from the moment of transection until the first arrest of bleeding and blood was collected and quantified. The observation was stopped at 6 min if bleeding continued.

Tail Vein Transection (TVT) or Tail Artery Transection (TAT) bleeding models. TVT and TAT assays were performed essentially as described[12]. Briefly, mice were anesthetized with 2% isoflurane. The tail was placed into a tube filled with pre-warmed saline (37°C). Ten minutes after, using a specially designed transection template, the tail was cut at 2.5 mm diameter through a 0.5 mm deep incision transecting either the left lateral vein or the ventral artery, for TVT or TAT bleeding models respectively. Immediately after injury, the tail was re-immersed in the saline and the

bleeding was monitored during 60 min. In both bleeding models, if the mouse was not bleeding at 15, 30 and 45 min post injury, the wound was challenged by gently wiping it twice with a saline wetted gauze swab in the distal direction. Immediately after the challenge, the tail was re-submerged into the saline. At 60 min, mice were killed by cervical dislocation while still under full anesthesia.

Blood loss quantification. Blood loss was determined by collecting blood cells via a centrifugation at 1500 g for 25 min. After lysis of blood cells with a known volume of water, hemoglobin content was determined by measuring absorbance at 575 nm[13]. A standard curve in which known volumes of mouse blood were used to extract hemoglobin served to calculate blood loss volumes.

Real-time in vivo imaging of thrombus formation. Mice were anesthetized by the intraperitoneal injection of 100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine in saline. Then, Alexa Fluor 647–conjugated antibody against glycoprotein IX (GPIX) (0.12 mg/kg mouse, Emfret Analytics) and FITC conjugated-human-fibrinogen (10mg/kg mouse, Invitrogen life technology) were injected into the retro-orbital sinus. The mesentery was gently exteriorized and small vessels were visualized by intravital microscopy using an upright fluorescence macroscope (MacroFluo, Leica Microsystems) equipped with a thermostated heating plate and a 5X objective, and connected to a sCMOS camera (Orca-Flash-4.0, Hamamatsu Photonics) for image acquisition using Metamorph software (Molecular Devices). A filter paper strip (1×2 mm) was immersed in a ferric chloride solution (10% $FeCl_3$; Sigma-Aldrich) and applied to the surface of the selected vessels. After 3 minutes of exposure, the filter paper was removed. Thrombus formation following injury was examined in real-time by monitoring the accumulation of fluorescence for 50 min.

Rotational Thromboelastometry (ROTEM). Whole blood coagulation profiles were recorded by a ROTEM® Coagulation Analyzer (Tem International). Pre-warmed ROTEM® cups were prepared with 300 µL of citrated human or murine whole blood in the presence of tissue-plasminogen activator at 0.5 or 1µg/ml, respectively (r-tPA, Alteplase, Boehringer) to induce fibrinolysis. Human blood samples were preincubated with 100 µg/mL irrelevant IgG or 100 µg/mL polyclonal anti-PN-1 antibody at RT for 15min. Clotting was initiated by adding 20 µL of extrinsically activated ROTEM® assay reagent (ExTEM®, Tem International) pre-diluted at 1:50 for murine samples or undiluted for human samples, and 20 µL of buffered concentrated calcium chloride solution (Star-TEM® reagent, Tem International) according to the manufacturer's recommendation. Clotting time (CT, s),

maximum clot firmness (MCF, mm), lysis onset time (LOT, s), lysis time (LT, s) and maximum clot elasticity (MCE) were assessed using ROTEM® software.

Statistical analyses. Results are shown as means ± standard error of the mean (SEM). The Shapiro-Wilk test was applied to analyze the data distribution and determine the use of parametric or non-parametric tests. Statistical analysis of differences between groups was performed using the appropriate test as indicated. A *P* value less than to 0.05 was considered significant.

Data sharing statement. Supplemental data may be found in a data supplement available on the *Blood* web site with the online version of this article.

RESULTS

PN-1 inhibition enhances thrombin generation in platelet-rich plasma (PRP) from patients with mild and moderate hemophilia A and B but has no such effect in PRP from severely affected patients.

We determined thrombin generation in PRP using the calibrated automated thrombin generation assay (TGA). In this assay, we tested PRP from mild and moderate hemophilia patients, in the presence of a polyclonal anti-PN-1 antibody that abolishes the protease inhibitory activity of PN-1 (Figure 1). The mean values of TGA parameters: lag time (LT), thrombin peak, Time to Peak (TTP), Endogenous Thrombin Potential (ETP) and calculated velocity are summarized in Table 1. Mild and moderate hemophilia A patients displayed a significantly higher thrombin peak height and a reduced time to peak, in the presence of the neutralizing anti-PN-1 antibody compared to values obtained with the irrelevant antibody (Figure 1A-B and Table 1A). Increased peak and velocity of thrombin generation were also observed in PRP from hemophilia B patients in the presence of the neutralizing anti-PN-1 antibody (Figure 1C-D and Table 1B). Even if the endogenous thrombin potential was not modified, our data clearly indicate that blocking PN-1 can significantly shorten the thrombin burst in mildly and moderately affected patients, as illustrated by the increased velocity (Table 1).

TGA was also performed in platelet-poor plasma (PPP) from hemophilia A and B patients, in the presence of the polyclonal anti-PN-1 antibody. As expected, the anti-PN-1 antibody had no effect on the mean values of TGA in these patients, confirming the platelet origin of PN-1 (Supplemental Table 1).

Moreover, TGA was performed on PRP from severe hemophilia A and B patients (Figure 2 and Table 2A). The anti-PN-1 antibody had no significant effect on thrombin generation parameters either in the PRP (Table 2) or in the PPP of these patients (supplemental Table 1). Thus, a minimum of generated thrombin was required to observe a significant improvement of coagulation in the presence of the neutralizing anti-PN-1 antibody.

PN-1 inhibition enhances thrombin generation in PRP from severe hemophilia A and B patients, after collagen-induced platelet activation.

Thrombin generation is known to be greatly increased when TGA is performed in PRP incubated with collagen[14]. Indeed, under this condition, there is a strong induction of platelet activation and therefore of platelet release. We thus performed TGA in PRP from severe hemophilia

patients in the presence of collagen to accentuate platelet PN-1 release. Interestingly, under this condition, blocking PN-1 can increase thrombin generation (Figure 3 and Table 2B). These results underline the regulatory role of PN-1 released after platelet activation on thrombin generation.

PN-1-deficiency or PN-1 blocking enhances thrombin generation in PRP from mice with F8-deficiency after collagen-induced platelet activation.

To support our data obtained with PRP from severe hemophilia A patients, TGA was also performed on PRP from WT, PN-$1^{-/-}$, $F8^{-/-}$, $F8^{-/-}$/PN-$1^{-/-}$ and $F8^{-/-}$ mice preincubated with the anti-PN-1 antibody. The mean values of TGA parameters are summarized in Table 3. As previously described, TF-triggered thrombin generation was accelerated and increased in the PRP from PN-$1^{-/-}$ compared to WT[11]. As expected, thrombin generation was clearly abnormal in PRP from $F8^{-/-}$ mice with decreases in thrombin peak height, velocity and ETP compared to WT and PN-$1^{-/-}$ mice. Under our experimental conditions, no significant differences in the mean values of TGA parameters were observed between $F8^{-/-}$, and $F8^{-/-}$/PN-$1^{-/-}$ mice (Figure 4A-B; Table 3) or between PRP from $F8^{-/-}$ preincubated with the anti-PN-1 antibody *vs* $F8^{-/-}$ preincubated with the irrelevant antibody (Figure 4E-F; Table 3).

To confirm the role of PN-1 released after platelet activation on thrombin generation, we also performed TGA on PRP from $F8^{-/-}$, $F8^{-/-}$/PN-$1^{-/-}$ and $F8^{-/-}$ mice preincubated with the anti-PN-1 antibody, in the presence of collagen to accentuate platelet PN-1 release. Thrombin generation was significantly increased in PRP from $F8^{-/-}$/PN-$1^{-/-}$ compared with PRP from $F8^{-/-}$, in response to TF and collagen activation (Figure 4C-D; Table 4). Neutralizing PN-1 with the anti-PN-1 antibody in PRP from $F8^{-/-}$ mice, also significantly increased thrombin generation in response to TF and collagen activation (Figure 4G-H, Table 4).

PN-1-deficiency limits tail bleeding in mice with F8-deficiency.

To determine the *in vivo* efficacy of PN-1-deficiency for reducing bleeding in hemophilic mice, blood loss was measured in three different models: the classical tail clip assay, and the tail vein and artery transection bleeding models. In the classical tail clip model, the bleeding phenotype of hemophilia was totally rescued in $F8^{-/-}$/PN-$1^{-/-}$ mice (Figures 5A-B). Indeed, bleeding volumes and times were similar between $F8^{-/-}$/PN-$1^{-/-}$ and WT mice, whereas $F8^{-/-}$ mice displayed, as expected, a prolonged bleeding time and an increased blood loss. The tail tip bleeding model is a common and easy test to evaluate hemostasis in mice. However, caution must be taken in drawing broad conclusions when using only one bleeding model. We thus performed the complementary tail vein

transection bleeding model, a highly sensitive hemostasis model[12]. In this model, blood loss and bleeding time were significantly reduced in $F8^{-/-}$/PN-$1^{-/-}$ mice compared with $F8^{-/-}$ mice (Figures 5C-D). The results obtained with the tail artery transection model showed the same significant differences: reduced blood loss and bleeding time in $F8^{-/-}$/PN-$1^{-/-}$ mice compared with $F8^{-/-}$ mice (Figures 5E-F). Taken together, these results demonstrate that PN-1-deficiency reduces blood loss and thus can provide protection against bleeding.

PN-1-deficiency does not lead to excessive thrombosis in mice with F8-deficiency in a mesenteric vessel ferric chloride-induced injury model.

The impact of PN-1-deficiency on clotting was also assessed in vivo using the ferric chloride mesenteric vessel injury. In this model, we monitored thrombus formation over time, by visualizing platelets (red) and fibrin (green) depositions, using intravital microscopy. Representative images from recorded videos are presented in Figure 6A. Thirty minutes after mesenteric vessel injury, occlusive thrombi were observed both in venules and arterioles of WT mice (Figure 6A). As expected, in $F8^{-/-}$ mice, venules showed very few, if any, platelet-fibrin depositions during the entire period of recording, and none of the arterioles presented thrombi. In contrast, $F8^{-/-}$/PN-$1^{-/-}$ mice showed small subocclusive thrombi in the mesenteric vessels. Fluorescence quantification over time showed that platelet accumulation and fibrin deposition on mesenteric arterioles were significantly increased in $F8^{-/-}$/PN-$1^{-/-}$ mice compared to $F8^{-/-}$ mice, without reaching fluorescence levels observed in WT mice (Figures 6B-C). Regarding mesenteric venules, platelet accumulation was also significantly increased in $F8^{-/-}$/PN-$1^{-/-}$ mice compared to $F8^{-/-}$ mice, and fibrin accumulation tended to be increased; this tendency being significant only during the first fifteen minutes after injury (Figures 6D-E). However, even if thrombus formation was promoted in $F8^{-/-}$/PN-$1^{-/-}$ mice, injured vessels were never occluded.

PN-1-deficiency in mice with F8-deficiency or PN-1 inhibition in hemophilia A patients improves clot stability and limits clot lysis.

Clot stability is an important hemostatic aspect in the treatment of hemophilia A. We investigated the effect of PN-1-deficiency in mice or the effect of PN-1 blocking in hemophilia A patients, on blood clot stability and lysis, using the rotational thromboelastometry (ROTEM)-based assay, with tissue plasminogen activator (tPA) as the clot lysis trigger. Representative thromboelastogram profiles are shown in Figures 7A and 7D. Under our experimental conditions, ROTEM parameters were similar between WT and PN-$1^{-/-}$ mice (Table 5A). As expected, clots from

hemophilic mice displayed not only abnormal structure compared with WT, as illustrated by the very low maximum clot firmness (MCF), but also excessive susceptibility to fibrinolysis, as illustrated by the shortening of the lysis onset time (LOT) (Table 5A). Interestingly, both MCF and LOT were significantly increased in $F8^{-/-}$/PN-$1^{-/-}$ mice compared with $F8^{-/-}$ mice (Figure 7B-C; Table 5A). Similarly, clots from hemophilia A patients displayed improved MCF and LOT in the presence of the neutralizing anti-PN-1 antibody compared to values obtained with the irrelevant antibody (Figure 7E-F; Table 5B). These results demonstrate that in the context of hemophilia, PN-1-deficiency or neutralizing PN-1 does not induce excessive fibrinolysis.

DISCUSSION

Research in hemophilia management is currently undergoing major changes by developing novel strategies to rebalance the hemostatic system. Some new approaches consist of targeting negative clotting regulators like AT, TFPI, APC or PS. Neutralizing another anticoagulant serpin like PN-1 is a concept that has not yet been proposed up to now for hemophilia treatment. This is probably due to the fact that PN-1 is an underestimated negative regulator of thrombin generation. Yet, *in vitro* studies demonstrated that PN-1 inhibits thrombin at a rate approximately 100-fold faster than AT[7]. Thus, by directly inhibiting thrombin activity, PN-1 is able to regulate the pleiotropic activities of thrombin involved in the coagulation cascade, such as the direct activations of FVIII, FV, FXI and fibrin formation. The increased thrombin generation obtained in PRP from patients with hemophilia A incubated with a neutralizing anti-PN-1 antibody, and the *in vivo* improvement of the bleeding phenotype of $F8^{-/-}$/$PN\text{-}1^{-/-}$ mice observed in the different tail-bleeding assays clearly demonstrate that targeting PN-1 can improve hemostasis in hemophilia.

Our data with the TGA showed that blocking PN-1 improved TF-initiated thrombin generation in PRP from hemophilia patients displaying a minimum of 1% plasma FVIII. This result thus indicates that blocking PN-1 may be used in mildly or moderately affected hemophilia A patients, as an adjuvant therapy to reduce FVIII injections. When PN-1 was blocked, the total amount of thrombin generated was not increased, however thrombin formation was clearly accelerated and facilitated. Thus targeting PN-1 in the hemophilia context makes sense because the capacity for thrombin production in the initial stages of coagulation is essential for optimum hemostasis.

Importantly, neither blocking PN-1 in PPP from patients with hemophilia A nor PN1-deficiency in PPP from $F8^{-/-}$/$PN\text{-}1^{-/-}$ mice had an effect on TF-initiated thrombin generation, because PN-1 is not present in plasma but is principally expressed in platelets. We have previously demonstrated the anticoagulant properties of platelet PN-1[11]. In the context of hemophilia, such platelet properties are of particular interest. Indeed, platelets play a central role in the promotion and regulation of thrombin generation. In hemophilia, there is a failure of platelet-surface FX activation, leading to a decrease in platelet-surface thrombin generation and ineffective clot formation[15]. Therefore, PN-1 appears as a platelet-dependent factor, playing a non-negligible regulatory role in hemophilia via platelet-dependent thrombin generation. This was further confirmed by our data obtained when TGA was performed in PRP incubated with collagen, a strong agonist for platelet activation and release. We indeed observed that thrombin generation remained flat in PRP from $F8^{-/-}$

/PN-1$^{-/-}$ mice in the presence of TF alone, whereas it was improved in the presence of TF mixed with collagen. This data demonstrates that platelet PN-1 released from the α-granules has a direct impact on coagulation and thus regulates thrombin generation on platelets.

Drugs targeting other natural anticoagulant proteins have been developed for the treatment of hemophilia. A therapeutic RNAi targeting AT has indeed been developed. AT is a highly concentrated plasma protein and, therefore, targeting AT via inhibitory molecules will require caution to avoid uncontrolled rebalancing of the hemostatic system in hemophilia. A serpin engineered to specifically inhibit APC was also found to rescue hemostasis in a hemophilia mouse model. Because APC provides also cytoprotection through its antiapoptotic and anti-inflammatory effects, targeting APC should allow inhibition of its anticoagulant activity with preservation of its anti-inflammatory and cytoprotective effects. Targeting TFPI is another therapeutic strategy developed for hemophilia. However, TFPI displays different splicing forms (TFPIα and TFPIβ) that act in different ways, and its bio-distribution is complex. Targeting PN-1 would have the advantage of reinforcing thrombin generation, specifically on platelet surfaces, and thus could represent a safer approach for the treatment of hemophilia. When targeting PN-1, we can act in a spatially restricted area that corresponds to the site of injury where activated platelets are present. Another important positive point concerning PN-1 targeting strategy is the absence of thrombotic risk demonstrated by our data in the *in vivo* model of thrombosis. Indeed, although PN-1 deficiency improved hemostasis in hemophilic mice, the kinetics and extent of thrombus formation remained lower than that measured in WT mice.

The abnormal composition of hemophilic clots makes them unstable and prone to premature lysis. This abnormal clot fragility is explained by altered activation of FXIII by thrombin, leading to impaired crosslinking of fibrin and therefore to the formation of weaker clots[16]. The high susceptibility of hemophilic clots to fibrinolysis is explained by a defective downregulation of fibrinolysis due to the lack of thrombin-activatable fibrinolysis inhibitor (TAFI) activation by thrombin[17]. We thus assumed that PN-1-deficiency could favor thrombin effects on the hemophilic clot structure. Our data obtained by ROTEM confirmed this hypothesis, since clot strength and lysis onset time were significantly increased in F8$^{-/-}$/PN-1$^{-/-}$ mice compared with F8$^{-/-}$ mice. This increased lysis time may appear surprising since we have previously shown that PN-1 could also regulate fibrinolysis by inhibiting plasmin generation and activity. This apparently conflicting result is explained by the fact that the effect of PN-1-deficiency on clot stability in normal blood is not comparable to that in hemophilic blood. Indeed, in hemophilia, enhanced fibrinolysis results from defective clotting and abnormal fibrin. Thus, in this pathology, neutralizing PN-1 is able to correct

such secondary hyperfibrinolysis. Since various *in vitro* studies previously showed that PN-1 inactivates thrombin at a rate at least 1000-fold faster than plasminergic proteases[5,18,19], we can assume that in the context of hemophilia, the anti-thrombin activity of PN-1 is dominant compared to its anti-fibrinolytic activity.

This study provides the proof-of-concept that targeting PN-1 is an original and novel approach for future clinical care in hemophilia. Interestingly, the platelet strategy has become an approach chosen by several groups who try to use platelets as a target to deliver therapeutics for hemophilia A treatment[20]. Further studies would be justified to assess whether accentuating PN-1 targeting directly inside platelets could be a promising approach.

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AUTHORSHIP CONTRIBUTIONS

K.A., C.K. and L.V. designed and performed the experiments, analyzed the data; E.d.R. recruited the patients, contributed to the experimental design and proofread the manuscript; Y.B., O.C., P.L., V.A. and C.D. contributed to the experimental design and proofread the manuscript; M-C.B designed the study, supervised the research and analyzed the data; K.A., C.K. and M-C. B. wrote the manuscript.

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CONFLICT OF INTEREST DISCLOSURES

The authors declare no competing financial interests

ADDITIONAL INFORMATION

Supplemental information for this article includes 1 table.

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TABLES

Table 1: Parameters of thrombin generation in PRP from patients with mild and moderate Hemophilia A and B.

A.

	Patients with mild hemophilia A (n=10)			Patients with moderate hemophilia A (n=6)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT (min)	10.32 ± 2.92	10.33 ± 2.96	10.32 ± 3.02	11.98 ± 1.70	12.09 ± 1.70	11.89 ± 1.83
Peak (nM)	87.68 ± 32.70	87.16 ± 35.19	110.8 ± 42.10 **	75.05 ± 21.33	71.88 ± 24.37	96.25 ± 32.91 *
TTP (min)	23.23 ± 5.09	23.34 ± 5.07	21.55 ± 4.68***	25.41 ± 4.63	25.73 ± 4.82	22.92 ± 4.30 *
ETP (nM.min)	1485 ± 381.0	1474 ± 382.2	1442 ± 374.2	1341 ± 448.0	1288 ± 452.6	1299 ± 410.6
Velocity (nM/min)	7.26 ± 3.63	7.07 ± 3.64	10.99 ± 6.63**	5.77 ± 2.24	5.56 ± 2.67	9.19 ± 4.65 *

B.

	Patients with mild hemophilia B (n=5)			Patients with moderate hemophilia B (n=3)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT (min)	11.53 ± 1.55	11.33 ± 1.76	11.41 ± 1.87	14.62 ± 2.09	15.29 ± 3.57	15.74 ± 3.90
Peak (nM)	75.28 ± 11.50	77.49 ± 12.65	97.73 ± 16.28 *	29.92 ± 6.12	31.07 ± 5.41	43.09 ± 3.31
TTP (min)	22.31 ± 3.76	21.88 ± 3.91	20.69 ± 2.78	27.11 ± 2.93	30.01 ± 6.32	29.73 ± 3.53
ETP (nM.min)	1368 ± 411.2	1395 ± 374.4	1292 ± 303.4	775,6 ± 173.0	1013 ± 491.1	813.1 ± 264.9
Velocity (nM/min)	7.44 ± 1.92	7.51 ± 1.98	10.60 ± 1.73 *	2,47 ± 0.80	2.33 ± 0.85	2.80 ± 0.44

Means ± SD; A non-parametric one-way ANOVA test followed by a multiple comparison test was used; **P*<0.05, ***P*<0.01, ****P*<0.001 *vs* "PRP + irrelevant IgG".

Table 2: Parameters of thrombin generation in PRP from severe hemophilia A and B patients (A) without or (B) with collagen-induced platelet activation.

A.

	Patients with severe hemophilia A (n=8)				Patients with severe hemophilia B (n=4)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1		PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT (min)	12.82 ± 6.87	13.05 ± 7.03	13.40 ± 7.36	LT (min)	8.21 ± 6.0	8.90 ± 3.77	8.44 ± 3.73
Peak (nM)	20.21 ± 12.18	19.19±10.44	20.96 ± 11.76	Peak (nM)	9.03 ± 7.07	9.19 ± 6.42	11.97 ± 6.24
TTP (min)	40.40 ± 22.26	41.45 ± 22.95	39.15 ± 23.94	TTP (min)	24.25 ± 7.0	24.21 ± 6.39	28.94 ± 2.45
ETP (nM.min)	230.9 ± 336.8	235.9 ± 323.6	360.5 ± 383.5	ETP (nM.min)	254.9 ± 198.9	272.3 ± 149.3	398.2 ± 140.8
Velocity (nM/min)	0.74 ± 0.51	0.67 ± 0.45	0.87 ± 0.61	Velocity (nM/min)	0.51 ± 0.43	0.59 ± 0.36	0.65 ± 0.48

B.

	Patients with severe hemophilia A (n=6)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT (min)	12.48 ± 4.61	12.14 ± 4.72	11.23 ± 4.70
Peak (nM)	22.59 ± 11.66	23.59 ± 11.65	31.49 ± 16.20 *
TTP (min)	40.30 ± 5.67	39.90 ± 5.49	34.94 ± 6.31*
ETP (nM.min)	1060 ± 587.0	1103 ± 579.0	1172 ± 581.9
Velocity (nM/ min)	0.81 ± 0.39	0.88 ± 0.41	1.39 ± 0.81 *

	Patients with severe hemophilia B (n=3)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT (min)	8.85 ± 1.08	8.39 ± 1.08	8.30 ± 1.75
Peak (nM)	19.56 ± 6.64	19.56 ± 6.11	38.95 ± 10.62
TTP (min)	29.59 ± 0.92	28.30 ± 1.45	26.04 ± 2.93 *
ETP (nM.min)	1046 ± 243.2	926.1 ± 300.3	1429 ± 194.0*
Velocity (nM/ min)	1.05 ± 0.31	0.99 ± 0.32	2.23 ± 0.78

Means ± SD; A non-parametric one-way ANOVA test followed by a multiple comparison test was used; *$P<0.05$ *vs* "PRP + irrelevant IgG".

Table 3: Parameters of thrombin generation in murine PRP.

	WT (n=7)	PN-1$^{-/-}$ (n=6)	F8$^{-/-}$(n=7)	F8$^{-/-}$/PN-1$^{-/-}$ (n=6)
LT , min	5.47 ± 1.82	5.12 ± 2.07	7.34 ± 2.84	6.26 ± 1.23
Peak, nM	10.36 ± 4.47	19.53 ± 9.29	3.05 ± 2.44	3.30 ± 3.69
TTP, min	12.80 ± 2.86	11.12 ± 3.02	11.96 ± 1.98	11.71 ± 2.02
ETP, nM.min	179.5 ± 47.62	222.3 ± 120.4	31.11 ± 35.25	25.95 ± 10.45
Velocity, nM/ min	1.64 ± 0.79	3.35 ± 2.15	0.44 ± 0.26	0.58 ± 0.41

	F8$^{-/-}$(n=10)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT , min	2.43 ± 1.06	5.63 ± 7.21	2.78 ± 1.18
Peak, nM	2.38 ± 1.71	2.17 ± 1.82	2.40 ± 1.97
TTP, min	7.05 ± 3.38	9.51 ± 7.45	7.12 ± 3.40
ETP, nM.min	29.55 ± 23.69	25.83 ± 23.19	27.10 ± 22.17
Velocity, nM/ min	0.48 ± 0.37	0.42 ± 0.35	0.47 ± 0.28

Thrombin generation was triggered by TF. Means ± SD; A Mann-Whitney test was used in the left-hand table. A non-parametric one-way ANOVA test followed by a multiple comparison test was used in the right-hand table.

Table 4: Parameters of thrombin generation in murine PRP after collagen-induced platelet activation.

	WT (n=22)	PN-1$^{-/-}$ (n=12)	F8$^{-/-}$(n=13)	F8$^{-/-}$/PN-1$^{-/-}$ (n=15)
LT , min	4.92 ± 1.30	5.14 ± 0.83	3.57 ± 0.75	3.63 ± 0.88
Peak, nM	27.06 ± 7.43	27.41 ± 9.81	5.50 ± 2.65	8.50 ± 3.74 *
TTP, min	9.57 ± 1.73	10.39 ± 1.87	10.55 ± 1.53	10.81 ± 1.11
ETP, nM.min	373.8 ± 134.4	361.8 ± 136.0	65.47 ± 28.04	114.3 ± 61.90 *
Velocity, nM/ min	5.89 ± 2.08	5.78 ± 3.17	0.99 ± 0.63	1.05 ± 0.61

	F8$^{-/-}$(n=10)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
LT , min	3.36 ± 0.74	4.67 ± 3.57	3.67 ± 0.55
Peak, nM	7.18 ± 6.28	7.16 ± 6.29	9.73 ± 8.05 **
TTP, min	9.12 ± 1.74	9.93 ± 2.84	8.36 ± 0.94
ETP, nM.min	88.45 ± 74.25	79.43 ± 65.34	87.30 ± 66.90
Velocity, nM/ min	1.37 ± 1.38	1.41 ± 1.37	2.3 ± 1.98 **

Thrombin generation was triggered by TF and collagen. Means ± SD; A Mann-Whitney test was used in the left-hand table; *P<0.05 *vs* $F8^{-/-}$ mice. A non-parametric one-way ANOVA test followed by a multiple comparison test was used in the right-hand table; **P<0.01 *vs* "PRP + irrelevant IgG".

Table 5: Parameters of thromboelastography in PRP from hemophilia mice and patients.

A.

	WT (n=9)	$PN\text{-}1^{-/-}$(n=9)	$F8^{-/-}$ (n=9)	$F8^{-/-}/PN\text{-}1^{-/-}$ (n=9)
CT, s	216.8 ± 33.21	225.7 ± 26.99	246.6 ± 39.84	241.2 ± 60.42
MCF, mm	56.56 ± 9.14	56.33 ± 6.44	19.33 ± 15.39	35.56 ± 16.61 *
LOT, s	2139 ± 555.0	2383 ± 1001	796.3 ± 583.3	1498 ± 1019 *
LT, s	3079 ± 896.2	3609 ± 1557	2066 ± 479.1	2639 ± 1393
MCE	137.6 ± 42.37	133.2 ± 30.32	28.89 ± 31.61	65.78 ± 51.10

B.

	Patients with severe hemophilia A (n=5)		
	PRP alone	PRP + irrelevant IgG	PRP + anti-PN-1
CT, s	90.20 ± 12.81	90.80 ± 7.64	88.70 ± 7.13
MCF, mm	25.25 ± 11.52	23.17 ± 7.42	30.00 ± 9.93 *
LOT, s	222.80 ± 50.41	223.60 ± 30.62	295.70 ± 64.69 *
LT, s	358.40 ± 91.40	387.20 ± 102.10	480.10 ± 134.10
MCE	30.20 ± 16.39	26.90 ± 7.89	38.90 ± 15.52 *

Means ± SD; A two-way ANOVA test in table A or a non-parametric one-way ANOVA test in table B followed by a multiple comparison test were used; *P<0.05 *vs* $F8^{-/-}$ mice or *vs* « PRP + irrelevant IgG ».

FIGURE LEGENDS

Figure 1. PN-1 inhibition enhances thrombin generation in platelet-rich plasma (PRP) from mild and moderate hemophilia A and B patients.

Peaks were measured in PRP from mild and moderate hemophilia A patients (A, B) or hemophilia patients B patients (C, D), incubated with 100 μg/mL irrelevant IgG antibody or 100 μg/mL anti-PN-1 antibody. Each dot represents one patient. Values are means ± SEM. A non-parametric one-way ANOVA test followed by a multiple comparison test was used; **P*<0.05, ***P*<0.01.

Figure 2. PN-1 inhibition has no effect thrombin generation in platelet-rich plasma (PRP) from severe hemophilia A and B patients.

Representative thrombin generation profiles from a severe hemophilia A patient (A) and a severe hemophilia B patient (C). Peaks were measured in PRP from severe hemophilia A patients (B) and severe hemophilia patients B patients (D), incubated with 100 μg/mL irrelevant IgG antibody or 100 μg/mL anti-PN-1 antibody. Each dot represents one patient. Values are means ± SEM. A non-parametric one-way ANOVA test followed by a multiple comparison test was used.

Figure 3. PN-1 inhibition enhances thrombin generation in platelet-rich plasma (PRP) from severe hemophilia A and B patients after collagen-induced platelet activation.

Representative thrombin generation profiles from a severe hemophilia A patient (A) and a severe hemophilia B patient (C), after TF and collagen activation. Peaks were measured in PRP from severe hemophilia A patients (B) and severe hemophilia patients B patients (D), incubated with 100 μg/mL irrelevant IgG antibody or 100 μg/mL anti-PN-1 antibody. Each dot represents one patient. Values are means ± SEM. A non-parametric one-way ANOVA test followed by a multiple comparison test was used; **P*<0.05.

Figure 4. PN-1-deficiency enhances thrombin generation in PRP from mice with an F8-deficiency after collagen-induced platelet activation.

Representative thrombin generation profiles and thrombin peaks obtained after TF activation (A-B; E-F), or after TF and collagen activation (C-D;G-H) in PRP from $F8^{-/-}$, $F8^{-/-}$/$PN\text{-}1^{-/-}$ and $F8^{-/-}$ mice in the presence of 100 μg/mL irrelevant IgG antibody or 100 μg/mL anti-PN-1 antibody. Each dot represents one mouse. Values are means ± SEM. A Mann-Whitney test was used for B and D;

*P<0.05. A non-parametric one-way ANOVA test followed by a multiple comparison test was used for F and H; *P<0.05, **P<0.01.

Figure 5. PN-1-deficiency limits tail bleeding in mice with an F8-deficiency.
Tail bleeding times and volumes in WT, PN-$1^{-/-}$, $F8^{-/-}$ and $F8^{-/-}$/PN-$1^{-/-}$ mice in a classical tail clip model (A, B), in a tail vein transection model (C, D) and in a tail artery transection model (E, F). Each dot represents one mouse. A non-parametric one-way ANOVA test followed by a multiple comparison test was used; *P<0.05, **P<0.01, ***P<0.001.

Figure 6. PN-1-deficiency effect in mice with an F8-deficiency during the mesenteric vessel ferric chloride-induced injury model.
Quantitative analysis of platelet accumulation and fibrin generation after deposit of $FeCl_3$ to mesenteric vessels. Mice were injected with Alexa 647-labeled antibodies against GPIX and with AF488-labeled fibrinogen to monitor platelet accumulation and fibrin generation respectively. Representative images obtained 30 min after the start of recording (A). Platelet (B, D) and fibrin accumulation (C, E) in arterioles (B-C) and in venules (D-E). The mean of fluorescence intensity ± SEM is shown (n=10-12). A two-way ANOVA test followed by a multiple comparison test was used; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 7. PN-1-deficiency in mice with F8-deficiency or PN-1 blocking in blood of hemophilia A patients improves clot stability and limits clot lysis.
Representative whole blood thromboelastogram (ROTEM®) profiles for hemophilic mice (A) or hemophilic patient (D). The maximum clot firmness corresponds to the maximum amplitude reached during the test (B, E), and the lysis on time corresponds to the time span from clotting time to the start of significant lysis (C, F). Each dot represents one mouse (B, C) or one patient (E, F). Values are means ± SEM. A two-way ANOVA followed by a multiple comparison test was used; *P<0.05, **P<0.01, ****P<0.0001.