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## **Neutralizing Protease Nexin-1 in hemophilia patients on emicizumab improves thrombin generation**

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Running title : Blocking PN-1 in patients on emicizumab

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Hemophilia A (HA) is one of the most common inherited bleeding disease due to FVIII deficiency. In the past decade, the treatment of patients with severe HA has undergone an explosion of innovation, including the use of emicizumab (Hemlibra®, Roche-Chugai), a recombinant humanized bispecific antibody that partially mimics FVIII (1). Emicizumab is now approved for bleeding prophylaxis in severe congenital HA with and without inhibitors as well as in moderate HA with a severe bleeding phenotype. Its impact on bleeding prevention has been confirmed by real-world data (2). Unlike recombinant FVIII (rFVIII) which is injected intravenously (2-3 days per week), a maintenance dose of emicizumab is administered subcutaneously every week or once every 2-4 weeks, and is efficient in patients with anti-FVIII immune responses, a serious complication of rFVIII treatment. Although emicizumab significantly reduces bleeding rates, it does not completely normalize hemostasis. In fact, some concerns remain about the net benefit of emicizumab (3). Patients can still experience breakthrough bleeding, and require adjunctive procoagulant therapy during invasive surgical procedures (4). Moreover, combining emicizumab treatment with activated prothrombin complex concentrate (aPCC) (5) may increase the risk of thrombotic microangiopathy and thromboembolic events, so close monitoring is required in this condition (6). Here, we investigated whether inhibition of a moderate natural anticoagulant named Protease Nexin-1 (PN-1) could provide another means to safely manage bleeding in patients on emicizumab.

PN-1 is encoded by the *SERPINE2* gene and is an antiprotease able to inhibit various serine proteases such as proteases of blood coagulation (thrombin, activated FXI) and fibrinolysis (tissue plasminogen activators (tPA), urokinase (uPA) and plasmin (7)). PN-1 is not detected free in the bloodstream but is expressed by multiple cells, including blood and vascular cells (7). PN-1 is largely present in  $\alpha$ -granules of platelets and is released after their activation. Platelet PN-1 has been shown to be involved in the regulation of thrombus formation. Indeed, thrombus formation is accelerated in PN-1-knock-out (PN-1-KO) mice after vascular injury (8). Interestingly, double KO mice for PN-1 and FVIII display reduced bleeding compared to FVIII-KO mice. Moreover, a PN-1-blocking antibody has been shown *in vitro* to increase thrombin generation in platelet rich plasma (PRP) from patients with hemophilia (9). These previous data suggested that neutralizing a moderate natural anticoagulant protein like PN-1 could help to restore hemostatic balance in hemophilia.

In this report, we describe the *in vitro* evaluation of a blocking anti-PN-1 antibody on thrombin generation in PRP and on blood clot stability, in patients with severe HA who are currently on emicizumab (the mean  $\pm$  SD emicizumab blood concentration was  $68 \pm 20$   $\mu\text{g/mL}$ ,  $n=8$ ). Emicizumab plasma levels were measured using a diluted FVIII assay calibrated to emicizumab (CK-Prest<sup>®</sup>, STAGO, r2diagnostics). Thrombin generation was measured in PRP with the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope<sup>®</sup> BV), as previously described (9). Briefly, 80  $\mu\text{L}$  PRP preincubated for 15 minutes at 37°C, with 150  $\mu\text{g/mL}$  Irrelevant IgG (Immuno-research), or in the presence of a polyclonal anti-PN-1 antibody (150  $\mu\text{g/mL}$ ) which significantly abolishes the protease inhibitory activity of PN-1 at this concentration (8), was added to 20  $\mu\text{L}$  PRP reagent containing Tissue Factor (3 pM) and incubated for 15 minutes at 37°C. The thrombin generation assay (TGA) was then initiated by adding 20  $\mu\text{L}$  FLUCA reagent (Fluca kit, TS50, Thrombinoscope<sup>®</sup> BV) which contains  $\text{CaCl}_2$  and the fluorogenic substrate Z-Gly-Gly-Arg-AMC. Fluorescence was measured during 60 min and analyzed by the dedicated Thrombinoscope software. The mean values of TGA parameters are summarized in Table 1. Unexpectedly, patients on emicizumab displayed an increased lag time in the presence of the neutralizing anti-PN-1 antibody compared to values obtained with the irrelevant antibody. Because activated platelets are important for this priming step generating the small amount of thrombin, we can speculate that this increased lag time may be due to a steric hindrance related to the presence of both emicizumab and the anti-PN-1 polyclonal antibody. However, most importantly, patients on emicizumab also displayed a significantly higher peak and a reduced time to peak ( $t_{\text{tpeak}}$ ), in the presence of the neutralizing anti-PN-1 antibody (Table 1). Thus, even though the TGA parameters remain below those observed for PRP samples from healthy donors, our data show that PN-1 blockade can significantly accelerate the thrombin burst in patients on emicizumab, as illustrated by the increased velocity (Table 1). There are thus additive effects between emicizumab and PN-1 blockade on the rate of thrombin formation.

Because clot stability is also an important feature to manage in the treatment of hemophilia patients (10), and since PN-1 is also known to inhibit fibrinolytic proteases, we analyzed the effect of PN-1 blockade on blood clot stability and lysis in blood samples from HA patients currently on emicizumab. For this purpose, we used the rotational thromboelastometry (ROTEM)-based assay, with or without rtPA as the

clot lysis trigger, as previously described (9). Briefly, 300  $\mu$ L of citrated human whole blood was preincubated with 150  $\mu$ g/mL irrelevant IgG or 150  $\mu$ g/mL polyclonal anti-PN-1 antibody for 15 minutes at room temperature. Clotting was initiated by adding 20  $\mu$ L of extrinsically activated ROTEM® assay reagent (ExTEM®, Tem International), and 20  $\mu$ L of buffered CaCl<sub>2</sub> solution (Star-TEM® reagent, Tem International). For fibrinolysis studies, rtPA (Alteplase, Boehringer) (0.1  $\mu$ g/mL final concentration) was added just before clotting initiation. ROTEM parameters shown in Table 2 were assessed using ROTEM® software. Clot ROTEM parameters were similar in the presence of the neutralizing anti-PN-1 antibody and with the irrelevant antibody (Table 2A). They were also similar in the presence of rtPA (Table 2B). These results show that blocking PN-1 does not counteract the restorative effect of emicizumab on blot clot stability. These data confirm that the role of PN-1 on t-PA initiated fibrinolysis has no significant impact in hemophilia condition (9). In fact, in this context the antifibrinolytic activity of PN-1 is negligible compared to its antithrombin activity.

In conclusion, our results suggest that targeting PN-1 could be a potential strategy to improve hemostasis in HA patients receiving emicizumab. Because the total of thrombin produced remains lower than in controls, our data also suggest that addition of a blocking PN-1 product to emicizumab treatment should not entail any increased thrombotic risk. Although further studies are necessary to confirm these results, this strategy is original since it involves a replacement therapeutic approach based on the inhibition of a natural anticoagulant that is not a key anticoagulant protein like antithrombin or tissue factor pathway inhibitor.

## **AUTHOR CONTRIBUTIONS**

V.A., L.V. and S.L. performed the experiments and analyzed the data; V.A., Y.B and M-C.B. conceived and designed the study, analyzed and interpreted the data, drafted the research letter; C.F. and E.d.R. recruited the patients, analyzed and interpreted the data. All authors critically revised the research letter and gave final approval to its submission for publication.

## **FUNDING INFORMATION**

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## **CONFLICT OF INTEREST STATEMENT**

M-C.B. has patent applications related to PN-1 targeting in hemophilia. The other authors have no relevant conflict of interest to declare.

## **ETHICS STATEMENT**

The study was approved by the ethics evaluation committee of Inserm (IORG0003254 -FWA00005831)

## **PATIENT CONSENT STATEMENT**

All patients gave their written consent before participation to the study.

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**Table 1:** Parameters of thrombin generation in platelet rich plasma (PRP) from patients with severe Hemophilia A (HA) on current emicizumab treatment (n = 8), and from healthy donors (n = 7).

	<b>PRP HA on emicizumab + Irr IgG</b>	<b>PRP HA on emicizumab + Anti-PN-1 IgG</b>	<b>PRP control healthy donor + Irr IgG</b>
<b>Lag Time (min)</b>	13.5 ± 1.7	15.6 ± 3.1 **	6.0 ± 0.8
<b>Peak (nM)</b>	73.0 ± 19.5	89.3 ± 28.5 **	118.4 ± 16.1
<b>ttPeak (min)</b>	30.2 ± 4.7	28.8 ± 5.5 *	15.2 ± 3.7
<b>ETP (nM.min)</b>	1264 ± 233	1178 ± 218	1630 ± 271
<b>Velocity (nM/min)</b>	4.8 ± 2.2	7.5 ± 3.9 **	21.7 ± 9.4

Means ± SD; A parametric paired t-test was used; \* $P < 0.05$ , \*\* $P < 0.01$ , vs “PRP HA on emicizumab + irrelevant IgG (Irr IgG)”. Time to Peak (ttPeak); Endogenous Thrombin potential (ETP).



**Table 2:** Parameters of thromboelastometry in blood from hemophilia patients with severe Hemophilia A on current emicizumab treatment, in the absence (A) or presence (B) of rtPA.

<b>Table 2A (no rtPA)</b>	<b>Blood + Irr IgG</b>	<b>Blood + Anti-PN-1 IgG</b>
<b>Clotting Time (s)</b>	71 ± 34	74 ± 34
<b>Clotting formation time (s)</b>	85 ± 37	81 ± 36
<b>MCF (mm)</b>	61 ± 9	59 ± 7
<b>MCE</b>	165 ± 63	153 ± 50
<b>Table 2B (with rtPA)</b>	<b>Blood + Irr IgG</b>	<b>Blood + Anti-PN-1 IgG</b>
<b>Clotting Time (s)</b>	62 ± 21	66 ± 23
<b>Clotting formation time (s)</b>	62 ± 14	61 ± 13
<b>MCF (mm)</b>	48 ± 17	46 ± 17
<b>MCE</b>	111 ± 71	102 ± 67
<b>Lysis Onset Time (s)</b>	1011 ± 552	910 ± 500
<b>Lysis Time (s)</b>	1832 ± 1164	1456 ± 780

Means ± SD, n=6; A non-parametric paired t-test was used.; irrelevant IgG (Irr IgG).  
Maximal Clot Formation (MCF); Maximal Clot Elasticity (MCE).

## Highlights

- Blocking PN-1 has been shown to control bleeding and improve hemostasis in hemophilia
- PN-1 blockade can accelerate the thrombin burst in blood from patients on emicizumab
- PN-1 blockade has no impact on fibrinolysis in blood from patients on emicizumab
- Patients receiving emicizumab may benefit from PN-1 blockade to improve hemostasis