




METHODOLOGICAL ARTICLE

Novel ELISA for the specific detection of protease NEXIN-1 in human biological samples

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Abstract

Introduction: Serpin E2 or protease nexin-1 (PN-1) is a glycoprotein belonging to the serpin superfamily, whose function is closely linked to its ability to inhibit thrombin and proteases of the plasminergic system.

Objectives: In the absence of specific quantitative methods, an ELISA for the quantification of human PN-1 was characterized and used in biological fluids.

Methods: The ELISA for human PN-1 was developed using two monoclonal antibodies raised against human recombinant PN-1. PN-1 was quantified in plasma, serum, platelet secretion from controls and patients with hemophilia A and in conditioned medium of aortic tissue.

Results: A linear dose–response curve was observed between 2 and 35 ng/mL human PN-1. Intra- and interassay coefficients of variation were 6.2% and 11.1%, respectively. Assay recoveries of PN-1 added to biological samples were ~95% in plasma, ~97% in platelet reaction buffer, and ~93% in RPMI cell culture medium. Levels of PN-1 secreted from activated human platelets from controls was similar to that of patients with hemophilia A. PN-1 could be detected in conditioned media of aneurysmal aorta but not in that of control aorta.

Conclusion: This is the first fully characterized ELISA for human serpin E2 level in biological fluids. It may constitute a relevant novel tool for further investigations on the pathophysiological role of serpin E2 in a variety of clinical studies.

Laurence Venisse and Déborah François contributed equally to this work.

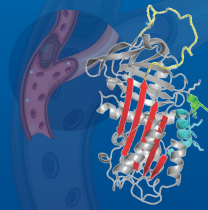
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A Novel ELISA for the Detection of Human Protease Nexin-1

Protease nexin-1 (PN-1) is a glycoprotein, high levels of which are associated with many diseases including human cancers and ischemic and fibrotic diseases




There are few specific methods to accurately quantify human PN-1


Can a specific ELISA be used for the reliable quantification of human PN-1 in biological fluids?




Monoclonal antibodies (mAb) against human PN-1 developed using the hybridoma technique



ELISA for human PN-1 with mAbs MA-57B11 for capture and MA-55F11 for detection constructed



Collected biological samples used in the novel ELISA



Platelet-rich plasma and serum from controls and patients with hemophilia A, and conditioned medium of aneurysmal aorta obtained


The minimum detectable value for PN-1 was 2.5 ng/mL

The novel ELISA method was:

✓ Sensitive
✓ Specific
✓ Reproducible
✓ Accurate

This novel ELISA can be helpful in understanding the role of PN-1 in pathophysiology. Given the cellular localization of PN-1, its abnormal presence in plasma or body fluids should be useful to compare samples from healthy or physiological conditions with samples from pathological conditions

Novel ELISA for Specific Detection of Protease Nexin-1 in Human Biological Samples
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KEYWORDS

ELISA, hemophilia A, human, platelets, serpin

Essentials

- Protease nexin-1 (PN-1) is a cellular serpin with antithrombotic and antifibrinolytic properties.
- There are very few specific methods to quantify human PN-1 in biological fluids.
- We developed a specific ELISA to detect human PN-1 in plasma, platelet release, or culture medium.
- Measured levels of PN-1 will facilitate studies on its impact in human pathologies.

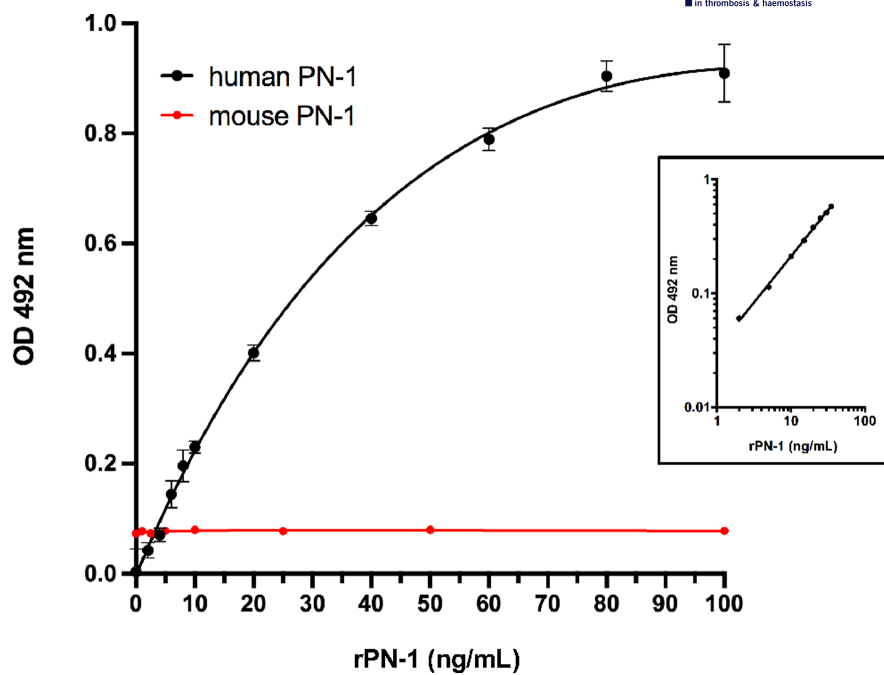
1 | INTRODUCTION

The serpins comprise a superfamily of proteins that share a conserved tertiary structure. Most serpins are serine protease inhibitors involved in a wide range of biological processes such as coagulation, fibrinolysis, or inflammation. Unlike SERPINE1/plasminogen activator type 1 (PAI-1) and SERPINC1/antithrombin (AT), SERPINE2/Protease nexin-1 (PN-1) is a non-circulating protein. It is expressed by multiple cell types, including vascular and blood cells. The best-known function of PN-1 is related to its platelet expression. Platelet PN-1 plays a role in the regulation of thrombus formation.¹ Indeed, when released from the alpha granules during platelet activation, it can inhibit not only thrombin activity through the formation of an irreversible complex with the protease,^{2,3} but also thrombin generation by directly inhibiting activated factor XI (FXIa),⁴ an important factor for the contact system, known to maximize thrombin generation. PN-1 has also been shown to inhibit proteases of the plasminergic system, tissue-type plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and plasmin.^{2,3} However, the anti-thrombin activity of PN-1 is assumed to be dominant compared to its antifibrinolytic activity since in vitro studies previously showed that PN-1 could inactivate thrombin at a rate at least 1000-fold faster than plasminergic proteases.

PN-1 is a unique serpin in hemostasis since it does not circulate in blood. Rather, it is considered as a cellular serpin that can be secreted from various cells producing it, in particular cultured fibroblasts,⁵ vascular endothelial cells,⁶ and smooth muscle cells,⁷ as well as platelets.⁸ When secreted, PN-1 can either be found in the conditioned medium or remains associated with the cell surface, depending on the cell type. PN-1 indeed has a high affinity for heparin-like glycosaminoglycans and extracellular matrix proteins like vitronectin, explaining its pericellular localization.⁹ Human PN-1 has usually been revealed either by checking complexes formed with radiolabeled target proteases or by using enzymatic assays based on the measurement of its thrombin or u-PA inhibitory activity, or by western blot using specific antibodies. In this way, PN-1 has been detected in a conditioned medium of fibroblasts¹⁰ and in supernatants of isolated activated platelets.¹ It has also been shown to be mainly retained at the cell surface of vascular smooth muscle cells and shed by heparin from the surface of these cells.¹¹ However, there are no well-characterized and specific assays available to quantify human PN-1 in fluids, and therefore studies from patients are challenging.

In the present study, we describe a novel ELISA using monoclonal antibodies to specifically capture and detect human PN-1 in biological fluid samples.

FIGURE 1 Standard curve for the detection of recombinant PN-1 by ELISA. Increasing concentrations of recombinant human (black curve) and mouse (red curve) PN-1 was added to MA-57B11-coated wells. Bound PN-1 was detected using the MA-55F11-HRP as described in methods. Each point of the curve represents mean \pm SD of four independent experiments, each performed in duplicate. HRP, horseradish peroxidase; PN-1, protease nexin-1



2 | MATERIALS AND METHODS

2.1 | Production of monoclonal antibodies toward human PN-1

BALB/c mice were immunized by subcutaneous injection of 10 μ g of human recombinant PN-1 in complete Freund's adjuvant, followed 2 weeks later by intraperitoneal injection of 10 μ g of human recombinant PN-1 in incomplete Freund's adjuvant. After an interval of at least 6 weeks, the mice were boosted intraperitoneally with 10 μ g of human recombinant PN-1 in saline on days 4 and 2 before the cell fusion. Monoclonal antibodies (mAbs) against human PN-1 were produced using the standard hybridoma technique by Galfrè and Milstein.¹² Recombinant *Escherichia coli*-expressed human PN-1 (rPN-1) was produced and purified as previously described¹³ and used as antigen.

2.2 | Construction of an ELISA for human PN-1

The mAbs were pairwise tested for their reactivity toward human PN-1 using one mAb for capture and another one, conjugated to horseradish peroxidase (HRP) for detection. mAb-HRP conjugates were produced as described by Nakane and Kawaoi.¹⁴ This resulted in the selection of the mAbs MA-57B11 for capture and MA-55F11 for detection.

Microton-Med half-well high binding plates (Greiner bio-one, Courtaboeuf, France) were coated for 72 hours at 4°C, with 0.5 μ g MA-57B11/well. After washing with phosphate buffered saline with Tween (PBS-T) buffer (PBS, 0.1%; bovine serum albumin [BSA], 0.002%; Tween 20), wells were saturated for 2 hours at room temperature (RT) with 100 μ L of PBS containing 1% BSA (Sigma,

Saint-Quentin Fallavier, France). After four washes with PBS-T buffer, the wells were incubated 4 minutes at RT with 100 μ L of a solution containing 100 g/L of mannitol and 20 g/L of saccharose, then emptied before storing at -20°C. Immediately before use, plates were washed four times with PBS-T buffer. Calibration was performed using different concentrations of rPN-1 (0-100 ng/mL) diluted in PBS-T buffer. Biological samples diluted or not in PBS-T buffer were applied to wells overnight at 4°C in a moist chamber. After washing in PBS-T buffer, bound PN-1 was probed with MA-55F11-HRP (0.4 μ g/mL in PBS-T buffer) for 2 hours at RT, and detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine (Biotechne, Rennes, France) during 15 minutes at RT. The reaction was stopped with 25 μ L of 3 M H₂SO₄ and absorbance read at 492 nm.

2.3 | Samples

Human blood taken from healthy adult donors was provided by blood donation of the French Blood Establishment. Blood from patients with hemophilia A was provided by the Hemophilia Center (Hôpital André Mignot, Le Chesnay, France), obtained after informed consent, and with an agreement from the Ethic Evaluation Committee and the Institutional Review Board of the French Institute of Medical Research and Health. None of the patients with hemophilia A were on prophylactic treatment at the time of blood sampling.

Blood was drawn by venipuncture using a vacutainer system (Becton Dickinson, Franklin Lakes, NJ, USA) containing trisodium citrate (1:9) as anticoagulant for plasma preparations. Platelet-rich plasma (PRP) was obtained by centrifugation at 120 g for 15 minutes at RT. Platelet-poor plasma (PPP) was obtained by a double centrifugation: after a first centrifugation of blood at 1200 g for 12 minutes, plasma was subsequently centrifuged at 10000 g for 5 minutes to

remove residual platelets and cell fragments. PRP was adjusted to 3×10^8 platelets/mL with autologous PPP. Washed platelets were isolated as previously described¹⁵ and adjusted at 3×10^8 platelets/mL. Platelet preparations were activated by 100 μ M of thrombin receptor activating peptide (TRAP; the protease-activated receptor-1 activating peptide, SFLLRN; NeoMPS, Strasbourg, France) for 30 minutes at 37°C. Control samples were obtained by incubation of platelet preparations under the same conditions but in the absence of TRAP. At the end of the incubation, samples were centrifuged and the supernatants were removed for PN-1 quantification by ELISA. Serum was collected in red-top (no additive) blood collection tubes (Becton Dickinson Vacutainer Serum), incubated at room temperature for at least 30 minutes to induce clotting, and then processed within 4 hours of collection. Processing consisted of centrifugation at 1200 g for 10 minutes at RT. Before testing in the ELISA assay, plasma and serum samples were routinely diluted 1:3 and 1:5 (v/v), respectively, to overcome the interference of the plasma or serum matrices.

Normal thoracic aortas were obtained from organ transplant donors. Thoracic aortic aneurysms (TAAs) were collected during aortic surgery (Hôpital Bichat, Paris, France). Approval was obtained after informed consent, with the approval of the local ethical committee, and was in accordance with the principles of the Declaration of Helsinki. Aortic conditioned medium was obtained by incubation of small pieces of healthy aorta or aorta of TAA (24 hours at 37°C) in a standardized volume (6 mL/g of sample wet weight) of RPMI culture medium supplemented with antibiotics and antimycotics. Before testing in the ELISA assay, RPMI culture medium was diluted 1:3 (v/v) to overcome its interference matrix effect.

2.4 | Statistical analysis

Results are shown as means \pm standard error of the mean (SEM). Statistical analysis was conducted using Prism (GraphPad Software, San Diego, CA, USA). The nonparametric Mann-Whitney test was used; differences between groups were considered statistically significant at $P < .05$.

3 | RESULTS

3.1 | Generation of anti-PN-1 monoclonal antibodies

Hybridoma cell lines generated by fusion of spleen cells from the immunized mice and myeloma cells yielded 20 monoclonal antibodies that were purified, conjugated to HRP, and pairwise tested for their suitability for the quantification of human PN-1 by ELISA. One combination constituted of MA-57B11 for capture and MA-55F11-HRP for tagging was selected for its high reactivity toward rPN-1.

3.2 | Linearity, sensitivity, and specificity

Dose-response was linear for human rPN-1 between 2 and 35 ng/mL (Figure 1). The correlation coefficient was higher than 0.97 within the linear portion of the curve. The ELISA revealed no reactivity toward murine rPN-1 (Figure 1). The analysis of the standard curve indicated

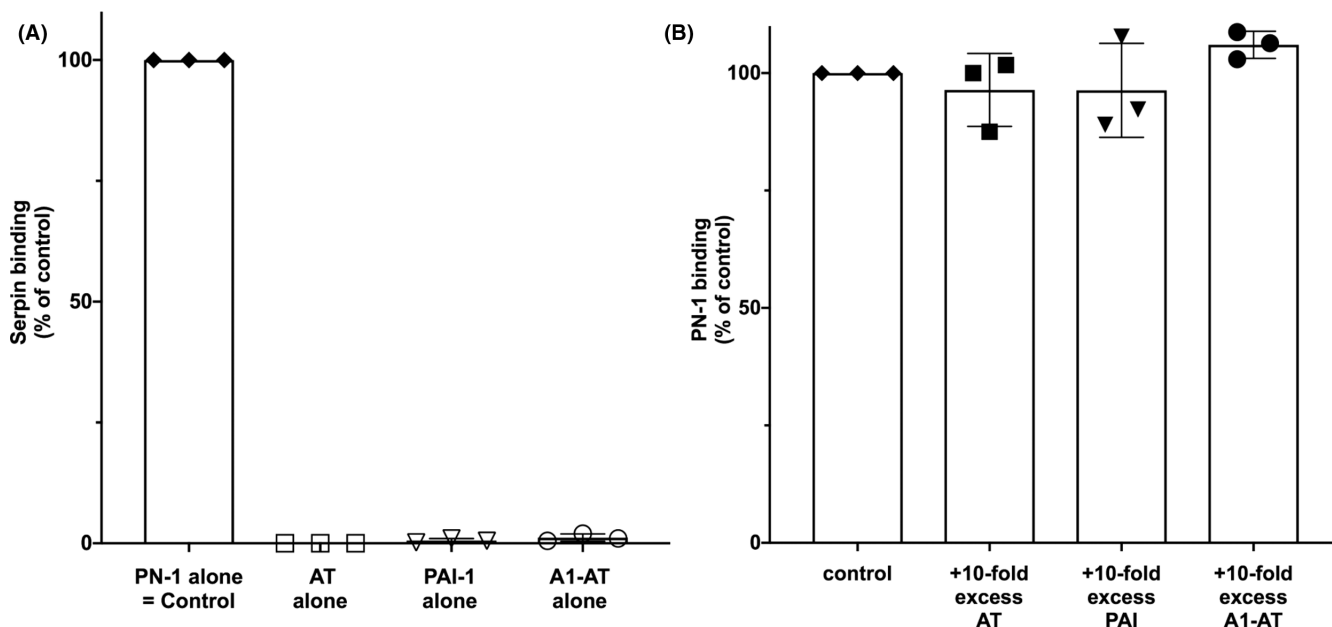
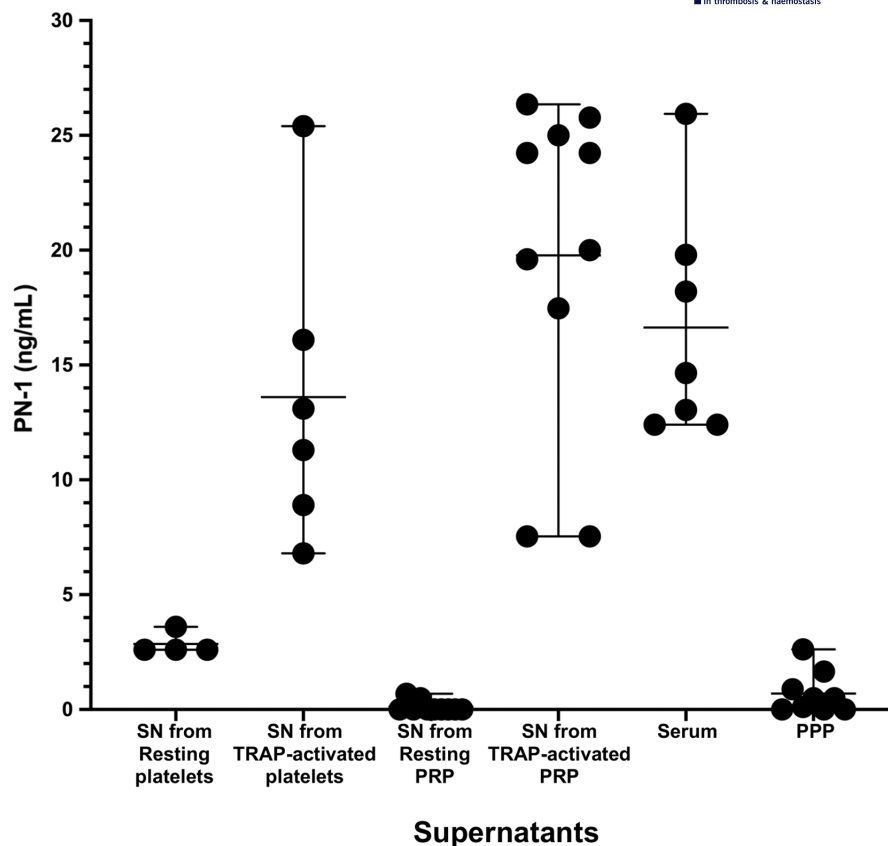


FIGURE 2 Specificity of the ELISA for PN-1. (A) High concentration (100 ng/mL) of recombinant serpins (PN-1 or AT or PAI-1) were applied to MA-57B11-coated wells overnight at 4°C, or (B) recombinant PN-1 (4.5 ng/mL corresponding to 0.11 nmol/L) was applied to MA-57B11-coated wells in the absence or presence of a 10-fold excess (1.1 nmol/L) of AT or PAI-1 overnight at 4°C. After washing, bound serpin was detected as described in methods. Data are means \pm standard deviation of three independent experiments. A1-AT, α 1-antitrypsin; AT, antithrombin; PAI-1, plasminogen activator type 1; PN-1, protease nexin-1

FIGURE 3 PN-1 dosage in serum and supernatants of different types of platelet preparations and in PPP from healthy blood donors. PN-1 secreted by platelets was quantified in the SNs from resting washed platelets ($n = 4$) and TRAP-activated washed platelets ($n = 6$), and in the SNs from resting PRP ($n = 10$) and TRAP-activated PRP ($n = 10$). PN-1 was also measured in the serum ($n = 7$), and in the PPP ($n = 9$). LOD = 2.5 ng/ml. Data are represented as means with range. LOD, Limit of detection; PN-1, protease nexin-1; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SN, supernatants; TRAP, thrombin receptor activating peptide



that the minimum level at which PN-1 can be reliably detected is 2.5 ng/mL (with a signal-to-noise ratio of 2:1). We also checked the effect of three other human serpins present in plasma, namely AT, PAI-1, and α 1-antitrypsin, alone at a high concentration or at a 10-fold excess over PN-1, to verify the specificity of the ELISA. None of these serpins are detected alone (Figure 2A). Moreover, none of them affected PN-1 detection (Figure 2B). These data confirmed the high specificity of the ELISA toward human PN-1.

3.3 | Variability and recovery

Inter- and intra-assay variability of the ELISA were evaluated using four different aliquots of rPN-1 to run the calibration curve four times on four occasions. Each point of each calibration curve (corresponding to a dilution of rPN-1) was done in duplicate. The intra- and interassay coefficients of variation were around 6% and 11%, respectively. The detailed analysis of the variations is presented in the Supporting Information.

Assay recovery was performed by adding rPN-1 in different types of biological samples initially devoid of PN-1. Addition of rPN-1 at a final concentration of 5 and 25 ng/mL yielded recoveries of $96.2 \pm 6.6\%$ and $93.7 \pm 6.0\%$ in human PPP, $100 \pm 0\%$ and $94.4 \pm 2.8\%$ in platelet reaction buffer and $81.0 \pm 10.0\%$ and $106.2 \pm 6.1\%$ in RPMI culture medium, respectively (mean \pm SD, $n = 3-5$).

3.4 | Quantitation of human PN-1 levels in various biological samples

Because PN-1 is a serpin present in alpha granules of platelets, its concentration was determined in supernatants from different types of platelet preparations (Figure 3). We measured PN-1 in the supernatants of TRAP-activated washed platelets prepared from six healthy blood donors. PN-1 levels in platelet-secreted fractions were 13.6 ± 2.7 ng/mL (mean \pm SEM; range, 6.8-25.4 ng/mL; $n = 6$). PN-1 concentration in supernatants from resting platelets was below the minimum level at which it could be reliably detected (Figure 3). It has to be noticed that PN-1 concentration measured in thrombin-activated platelet secreted fractions was three times lower than PN-1 concentration measured in TRAP-activated platelet secreted fractions, indicating that PN-1-thrombin complexes are much less detected than free PN-1 in the ELISA. We also measured PN-1 concentration in supernatants of TRAP-activated PRP from healthy blood donors. We detected PN-1 at 19.8 ± 2.2 ng/mL (mean \pm SEM; range, 7.5-26.3 ng/mL; $n = 10$). As expected, PN-1 was detected neither in PPP nor in supernatants of resting PRP (Figure 3). Because PN-1 is secreted during platelet activation, we also measured PN-1 concentration in serum samples (Figure 3). In serum obtained from seven healthy blood donors, levels of PN-1 were 16.6 ± 1.9 ng/mL (mean \pm SEM; range, 12.4-25.9 ng/mL).

Platelet PN-1 has been shown to regulate thrombus formation¹ and proposed as a target for hemophilia treatment.¹⁶ We thus

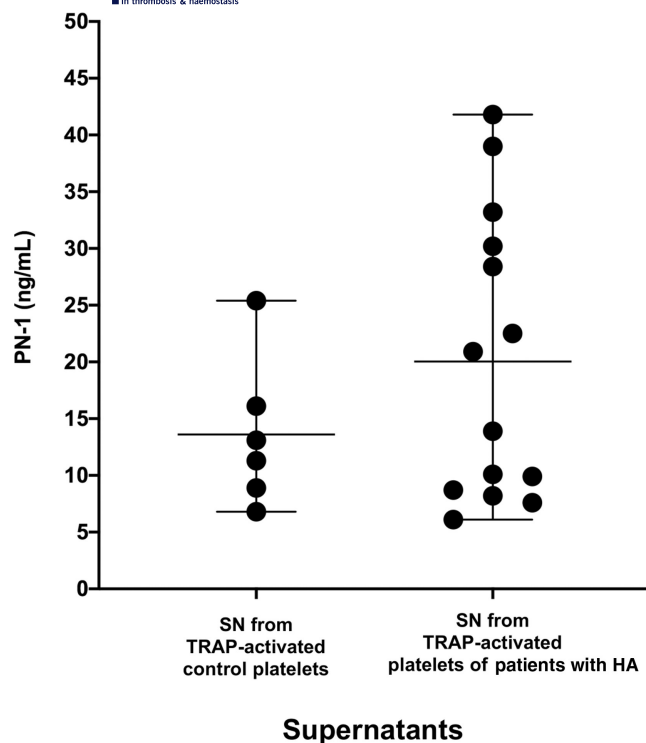


FIGURE 4 PN-1 dosage in SNs of platelets from patients with HA. PN-1 secreted by platelets from control donors ($n = 6$) and patients with HA ($n = 14$) were quantified in the SN from TRAP-activated washed platelets. LOD = 2.5 ng/mL. Data are represented as means with range. HA, hemophilia A; LOD, Limit of detection; PN-1, protease nexin-1; SN, supernatant; TRAP, thrombin receptor activating peptide

determined PN-1 levels in the supernatants of TRAP-activated washed platelets from patients with hemophilia A (HA) (Figure 4). The level of PN-1 (mean \pm SEM) was 20.0 ± 3.3 ng/mL ($n = 14$; range, 6.1–41.8 ng/mL) and was not significantly different from the level observed in the supernatants of TRAP-activated control platelets.

PN-1 is also recognized as a regulator of protease activities in the vascular wall. Immunostaining experiments previously demonstrated that PN-1 was overexpressed in the arterial wall of patients with a TAA compared with control aortas.¹⁷ We confirmed this data by ELISA quantification of PN-1 released into conditioned medium from aorta incubated in RPMI (Figure 5). Indeed, no PN-1 was detected in conditioned medium from healthy aortas, whereas the aorta from patients with TAA released measurable PN-1 ranging from 3.0 to 23.5 ng/mL ($n = 6$).

4 | DISCUSSION

PN-1 has been shown to be overexpressed in various diseases from human cancers to ischemic and fibrotic diseases. These data were usually obtained in tissue or cellular samples and often supported by immunohistochemical staining or by microarray analysis.¹⁸ An increase of PN-1 level in plasma or other body fluids relatively easy to obtain has never been described. A major reason

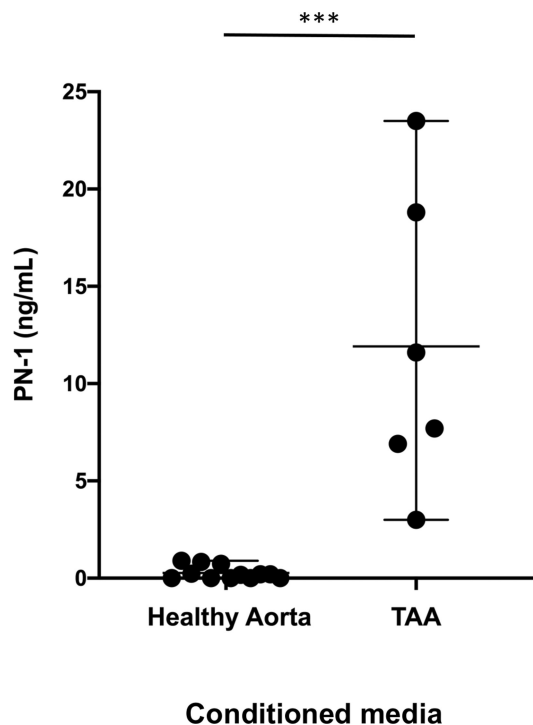


FIGURE 5 PN-1 dosage in tissue-conditioned media of aorta. PN-1 was quantified in tissue-conditioned media from healthy human aortas ($n = 12$ different donors) and TAAs ($n = 6$ different donors). LOD = 2.5 ng/mL. Data are represented as means with range. The nonparametric Mann-Whitney test was used: $***P < .001$. LOD, Limit of detection; PN-1, protease nexin-1; TAA, thoracic aortic aneurysm

is the absence of specific methods for quantification of human PN-1. Very few specific antibodies directed against PN-1 are available since many of those commercially available cross-react with PAI-1. Human PN-1 is indeed the phylogenetically closest relative of PAI-1. It shares 41% homology with human PAI-1 and 32% with human AT.¹⁹ To our knowledge, sparse immunological assays are available for the sensitive and specific quantification of human PN-1. In this study, we describe a novel ELISA that can be used to measure the concentration of human PN-1 in biological samples. ELISA has the advantage of being easy to perform and suitable for clinical studies.

The obtained data demonstrate that the assay is sensitive, specific, and reproducible, allowing the detection and quantification of PN-1 in the lower ng/mL range. It is noteworthy that even though the murine sequence of PN-1 has 84% homology with the corresponding human protein, only human PN-1 is detected in this ELISA. The recoveries demonstrate an adequate accuracy of the assay, with the lowest recovery observed in RPMI cell culture medium. Because the matrix effect of plasma is known to alter the readout, plasma samples were routinely diluted 1:3 (v/v), which resulted in a good recovery percentage, above 95%.

The mean recovery of added PN-1 in platelet reaction buffer is also $>95\%$. Therefore, the determination of PN-1 secreted by activated washed platelets is allowed using the ELISA. From data

obtained in the supernatants of TRAP-activated washed platelets (at 3×10^8 platelets/mL), it can be calculated that human platelets contain 45 pg PN-1/ 10^6 platelets. This value is ≈ 15 -fold lower than that reported for PAI-1 in human platelets (≈ 700 pg PAI-1/ 10^6 platelets).^{20,21} However, only 10% of platelet PAI-1 is assumed to be in the active form.^{20,21} Therefore, levels of both serpins released from activated platelets locally rise very significantly through accumulation within the thrombus and must both play a role in thrombus stabilization. The respective role of each serpin in thrombus lysis was unclear since PN-1 can inhibit not only u-PA and t-PA but also plasmin. PAI-1 inactivates t-PA at an ≈ 300 -fold faster rate than PN-1,²² but both PAI-1 and PN-1 have been shown to be highly present within the thrombus.²³ In contrast, the role played by platelet PN-1 in thrombus formation is unequivocal. Indeed, PN-1 is the most effective inhibitor of thrombin, even more effective than AT.² PN-1 is also an effective inhibitor of FXIa. Moreover, thrombus formation has been shown to be accelerated and facilitated in PN-1-deficient mice.¹ Interestingly, we did not see any significant difference between the levels of PN-1 secreted by activated platelets from controls versus patients with HA, reinforcing the previously reported idea that targeting PN-1 is a potential strategy to control bleeding in patients with HA by favoring blood clot formation.¹⁶

In the present study, we also confirmed the overexpression of PN-1 observed previously by immunohistochemistry or western blot in aortic tissues from patients with a TAA.¹⁷ By ELISA, we could indeed easily detect PN-1 released in the conditioned media of TAA samples, whereas it was barely detectable in that of control aorta. Thus, although PN-1 is retained at the cell surface of vascular cells and within the extracellular matrix of the vessel wall, PN-1 can be detected in the conditioned media of pathological aorta, indicating that overexpressed PN-1 is diffusible.

In conclusion, we developed and validated a novel ELISA for the determination of human PN-1 in body fluids. Given its cellular localization, the abnormal presence of PN-1 in the circulation or in body fluids may reflect not only disturbed blood cell activation but also vascular damage. We thus believe that any condition in which coagulation is activated is particularly susceptible to translate into increased PN-1 concentration due to its secretion by activated platelets. Therefore, this novel ELISA could be helpful for understanding the role of PN-1 in pathophysiology.

AUTHOR CONTRIBUTIONS

DF and EB generated and produced the monoclonal antibodies. LV and DF provided the experimental data, and conducted the analysis and interpretation of the data. EdR recruited the patients with hemophilia. PD contributed to the experimental design and proofread the manuscript. M-CB wrote the manuscript with the assistance of CM and YB. VA and M-CB did the concept and design of the experiments and supervised the project and preparation of the manuscript.

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RELATIONSHIP DISCLOSURE

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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

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