



Vascular pentraxin 3 controls arterial thrombosis by targeting collagen and fibrinogen induced platelets aggregation



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ABSTRACT

Aim: The long pentraxin PTX3 plays a non-redundant role during acute myocardial infarction, atherosclerosis and in the orchestration of tissue repair and remodeling during vascular injury, clotting and fibrin deposition. The aim of this work is to investigate the molecular mechanisms underlying the protective role of PTX3 during arterial thrombosis.

Methods and results: PTX3 KO mice transplanted with bone marrow from WT or PTX3 KO mice presented a significant reduction in carotid artery blood flow following FeCl₃ induced arterial thrombosis ($-80.36 \pm 11.5\%$ and $-95.53 \pm 4.46\%$), while in WT mice transplanted with bone marrow from either WT or PTX3 KO mice, the reduction was less dramatic ($-45.55 \pm 1.37\%$ and $-53.39 \pm 9.8\%$), thus pointing to a protective effect independent of a hematopoietic cell's derived PTX3. By using P-selectin/PTX3 double KO mice, we further excluded a role for P-selectin, a target of PTX3 released by neutrophils, in vascular protection played by PTX3. In agreement with a minor role for hematopoietic cell-derived PTX3, platelet activation (assessed by flow cytometric expression of markers of platelet activation) was similar in PTX3 KO and WT mice as were haemostatic properties. Histological analysis indicated that PTX3 localizes within the thrombus and the vessel wall, and specific experiments with the N-terminal and the C-terminal PTX3 domain showed the ability of PTX3 to selectively dampen either fibrinogen or collagen induced platelet adhesion and aggregation.

Conclusion: PTX3 interacts with fibrinogen and collagen and, by dampening their pro-thrombotic effects, plays a protective role during arterial thrombosis.

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1. Introduction

Pentraxin 3 (PTX3) is an essential component of the humoral arm of innate immunity and belongs to the pentraxin superfamily: soluble, multifunctional, and pattern recognition proteins [1]. Pentraxins share a common C-terminal pentraxin domain, which in the case of PTX3 is coupled to an unrelated long N-terminal domain [2]. PTX3 in humans, like CRP, correlates with other clinical markers of atherosclerosis [3] and is independently associated with the risk of developing vascular events [4–6]. PTX3 was detected in the myocardium and in the

vasculature under different pathological conditions (i.e. in the context of angiogenesis, vascular restenosis, atherosclerosis) [7], a finding paralleled by the observation of increased PTX3 plasma levels in patients with cardiovascular disorders [8,9]. These data prompted the research toward the investigation on the role of PTX3 as biomarker, player or both in the context of cardiovascular disease.

The high degree of conservation of PTX3 between mouse and human [1] supports the investigation of its pathophysiological role in mice. PTX3 deficiency has been associated with increased fibrin deposition in wounded tissues [10], increased inflammation as a result of defective control of P-selectin mediated neutrophil recruitment [11], increased atherosclerosis [12], and cardiac damage [13]. These observations point to a cardiovascular protective effect of PTX3, potentially associated with the tuning of inflammation during cardiovascular diseases [7], and, as a consequence, the increased levels observed in humans might represent an extreme attempt of

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the body to limit an excessive inflammatory response. This conclusion is further supported by the finding that genetically determined high levels of PTX3 do not influence the risk of acute myocardial infarction (AMI), suggesting that PTX3 concentration itself is unlikely to be even a modest causal factor for AMI [14].

We have recently shown that, after tissue injury, the local acid micro-environment activates PTX3 which orchestrates tissue repair [15] and that PTX3 deficiency is associated with defective repair of wound healing and with increased thrombosis [15]. The aim of this work is to investigate the mechanisms underlying the protective role of PTX3 during arterial thrombosis. In details, we characterized *in vivo* arterial thrombosis in PTX3 chimera mice generated by bone marrow transplantation and investigated the molecular mechanisms responsible for the phenotype observed taking advantage of PTX3/P-selectin double KO mice. *In vivo* and *in vitro* studies were then carried out to selectively investigate the contribution of platelets, leukocytes and vascular wall in the increased thrombus formation observed in PTX3 deficient mice.

2. Materials and methods

2.1. Animals

The generation of PTX3 KO animals was described in detail before [16]. P-selectin KO mice were from the Jackson Laboratories. P-selectin/PTX3 KO mice were generated by crossing the two animal models. All mice were on a C57BL/6J background (from more than 10 generations). The investigation conforms to the European Commission Directive 2010/63/EU and was approved by the Ethical Committee (Progetto di Ricerca 2009/3 and Progetto di Ricerca 2012/02).

2.2. Bone marrow transplantation

Wild-type (WT) or PTX3 KO mice were lethally irradiated with a total dose of 900 cGy. Two hours later, mice were injected in the tail vein with 5×10^6 nucleated bone marrow (BM) cells obtained by flushing of the cavity of a freshly dissected femur from a WT or PTX3 KO donor. Recipient mice received gentalyne (0.4 mg/mL in drinking water) starting 10 days before irradiation and maintained thereafter [11]. At 8 weeks after BM transplantation, the FeCl₃ injury model was carried out as described below.

2.3. FeCl₃ injury: experimental arterial thrombosis model

Experimental arterial thrombosis was induced as previously described [10]. Briefly, mice (8–12 week old) were anesthetized with ketamine chlorhydrate (75 mg/kg) and medetomidine (1 mg/kg; Virbic). The left carotid artery was dissected free and placed in the probe (model 0.7V, Transonic System) connected to transonic flow meter (Transonic T106). After blood flow stabilization (baseline flow constant for 7 min), filter paper imbibed with FeCl₃ (10–20%) was applied downstream of the probe to the top of the exposed carotid. After 3 min, the filter paper was removed, the carotid artery was washed with PBS, and the flow was recorded for 30 min. In a group of experiments, human recombinant PTX3 (hPTX3, 5 mg/kg per mouse [11,17]) or PBS (as negative control) was injected *iv* before the arterial thrombosis experiment. Data are presented as the percentage, compared to the basal level, of carotid artery blood flow during the 30 min of observation and time to carotid occlusion (less than 20% of carotid blood flow).

2.4. Platelet count, tail bleeding time and fibrinogen, PT and aPTT measurement

Platelet was counted optically from whole blood collected by orbital sinus bleeding into Unopette System reservoirs. Bleeding time was measured on tails of anesthetized mice, and immersed in saline solution

at 37 °C. After 3 min the last part of the tail, 3 mm from its end, was cut with a sharp scalpel blade, then immediately re-immersed in saline solution. Bleeding was followed visually, and time was determined as the interval (sec) from the tail transection to cessation of bleeding; 900 s was considered the cut off time for the purpose of statistical analysis [18]. Fibrinogen levels were measured as previously described [19], from plasma (PPP) obtained after centrifugation of blood [collected by cardiac venipuncture from anesthetized mice into 3.8% sodium citrate (1:10 vol:vol)] at 1000 g for 10 min. For prothrombin time (PT) test, PPP was mixed with Tissue Factor (TF) (containing phospholipid) at 37 °C and an excess of calcium chloride (25 mM) was added to initiate coagulation. Time was determined as the interval (sec) between calcium addition and clot formation. For activated partial thromboplastin time (APTT) test, PPP was incubated at 37 °C with phospholipid (cephalin) and a contact activator (e.g. Kaolin) was added followed by the calcium (all pre-warmed to 37 °C). Addition of calcium initiates clotting and time was determined (sec) as the interval taken for a fibrin clot to form.

2.5. Real time quantitative polymerase chain reaction

Total RNA was reverse transcribed as described [12,20,21]. 2 µL of cDNA was amplified by real time quantitative PCR with 1X Syber green universal PCR master mix (BioRad, Italy). The specificity of the Syber green fluorescence was tested as described [21]. PTX3 primers used have been described previously [12]. Each sample was analyzed in duplicate using the IQTM-Cycler (BioRad). The PCR amplification was related to a standard curve ranging from 10–11 mol/L to 10–14 mol/L and data were normalized for the housekeeping gene ribosomal protein L13a (RPL13a).

2.6. PTX3 plasma levels – ELISA assay

PTX3 plasma levels were measured with a sandwich ELISA using 2 anti-murine PTX3 mAb (2C3 and 6B11) as described [22]. The ELISA assay did not cross-react with the short pentraxins CRP and SAP.

2.7. P-selectin expression and integrin $\alpha_2\beta_3$ activation on platelet surface

Platelet rich plasma (PRP), isolated following centrifugation at $100 \times g$ with no break of citrated blood, from PTX3 KO and WT mice was stimulated with collagen (10 µg/mL, Mascia Brunelli, Italy) and U46619 (10 µM, Cayman Chemical, USA), an analog of thromboxane for 1 or 10 min, for P-selectin or integrin $\alpha_2\beta_3$, respectively, and then stained with the anti-CD42c FITC and anti-CD62P PE or $\alpha_2\beta_3$ PE antibodies (Emfret, Germany). Samples were fixed with PFA 4% and the expression of the platelet activation markers was evaluated through flow cytometry (BD FACS Calibur).

2.8. Platelet-leukocyte aggregates analysis

Citrated blood from PTX3 KO and WT mice was stimulated with ADP (40 µM, Sigma-Aldrich, Italy) for 30 s or 1 min and fixed with BD lysis buffer; samples were stained with the anti-CD45 PE (BD) and anti-CD42c FITC antibodies (Emfret, Germany) and analyze through flow cytometry (BD FACS Calibur).

2.9. Histology and immunofluorescence

Mouse carotid arteries and lungs were excised, perfused with PBS, fixed in 10% neutral formalin, processed and then embedded in paraffin as described [23]. Section of 5 µm were stained with hematoxylin and eosin, or DAPI and fluorescent antibodies against PTX3 and fibrin and visualized with *Axiovision* software [23] at $10 \times$ magnification. PTX3 protein expression in carotid arteries was analyzed as described [12,13].

2.10. Platelet aggregation

Citrated blood was diluted with HEPES-Tyrode buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 0.35% bovine serum (BSA), 1 mM MgCl₂, 2.7 mM KCl, 3.3 mM NaH₂PO₄) and centrifuged. PRP or washed platelets, obtained by serial centrifugations of PRP with the addition of PGI₂ and apyrase, were adjusted to 250 × 10³ platelets/μL, and aggregated by Born turbidimetric technique in presence of ADP (0.5 and 1 μM), collagen (0.5 and 1 μg/mL) and fibrinogen (0.25 mg/mL). PTX3, its C-terminal or N-terminal domain (1 μg/mL) was incubated for 1 h at RT with fibrinogen (IMMUNO AG, Italy – ratio 1:10) or overnight at 4 °C with collagen (Mascia Brunelli, Italy – ratio 1:1). As controls, same conditions were used for samples without PTX3 and its domains. All samples were incubated in an acid solution [10] (Diluent B, Mascia Brunelli, Italy), as collagen is soluble at acidic pH. To exclude unspecific binding to collagen, the ability of collagen preincubated with BSA (1 μg/mL, same experimental conditions used for PTX3) to stimulate aggregation was tested and resulted similar to that of collagen without preincubation with BSA (data not shown).

2.11. Static platelet adhesion assay

Glass coverslips were coated overnight at 4 °C with collagen (10 μg/mL) or fibrinogen (10 μg/mL, SIGMA-Aldrich, Italy), in the presence or absence of PTX3 (10 μg/mL). The coverslips were washed three times with PBS, then, unspecific binding sites were blocked with BSA 5 mg/mL (incubation 1 h at 37 °C), followed by three PBS washes. Next, washed platelets (2 × 10⁷ platelets/mL) were added to the coated coverslips for 1 h at 37 °C. Non-adherent platelets were removed by washing three times with PBS, adherent platelets were fixed with PFA 4% for 15 min followed by incubation with Triton X-100 0.1% on ice for three minutes. Platelets were then stained with phalloidin-Alexa 546 (1:100 in PBS, SIGMA) for 20 min in the dark and were visualized with the Axiovert software using the rhodamine light at 40× magnification.

2.12. Statistical analysis

Statistical analyses were performed with GraphPad Prism6 or with IBM-SPSS statistic 19. Data were analyzed by the Wilcoxon rank-sum test or by ANOVA with repeated measures for main effects of treatment time and genotype, followed by a Bonferroni post hoc analysis. Data are presented as mean ± SEM.

3. Results

3.1. PTX3 deficiency in vascular cells but not in hematopoietic cells is associated with increased arterial thrombosis.

PTX3 KO and WT present a similar blood flow in the carotid artery under basal conditions (0.67 ± 0.03 mL/min vs 0.60 ± 0.04 mL/min respectively, mean ± SEM are reported) but following FeCl₃ induce arterial thrombosis, PTX3 deficiency results in increased arterial thrombosis [10]. To disentangle the contribution of PTX3 produced by bone marrow (BM) cells or vascular cells in the increased arterial thrombosis observed in PTX3 KO mice, we performed the FeCl₃ arterial thrombosis experiment in irradiated PTX3 KO mice reconstituted with the BM of PTX3 KO or WT mice (PTX3 KO/BMT_PT3 KO or PTX3 KO/BMT_WT) and in irradiated WT reconstituted with the BM of PTX3 KO or WT mice (WT/BMT_PT3 KO or WT/BMT_WT) (Supplemental Fig. 1). Following the application of FeCl₃ (10%), WT transplanted with BM of PTX3 KO behaved similarly in terms of carotid blood flow to WT transplanted with BM of WT (Fig. 1A) without showing carotid occlusion during the 30 min after FeCl₃ injury (Fig. 1B). On the contrary, PTX3 KO transplanted with BM of WT showed a reduction of carotid artery blood flow (Fig. 1A) with carotid occlusion occurring, on average,

19 min after FeCl₃ application (Fig. 1B), a profile similar to that of PTX3 KO mice transplanted with the BM of PTX3 KO. Histological analyses confirmed the differences in carotid arterial thrombus formation (Fig. 1C). These data suggest a critical role in the modulation of thrombus formation for PTX3 produced in the vessel wall but not for the protein stored and acutely released by neutrophils during acute cardiovascular events [24]. Moreover, PTX3 plasma levels, after arterial thrombosis, were similar in WT mice transplanted with either WT or PTX3 KO bone marrow, while not detectable levels were observed in the plasma of PTX3 KO mice transplanted either with WT or with PTX3 KO bone marrow (Fig. 1D). In parallel with the latter observation, PTX3 plasma levels in WT mice during the 30 min following FeCl₃ injury were marginally altered (Supplemental Fig. II).

3.2. PTX3 protective activity is not dependent on P-selectin modulation

We next addressed whether PTX3 protective role could have been the consequence of the modulation of P-selectin. This protein is a key player of platelet–platelet, platelet–leukocyte and platelet–endothelium interaction during thrombosis [25] and it is targeted by PTX3 to dampen neutrophil recruitment [11]. Thus, increased P-selectin-dependent thrombotic response might represent a logical mechanism to explain the pro-thrombotic phenotype observed in PTX3 KO mice [10]. To address this issue, we generated P-selectin KO/PTX3 double KO mice and investigated carotid blood flow following arterial thrombosis compared to P-selectin KO, WT and PTX3 KO mice. Following the application of FeCl₃ (10%), P-selectin/PTX3 DKO showed increased arterial thrombosis similar to PTX3 KO mice while, at this FeCl₃ concentration, P-selectin KO mice and WT mice were protected toward arterial thrombosis (Fig. 2A). In P-selectin/PTX3 DKO and PTX3 KO mice, carotid occlusion was observed, on average, 17 min after FeCl₃ application, while no occlusion was observed in WT and P-selectin KO mice up to 30 min after FeCl₃ injury (Fig. 2B). The histological analysis confirmed the increased thrombosis in DKO mice (Fig. 2C). Of note, the lack of both proteins resulted in a thrombotic phenotype similar to that observed in PTX3 KO mice, while the only absence of P-selectin was not able to induce the formation of a thrombus, thus suggesting that the protection toward arterial thrombosis exerted by PTX3 might be independent on P-selectin modulation (Fig. 2A–C).

Given the partial resistance of P-selectin KO to FeCl₃ induced arterial thrombosis, we next investigated the effect of a more robust induction of arterial thrombosis in these mice (application of FeCl₃ 20%) to study whether PTX3 deficiency could affect thrombus formation and leukocyte recruitment in the context of P-selectin deficiency. In spite of larger thrombi observed in P-selectin/PTX3 DKO compared to P-selectin KO (Fig. 3A–C), leukocytes recruitment in the thrombus (Fig. 3D) and the percentage of circulating platelet–leukocyte aggregates, 30 min after FeCl₃ application (Fig. 3E), were not different between P-selectin KO and P-selectin/PTX3 DKO. The latter findings suggest also that the increased thrombus formation in DKO was not dependent on a different leukocyte recruitment during thrombosis. In line with this, ADP stimulation of whole blood from PTX3 KO and WT mice resulted in a similar percentage of platelet–leukocyte aggregates (Supplemental Fig. III). These observations limit the relevance of the P-selectin/PTX3 axis during arterial thrombosis and further suggest that PTX3 produced by hematopoietic cells, as the protein stored in neutrophils, does not contribute to the protection toward arterial thrombosis.

3.3. PTX3 deficiency does not affect platelet activation and hemostatic properties

A detailed characterization of PTX3 KO platelets was then performed. P-selectin expression and activated integrin α₂β₃ expression were similar in WT and PTX3 KO platelets, under basal conditions or

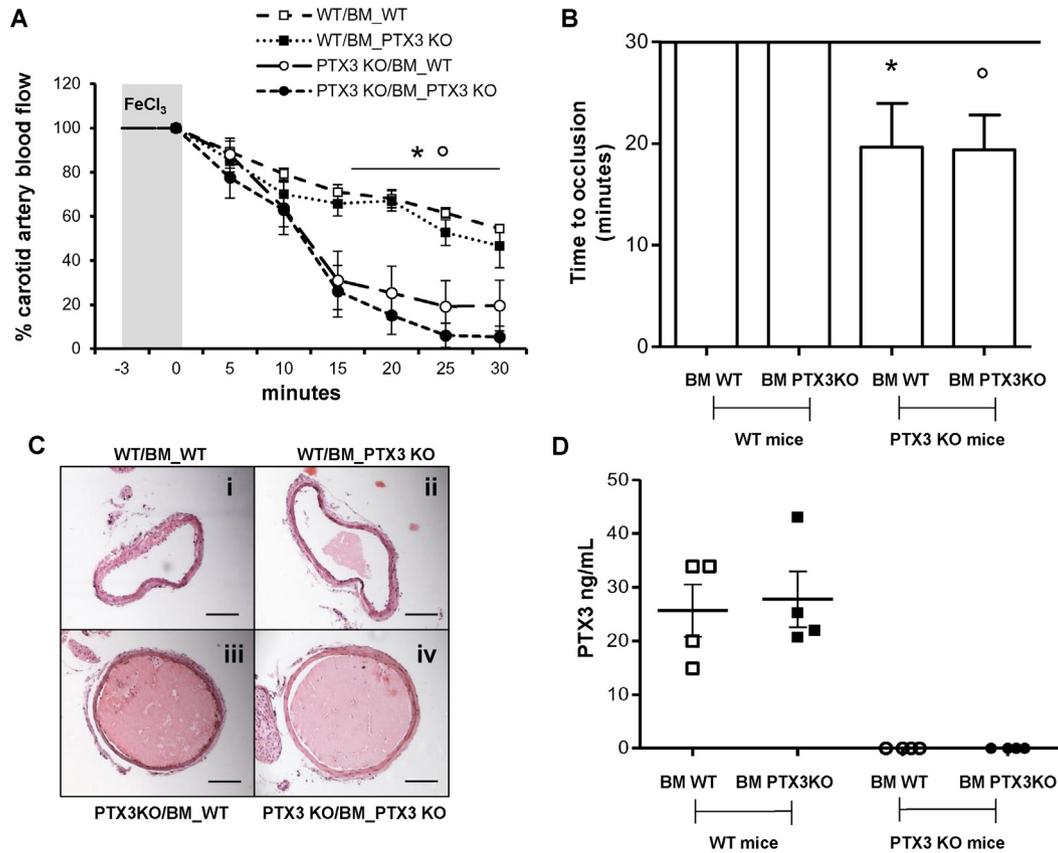


Fig. 1. PTX3 KO mice bone marrow transplanted with BM from WT and PTX3 KO show an enhanced thrombus formation *in vivo* compared to WT mice transplanted with BM from WT and PTX3 KO mice after topical application of FeCl₃ 10%. Panel A: carotid artery blood flow in BM transplanted (BMT) PTX3 KO and WT mice; data, expressed as relative percentage to the value before injury, are mean \pm SEM, * $p < 0.01$ WT/BMT_WT ($n = 5$) vs PTX3 KO/BMT_WT ($n = 6$) and PTX3 KO/BMT_PTX3 KO ($n = 5$), * $p < 0.05$ WT/BMT_PTX3 KO ($n = 6$) vs PTX3 KO/BMT_PTX3 KO ($n = 5$). Panel B: time to carotid occlusion during 30 min of observation after topical application of FeCl₃ 10%; data shown are mean \pm SEM, * $p < 0.01$ WT/BMT_WT ($n = 5$) vs PTX3 KO/BMT_WT ($n = 6$) and PTX3 KO/BMT_PTX3 KO ($n = 5$), * $p < 0.05$ WT/BMT_PTX3 KO ($n = 6$) vs PTX3 KO/BMT_PTX3 KO ($n = 5$). Panel C: representative hematoxylin and eosin (H/E) stained cross-sections of carotid arterial thrombi from BM transplanted mice, scale bar = 100 μ m. Panel D: PTX3 plasma levels after 30 min FeCl₃ treatment (10%); data were obtained from plasma with ELISA sandwich test and are mean \pm SEM.

after stimulation with collagen and a thromboxane analog (U46619) (Supplementary Fig. IV).

Furthermore, PTX3 KO and WT mice had similar number of circulating platelets (Supplementary Fig. V panel A), comparable plasma fibrinogen levels (Supplementary Fig. V panel B) and tail bleeding time (Supplementary Fig. VC). In addition, similar prothrombin time (PT) and activated partial prothrombin time (APTT) were measured in the two groups of mice under basal conditions (Supplementary Fig. V panels D and E). Finally, we excluded that the increased thrombosis observed in PTX3 KO mice was a consequence of an impaired expression of tissue factor (TF), indeed similar TF mRNA levels were detected in aortic tissue of PTX3 KO and WT mice (Supplementary Fig. V panel F).

3.4. PTX3 acts between the damaged vascular wall and the thrombus by dampening fibrinogen and collagen pro-thrombotic effects

We next focused our attention on vessel wall structure. PTX3 in WT mice after FeCl₃ application localized mainly between the damaged endothelium and the thrombus and co-localized with fibrin (Supplementary Fig. VI). Based on this and on the recent evidence that PTX3 deficiency induces fibrin deposition after tissue injury [10] we tested the hypothesis that PTX3 could interact with extracellular matrix components of the vessel wall thus limiting their pro-thrombotic potential.

We first observed that human recombinant PTX3 (hrPTX3) significantly prevented static adhesion of WT platelets on fibrinogen- and collagen-coated surface (Fig. 4A,B and D,E), and reduced platelets spreading on both coated surfaces (Fig. 4A,C and D,F).

Next, we tested the ability of PTX3 to interfere with collagen- and/or fibrinogen-induced platelet aggregation. Three different sets of experiments were performed. First we observed that the pre-incubation of fibrinogen with PTX3 significantly reduced platelet aggregation in the presence of collagen, an effect mainly dependent on PTX3 N-terminal domain (Fig. 5A). In addition, the pre-incubation of collagen with PTX3 attenuated platelet aggregation (in the presence of fibrinogen) but this effect was mainly dependent on PTX3 C-terminal domain (Fig. 5B). Finally we observed a further reduction of platelet aggregation when both fibrinogen and collagen were separately pre-incubated with PTX3 (Fig. 5C). Of note, the observation that N-terminal domain appeared to be more selective for fibrinogen while PTX3 C-terminal domain appeared to be more selective for collagen supports the hypothesis that PTX3 might dampen both fibrinogen and collagen effect, resulting in a synergistic control of their pro-thrombotic activity. (Fig. 5C).

3.5. Recombinant human PTX3 protects from arterial thrombosis

Data collected so far suggest that the protective effect of PTX3 on platelet aggregation could be the result of a synergistic control of collagen and fibrinogen pro-thrombotic activity. To extend these findings *in vivo*, we tested whether human recombinant PTX3 (hrPTX3) was able to revert the pro-thrombotic phenotype observed in PTX3 KO mice. Mice were injected *i.v.* with hrPTX3 (5 mg/kg) [11,17] followed by FeCl₃ (10%) injury. PTX3 KO mice injected with PBS showed carotid artery occlusion, on average, 17 min after FeCl₃ application, while hrPTX3 treatment completely prevented carotid artery occlusion in

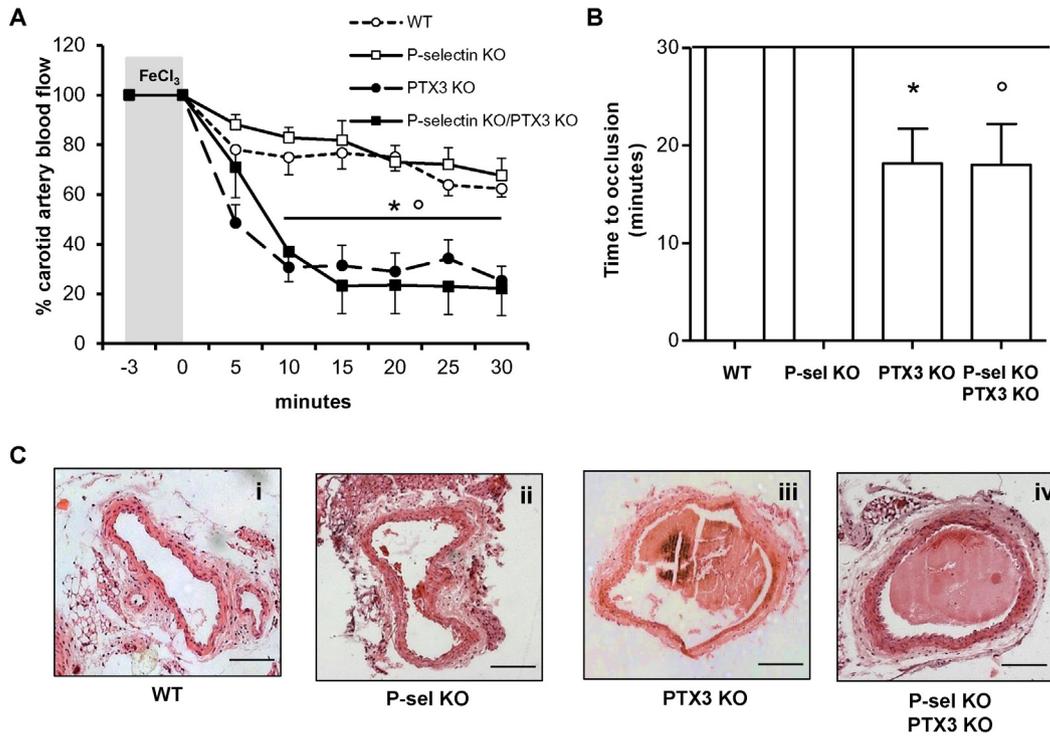


Fig. 2. The protective effect of PTX3 goes beyond the interaction with P-selectin. Panel A: carotid artery blood flow in WT, P-selectin KO, PTX3 KO and P-selectin KO/PTX3 KO mice after topical application of FeCl_3 (10%); data, expressed as relative percentage to the value before injury, are mean \pm SEM, $n = 5/\text{group}$, * $p < 0.01$ WT vs PTX3 KO and DKO, ° $p < 0.01$ P-selectin vs PTX3 KO and DKO. Panel B: time to carotid occlusion during 30 min of observation after topical application of FeCl_3 10%; data shown are mean \pm SEM; $n = 5/\text{group}$ * $p < 0.01$ WT vs PTX3 KO and P-selectin KO/PTX3 KO, ° $p < 0.01$ P-selectin KO vs PTX3 KO and P-selectin KO/PTX3 KO. Panel C: representative hematoxylin and eosin (H/E) stained cross-sections of carotid arterial thrombi from WT, P-selectin KO, PTX3 KO and P-selectin KO/PTX3 KO mice after FeCl_3 10% treatment, scale bar = 100 μm .

our experimental model (Fig. 6A). Similar data were observed when the effect of hrPTX3 injection was tested in wild type mice following FeCl_3 (20%) injury. In this group, carotid occlusion after FeCl_3 (20%) application occurred later in hrPTX3 injected mice compared to controls, within 24 min and 15 min respectively (Fig. 6B).

4. Discussion

In this work we have shown that PTX3 produced by vascular cells plays a protective role in arterial thrombosis by reducing collagen and fibrinogen induced platelet aggregation.

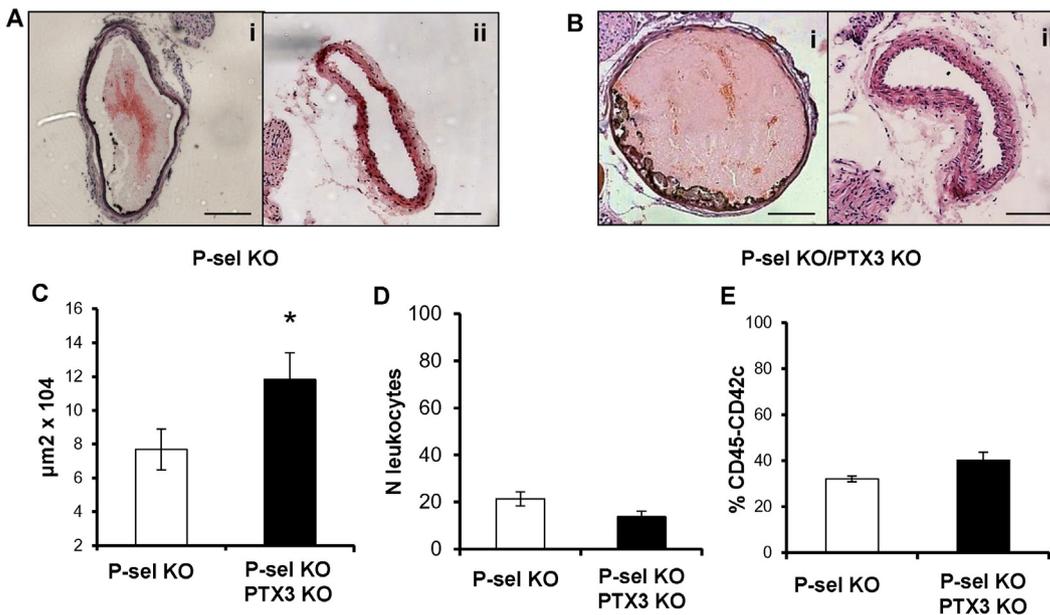


Fig. 3. PTX3 does not affect leukocyte recruitment during arterial thrombosis. Panels A–B: representative pictures of carotid arterial thrombi (Ai, Bi) and contralateral control carotid arteries (Aii, Bii) from P-selectin KO and P-selectin KO/PTX3 KO mice following FeCl_3 20% treatment, scale bar = 100 μm . Panel C: thrombi area of P-selectin KO and PTX3 KO/P-selectin KO mice following arterial thrombosis (FeCl_3 20%) for each animal, four different sections of the carotid artery (each every 50 μm) were analyzed. Mean \pm SEM is shown; $n = 4/\text{group}$, * $p < 0.05$. Panel D: number of leukocytes within thrombi from P-selectin KO, P-selectin KO/PTX3 KO after FeCl_3 20% treatment; four different sections of the carotid artery (each every 50 μm) were analyzed; mean \pm SEM is reported, $n = 4/\text{group}$. Panel E: percentage of platelet–leukocyte aggregates in whole blood from P-selectin KO, P-selectin KO/PTX3 KO after FeCl_3 20% treatment; data are shown as mean \pm SEM, $n = 4/\text{group}$.

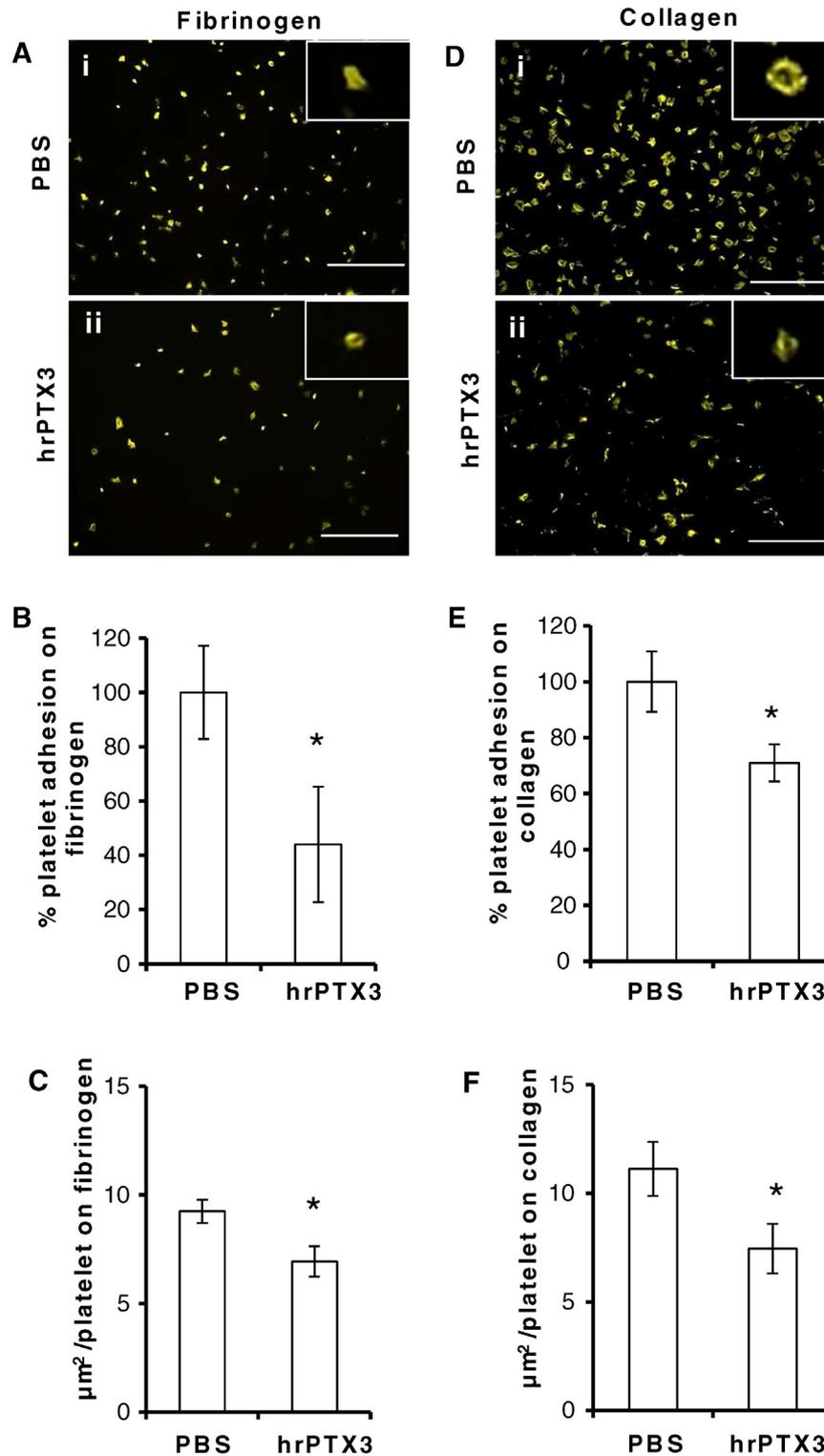


Fig. 4. The protective effect of PTX3 is mediated through the interaction with collagen and fibrinogen. Panel A: representative pictures of platelet adhesion on fibrinogen-coated coverslips; scale bar = 50 μm (magnification 40 \times , Axiovert). Panel B: platelet adhesion on fibrinogen-coated coverslips (10 $\mu\text{g}/\text{mL}$) incubated with PBS or hrPTX3 (10 $\mu\text{g}/\text{mL}$); data shown are mean of platelet adhesion expressed as relative percentage to fibrinogen alone \pm SEM; * $p < 0.01$, $n = 4$ /condition. Panel C: platelet spreading on fibrinogen-coated coverslips (10 $\mu\text{g}/\text{mL}$) incubated with PBS or hrPTX3 (10 $\mu\text{g}/\text{mL}$); data shown are mean of platelet area (μm^2) \pm SEM; * $p < 0.05$, $n = 7$ /condition. Panel D: representative pictures of platelet adhesion on collagen-coated coverslips (magnification 40 \times , Axiovert). Panel E: platelet adhesion on collagen-coated coverslips (10 $\mu\text{g}/\text{mL}$) incubated with PBS or hrPTX3 (10 $\mu\text{g}/\text{mL}$); data shown are mean of platelet adhesion expressed as relative percentage to collagen alone \pm SEM; * $p < 0.01$, $n = 7$ /condition. Panel F: platelet spreading on collagen-coated coverslips (10 $\mu\text{g}/\text{mL}$) incubated with PBS or hrPTX3 (10 $\mu\text{g}/\text{mL}$); data shown are mean of platelet area (μm^2) \pm SEM; * $p < 0.05$, $n = 7$ /condition.

In recent years, PTX3 has emerged as a key acute phase protein associated to inflammation in cardiovascular disorders, including heart failure, atherosclerosis, acute coronary syndromes and peripheral vascular

diseases [4,7,9]. Although PTX3 is a prognostic marker of acute myocardial infarction [26], genetically determined high levels of PTX3 do not increase the risk of acute myocardial infarction (AMI), suggesting that

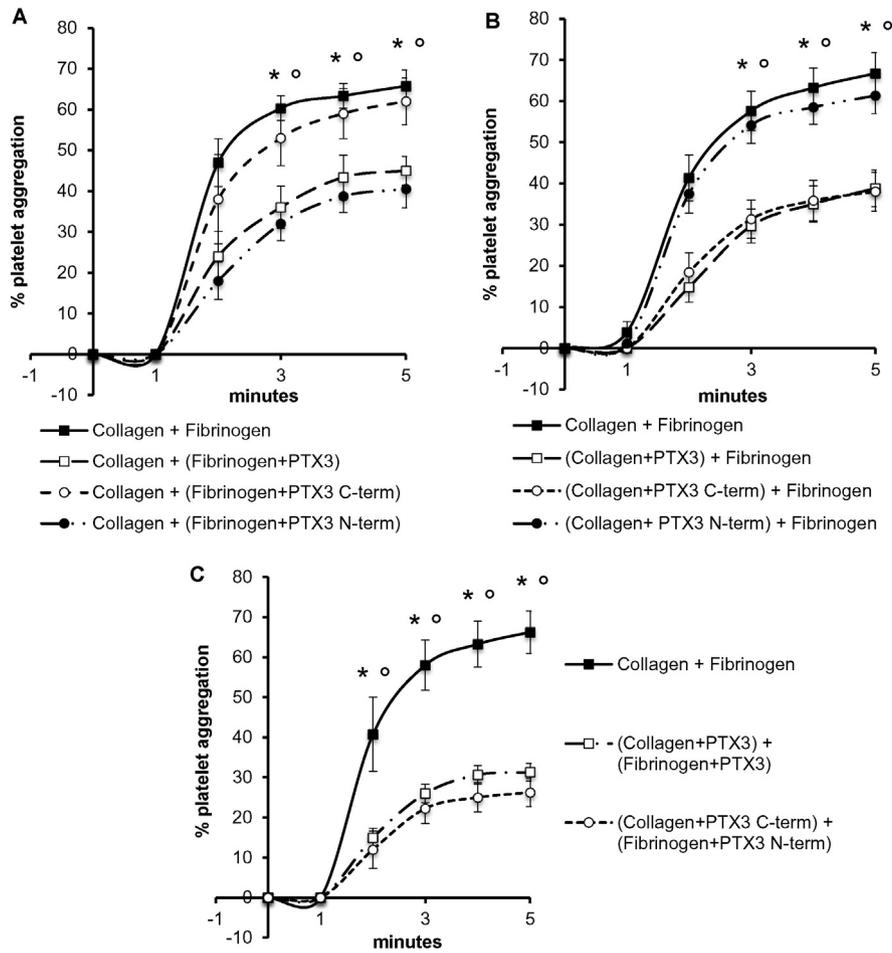


Fig. 5. PTX3 interacts with fibrinogen and collagen thus limiting platelet aggregation. Panel A: platelet aggregation following stimulation with collagen (1 µg/mL) in the presence of fibrinogen (0.25 mg/mL), fibrinogen pre-incubated with hrPTX3 (1 µg/mL, 1 h RT), fibrinogen pre-incubated with hrPTX3 C-terminal domain (1 µg/mL, 1 h RT) or fibrinogen pre-incubated with hrPTX3 N-terminal domain (1 µg/mL, 1 h RT) is shown. Data are presented as mean ± SEM; *p < 0.001, n = 4/group. Panel B: platelet aggregation in the presence of fibrinogen following stimulation with collagen (1 µg/mL), collagen pre-incubated with hrPTX3 (1 µg/mL, 18 h ON 4 °C), collagen pre-incubated with hrPTX3 C-terminal domain (1 µg/mL, 18 h ON 4 °C) and collagen pre-incubated with hrPTX3 N-terminal domain (1 µg/mL, 18 h ON 4 °C) is shown. Data are presented as mean ± SEM; *p < 0.001, n = 6/group. Panel C: platelet aggregation following stimulation with collagen (1 µg/mL) and fibrinogen (0.25 mg/mL), or following incubation with both collagen and fibrinogen pre-incubated with hrPTX3 (1 µg/mL, 18 h ON 4 °C and 1 µg/mL, 1 h RT respectively) and the proper controls is shown. Data are presented as mean ± SEM; *p < 0.001, n = 4/group.

PTX3 concentration itself is unlikely to be even a modest causal factor for AMI [14]. Furthermore, although PTX3 was initially described to induce tissue factor (TF) expression in endothelial cells and monocytes in vitro [27,28], later studies in vivo failed to confirm this findings [10]; and indeed we showed here that TF expression in the aorta of PTX3 KO and WT mice was similar, arguing

against a pro-thrombotic effect of PTX3. Moreover, data from animal models point to a protective function of the protein: PTX3 overexpression limits neointimal thickening after balloon injury of rat carotid artery [29], while PTX3 KO mice, following ischemia/reperfusion (I/R) injury, present a significantly larger infarcted area compared to WT mice [13]. PTX3 deficiency was also

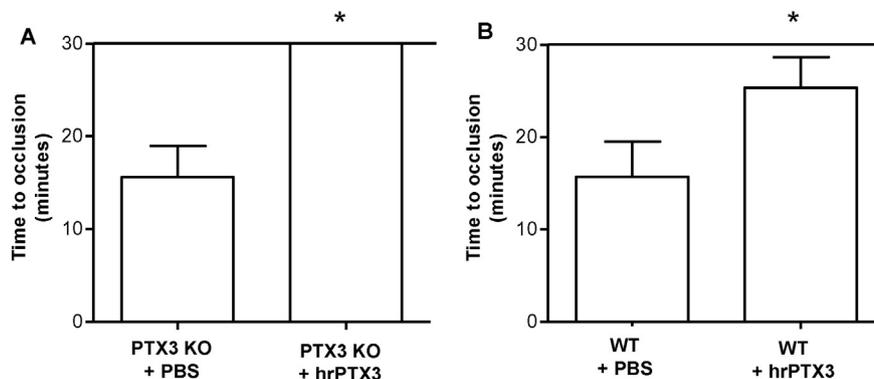


Fig. 6. Administration of human recombinant PTX3 limited carotid artery occlusion in PTX3 KO and in WT mice. Panel A: time to carotid occlusion during 30 min of observation after topical application of FeCl₃ 10% in PTX3 KO mice injected with hrPTX3 or PBS; data are shown as mean ± SEM; PTX3 KO + PBS n = 6, PTX3 KO + hrPTX3 n = 4, **p < 0.01. Panel B: time to carotid occlusion after topical application of FeCl₃ 20% in WT mice injected with hrPTX3 or PBS; data are shown as mean ± SEM; n = 8/group, *p < 0.05.

associated with increased atherosclerosis, increased macrophage accumulation and inflammation in the atherosclerotic lesions [12].

We have now extended these findings by identifying the mechanisms beyond PTX3 protective role in arterial thrombosis, a critical complication of atherosclerotic plaque rupture correlated with relevant clinical manifestations including myocardial infarction or stroke. First, we observed that PTX3 protective effects depend on the protein produced by vascular cells and not by hematopoietic cells as only the deficiency of PTX3 in the vasculature, but not in hematopoietic cells, was associated to a pro-thrombotic phenotype, similar to that of PTX3 KO mice. Next, we excluded that this effect was related to P-selectin, a target of PTX3 [11], which mediates the recruitment of leukocytes to the activated endothelium and is also relevant for platelet–leukocyte interaction. Indeed P-selectin/PTX3 DKO mice showed increased carotid artery occlusion, similar to PTX3 KO mice, following arterial thrombosis compared to P-selectin KO mice. This observation, coupled with those indicating: i) that PTX3 modulation of P-selectin activity is dependent on PTX3 produced by hematopoietic cells [11]; ii) that the protection toward arterial thrombosis is dependent on vascular produced PTX3 (see Fig. 1); and iii) that platelet activation is similar in PTX3 KO and WT mice (Supplementary Fig. IV), limits the role of PTX3 in dampening leukocyte–platelet interaction and leukocyte recruitment in the thrombus, but rather points toward a direct effect on vascular thrombogenicity.

PTX3, is produced by and stored in hematopoietic cells [11,15,30], but is also synthesized by several types of stromal cells including fibroblasts [31], smooth muscle cells [31], endothelial cells [2,22] and is a component of tissue extracellular matrix (ECM) [32]. PTX3 interacts with other components of the ECM, including the viscoelastic HA-rich matrix of the cumulus oophorus complex that surrounds the preovulatory follicle [32,33], and also plays a critical role during tissue repair following wound healing, a mechanism which is dependent on the acidic microenvironment which is generated following tissue injury [10]. These tissue-related functions of PTX3 appear to be independent of those associated with acute phase conditions, such as endotoxemic shock, sepsis and other inflammatory and infectious states, but also from myocardial infarction [9], where PTX3 increases in few hours and is contributed by neutrophils following the interaction with activated platelets [24].

Our data indicate that PTX3 N-terminal domain might target fibrinogen while the PTX3 C-terminal domain might target collagen, thus resulting in a synergistic effect of PTX3 in the prevention of thrombosis.

We might speculate that these interactions result from the profound homology between these pro-thrombotic molecules and highly conserved sequences of proteins belonging to the innate immune system: indeed, the first component of complement C1q and other related proteins as collectins and ficolins, which are targeted by PTX3 favoring the activation of complement system, possess either a collagen-like region or a fibrinogen like domain [34,35], further suggesting the intriguing hypothesis that tuning thrombosis could be part of the innate immune response. Furthermore, we also need to consider that the different functions ascribed to PTX3 might originate from the high glycosylation variability among PTX3 isolated from different cellular sources: this suggests that the glycosylation pattern might change depending on cell type and inducing stimulus, thus modulating the crosstalk between PTX3 with different ligands in different conditions (as complement components and P-selectin) [36].

Although it is tempting to speculate that the interaction of PTX3 through its N- and C-terminal domain with fibrinogen and collagen respectively may dampen the generation of the pro-thrombotic fibrin from fibrinogen and the exposure of collagen during vascular injury, an effect favored by local acidification occurring during tissue injury [10], we cannot exclude the possibility that PTX3 might also influence additional pathways in the atherothrombotic response, as the fibrinolytic process; indeed, PTX3 through its N-terminal domain was observed to bind plasminogen and, *in vitro*,

fibrin gel degradation mediated by plasmin and triggered by urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA) was potentiated in the presence of PTX3 [10]. The latter observation suggests the possibility that PTX3 perhaps might affect also thrombus resolution and additional studies need to be planned to address this issue.

It has to be acknowledged that the physio-pathological events occurring immediately after FeCl₃ injury are debated; initially it was proposed that red blood cells are the first adherent blood component toward FeCl₃ injured-endothelium [37], however a careful analysis revealed that, the first wave of erythrocytes adhesion, is followed after few minutes by a second phase associated to conventional platelet activation and adhesion [38]. Our experiments indicate that PTX3 deficiency affects carotid artery occlusion at least 10 min after FeCl₃ application, further supporting a role for PTX3 in the second phase of FeCl₃ induced arterial thrombosis, thus modulating platelet adhesion to damaged endothelium and fibrinogen.

Finally, the observation that the injection of recombinant PTX3 rescues, at least in part, the phenotype observed in PTX3 deficient mice and improves the outcome in wild type mice, supports the need for further studies aimed at addressing the pharmacological potential of PTX3 in the context of atherothrombosis.

Transparency document

The [Transparency document](#) associated with this article can be found, in the online version.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbdis.2016.03.007>.

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