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Novel insights in thrombosis pathophysiology using Mice with Impaired anticoagulation

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Silencing of Anticoagulant Protein C Evokes Low Incident but Spontaneous Atherothrombosis in Apolipoprotein E Deficient Mice

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ABSTRACT

Murine atherosclerosis models do not spontaneously develop atherothrombotic complications. We investigated whether disruption of natural anticoagulation allows pre-existing atherosclerotic plaques to progress towards an atherothrombotic phenotype. Upon lowering of plasma protein C levels with small interfering (si) RNA (si*Proc*) in 8 weeks Western-type diet (WTD) fed atherosclerotic apolipoprotein E deficient (*Apoe*$^{-/-}$) mice, one out of four mice displayed a large, organized, and fibrin- and leukocyte-rich thrombus on top of an advanced atherosclerotic plaque located in the aortic root. Although again at low incidence (3 in 25), comparable thrombi at the same location were observed during a second independent experiment in 9 weeks WTD-fed *Apoe*$^{-/-}$ mice. Mice with thrombi on their atherosclerotic plaques did not show other abnormalities and had equally lowered plasma protein C levels as si*Proc* treated *Apoe*$^{-/-}$ mice without thrombi. Fibrinogen and thrombin-antithrombin concentrations and blood platelet numbers were also comparable, and plaques in si*Proc* mice with thrombi had a similar composition and size as plaques in si*Proc* mice without thrombi. 7 out of 25 si*Proc* mice featured clots in the left atrium of the heart. Our findings indicate that siRNA-mediated silencing of *Proc* in *Apoe*$^{-/-}$ mice creates a condition that allows the occurrence of spontaneous atherothrombosis, albeit at a low incidence. Lowering natural anticoagulation in atherosclerosis models may help to discover factors that increase atherothrombotic complications.

INTRODUCTION

Atherothrombosis, characterized by superimposed luminal thrombus formation on a ruptured or eroded atherosclerotic plaque, is a major cause of acute coronary syndromes and cardiovascular death in humans (1). Pathological changes of the atherosclerotic plaque, such as thinning of the fibrous cap and the development of a large necrotic core, are known to make the plaque prone to rupture, and thereby expose triggers for thrombosis (2).

Murine models of atherosclerosis, such as hyperlipidemic apolipoprotein E deficient (*Apoe*$^{-/-}$) mice, have been instrumental to study atherosclerosis pathophysiology and the search for highly needed novel therapeutic targets. However, in these murine models the final stage of atherosclerosis i.e plaque rupture and the subsequent induction of atherothrombosis, does not occur spontaneously. Factors suggested to underlie the absence of spontaneous atherothrombosis include resistance of murine plaques to rupture, because of a different plaque composition, and differences in hemodynamics (3). Moreover, species differences in anticoagulation could contribute to the absence of progression to atherothrombosis in mice. The half-life of active coagulation factor IIa in mouse plasma is significantly shorter as compared to its human counterpart, pointing towards more potent natural anticoagulation in mice (4). In line, atherosclerotic plaques in hyperlipidemic mice with impaired hemostasis revealed markers of thrombotic events in carotid artery plaques (5, 6).

In the present study, we tested whether small interfering (si) RNA-mediated lowering of natural anticoagulants antithrombin (*Serpinc1*) and protein C (*Proc*) in *Apoe*$^{-/-}$ mice allowed plaques to progress towards an atherothrombotic phenotype. We found that lowering of *Proc* evokes low incident spontaneous atherothrombosis in *Apoe*$^{-/-}$ mice.

MATERIALS AND METHODS

All mice used in this experiment have the Apoetm1Unc mutation and were originally obtained from Jackson Laboratories (Bar Harbour (MA) USA) on a C57BL/6J background (catalogue nr. 002052), and were inbred in house at the Gorlaeus Laboratories (Leiden, the Netherlands). All animal experiments were performed in accordance with the national guidelines for animal experimentation. All experimental protocols were approved by the Ethics Committee for Animal Experiments of either Leiden University or the Leiden University Medical Center.

Experiment 1

Female *Apoe*$^{-/-}$ mice (C57BL/6J background, 6 weeks old, n=9 per group) were fed a Western-Type diet (WTD; Special Diet Services, Sussex, UK) for 2 weeks to exacerbate hyperlipidemia, followed by an intravenous injection (tail vein) with 5 mg/kg synthetic siRNAs (Ambion, Life Technologies, Carlsbad (CA), USA) targeting *Serpinc1* (si*Serpinc1*, cat. #S62673) or *Proc* (si*Proc*, cat. #S72192), or control siNEG (cat. #4404020), complexed with Invivofectamine 2.0 (Life Technologies) as described previously (7). Mice were sacrificed at 3 and 7 days post siRNA injection by anesthesia with a subcutaneous injection with a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg), followed by exsanguination and in situ perfused with PBS after which the heart, liver, lungs, spleen, and aorta were collected. Organs were either snap frozen or fixed for 24 h in 3.7% neutral buffered formalin (Formal-Fixx, Shandon Scientific Ltd, UK) for analysis.

Experiment 2

Female *Apoe*$^{-/-}$ mice (4 weeks old, n=4 per group) were fed a WTD for 8 weeks to induce atherosclerotic plaque development, followed by an intravenous injection (tail vein) with 8 mg/kg si*Proc*, *Serpinc1*, or control siNEG. Mice were sacrificed 2 days post siRNA injection as described above, after which blood and organs were collected and snap frozen or formalin-fixed for analysis.

Experiment 3

Female *Apoe*$^{-/-}$ mice (4-7 weeks old) were fed a WTD for 9 weeks to induce atherosclerotic plaque development, followed by an intravenous injection (tail vein) with 8 mg/kg si*Proc* (n=25) or control siNEG (n=3). Mice were sacrificed 7 days post siRNA injection as described above, after which blood and organs were collected and snap frozen or formalin-fixed for analysis.

Blood and plasma analysis

For the analysis of plasma (anti)coagulation proteins, a blood sample on sodium citrate (final concentration 0.32%) was drawn from the inferior caval vein upon sacrifice. Plasma was obtained by centrifugation and stored at −80°C until further use. Plasma protein C levels were determined using a polyclonal sheep anti-murine antibody (Haematologic Technologies Inc. Essex Junction (VT), USA). Plasma fibrinogen antigen levels were assessed with a commercial murine ELISA kit from Affinity Biologicals (Ancaster (ON), Canada). Thrombin-antithrombin levels were determined exactly according to the manufacturer's protocol (Siemens Healthcare, Frederick (MD), USA).

After blood sampling from the inferior caval vein, the mice were exsanguinated via orbital bleeding. Orbital blood was collected in EDTA-coated tubes. Whole blood cell and platelet counts were analysed using an automated XT-2000iV veterinary hematology analyser (Sysmex Europe GMBH, Norderstedt, Germany). Plasma from the orbital bleeding-obtained blood was

attained by centrifugation and stored at −20°C until further use. Plasma total cholesterol levels were measured by enzymatic colorimetric assay (Roche diagnostics, Almere, The Netherlands).

Hepatic gene expression analysis

siRNA-mediated hepatic silencing of *Serpinc1* and *Proc* silencing was routinely confirmed using quantitative PCR, with *Actb* as a reference gene (8).

Histology and immunohistochemistry

Formalin-fixed hearts were embedded in Sakura O.C.T. Compound™ (Sakura Finetek Europe B.V., Alphen aan de Rijn, The Netherlands), for sectioning. Serial sections (10 µm, 70 µm interval) of the aortic root were cut using the Leica CM3050S cryostat and routinely stained with hematoxylin and eosin for general histology. Plaque size per valve was determined by staining for neutral lipids using Oil-Red-O and hematoxylin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Corresponding sections were stained for collagen fibers using the Masson's Trichrome methods (Sigma-Aldrich). To determine the macrophage positive area, corresponding sections were immunohistochemically stained for CD68 (rat monoclonal anti-CD68 antibody 1:1000, AB53444, Abcam, Cambridge, UK). A goat α-rat alkali phosphatase antibody (1:100, A8438-1ML, Sigma-Aldrich) was used as second antibody. The sections were developed using ready-to-use substrate solution (BCIP/NBT Substrate system, code K0598, Dako, Heverlee, Belgium). Necrotic core area was determined as non-stained area within the atherosclerotic plaques. The percentage of collagen, macrophages and necrotic core in the plaques was determined by dividing the collagen- or CD68-positive or non-stained area by the total plaque surface area. All images were analysed by blinded computer aided morphometric analysis using the Leica DM-RE microscope and LeicaQwin software (Leica Ltd, Cambridge, UK). Martius Scarlett Blue staining (Atom Scientific, Manchester, UK) was performed as described by the manufacturer, with the exception of staining with Methyl Blue. Step involving MethylBlue was excluded, enabling a brighter and more specific signal for the red-stained fibrin. The aorta, liver, kidney, and lung were sectioned using Leica RM2235 microtome at 4 µm with an 36 µm interval for the aorta and 8 µm with an 40 µm interval for other organs. Serial sections were routinely stained with hematoxylin and eosin for general histology.

Data analysis

Statistical analysis was performed using Graphpad Instat (GraphPad Software, La Jolla (CA), USA). The significance of the differences was calculated using a non-parametric Mann Whitney U test. Probability values <0.05 were considered significant. All data are presented as median and range. Descriptive statistic and calculate proportions of the observations and the 95% confidence intervals (Wilson-score) were calculated using resources provided by the Open Source Epidemiologic Statistics for Public Health website:www.openepi.com/proprotions).

RESULTS

Mouse (C57BL/6J) hepatic *Serpinc1* and *Proc* expression can be effectively lowered using specific siRNAs (7). To investigate whether this siRNA approach also allowed successful downregulation of both anticoagulants in a hyperlipidemic background, *Apoe*$^{-/-}$ mice fed a Western-type diet (WTD) for 2 weeks were injected with si*Serpinc1*, si*Proc,* or siNEG (9 mice per group) and monitored for 7 days (experiment 1; median plasma total cholesterol levels 7.2 mg/mL (range: 4.4-12.7)) (supplemental figure 1). Within 4 days after siRNA injection, several si*Serpinc1* mice demonstrated lethargy, weight loss (1.5 g (0.1-2.4)), exophthalmos, periocular hemorrhages, and swelling of the mandibular area. Two mice died within this time frame. This phenotype has been described before in wild type C57BL/6J mice treated with si*Serpinc1* and si*Proc*, and is likely spontaneous venous thrombosis (7). In contrast to the *siSerpinc1* group, all WTD-fed *Apoe*$^{-/-}$ mice that received only si*Proc* remained grossly healthy for up to 7 days. Liver transcript analysis showed that the si*Serpinc1* mice had a *Serpinc1* transcript level of 6.0% (4.4-11.4; day 3; n=3) of the level in siNEG mice and 3.4% (3.2-3.7; n=3) compared to non-siRNA treated mice. *Proc* transcript levels in the si*Proc* mice were 21.2% of siNEG (17.3-27.5; day 7; n=4) and 10.7% (3.8-17.4, n=3) of non-siRNA treated mice.

To investigate whether inhibition of anticoagulation affects the atherosclerotic phenotype, *Apoe*$^{-/-}$ mice were fed WTD for 8 weeks to induce advanced atherosclerosis in the aortic root, prior to si*Serpinc1* or si*Proc* treatment. In this experiment (experiment 2; 4 mice per group; si*Serpinc1*, si*Proc*, and siNEG), mice were sacrificed 2 days after siRNA injection, which enabled us to include also si*Serpinc1* treated mice to study the impact on the atherosclerotic phenotype. Moreover, the siRNA dosage was increased to achieve a higher knockdown of *Proc.* Already within 2 days, one mouse in the si*Serpinc1* group demonstrated the characteristic venous thrombotic coagulopathy, while si*Proc* and siNEG mice again remained healthy. Histological analysis of the head, heart, kidneys, lungs, and liver of the remaining healthy si*Serpinc1* mice revealed no abnormalities (including no signs of coagulopathy). The early onset of spontaneous venous thrombosis in *Apoe*$^{-/-}$ mice precluded studies using si*Serpinc1* to inhibit *Serpinc1* for a longer time period.

Upon histological analysis, si*Proc* and siNEG mice appeared identical to the healthy si*Serpinc1* mice. However, one mouse in the si*Proc* group revealed an organized and large structure superimposed on one of the advanced atherosclerotic plaques in the aortic root, which was identified as a fibrin-positive thrombus (Martius Scarlet staining, figure 1). The thrombus consisted of layers of eosin positive-structures, and was infiltrated by leukocytes typically at the luminal side. Serial sections demonstrated that this thrombus was superimposed on the plaque for at least 320µm (supplemental figure 2).

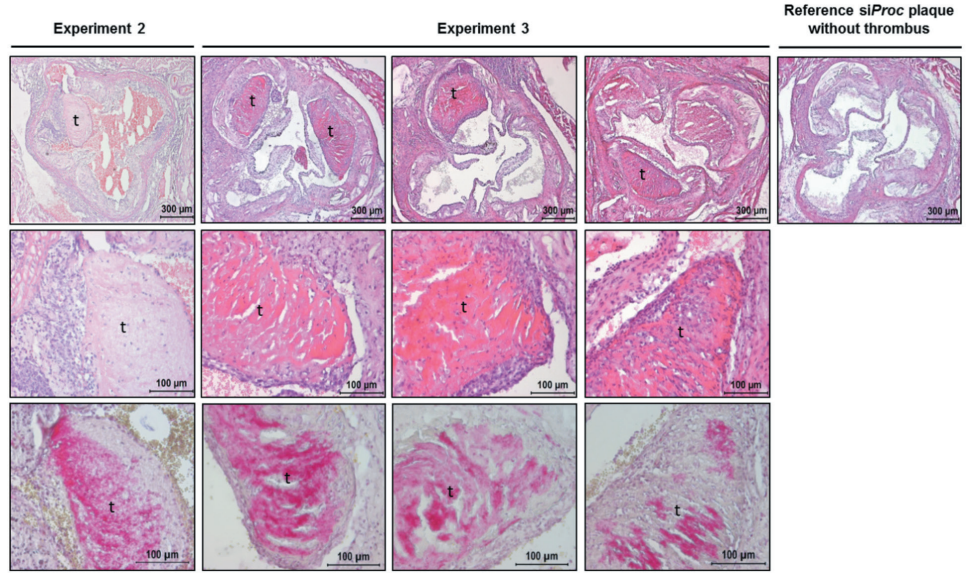

Figure 1 | siRNA-mediated inhibition of plasma protein C causes atherothrombosis in the aortic root in *Apoe*$^{-/-}$ mice. Microhistological images of aortic root atherothrombosis in *Apoe*$^{-/-}$ mice fed WTD for 8 weeks (1/4 mice; experiment 2) and 9 weeks (3/25 mice; experiment 3). Reference plaque without thrombus from si*Proc* mouse from experiment 3. Top 2 rows: Hematoxylin and eosin staining; bottom row: Martius Scarlet staining (red is indicative for fibrin). t = thrombus.

Although at a low incidence, the presence of an organized and large thrombus superimposed on an aortic root atherosclerotic plaque is unique and has, to our knowledge, not been reported before. To investigate the reproducibility of this finding, 25 mice were treated solely with si*Proc* (experiment 3). Silencing of *Proc* for 7 days in atherosclerotic *Apoe*$^{-/-}$ mice was again well-tolerated and no (macroscopic) abnormalities were seen. Importantly, 3 mice had organized and large fibrin-rich thrombi superimposed on atherosclerotic plaques in the aortic roots, with a similar size and composition as in experiment 2 (figure 1). Combining this result with experiment 2, a total of 4 out of 29 mice in 2 independent experiments showed atherothrombosis, resulting in a proportion of 13.8% with 95% confidence limits of 5.5%-30.5%).

Longitudinal sections of the aorta (arch, abdominal, and descending) demonstrated limited advanced atherosclerotic plaques at this location. In one mouse (out of 25), we observed Martius-Scarlett positive structures (indicative for fibrin) within an advanced atherosclerotic plaque in the aortic arch (supplemental figure 3). Lungs, kidneys, spleen, and liver of all si*Proc* mice were subjected to detailed microscopic analysis and did not exhibit any abnormalities or signs of thrombosis. Of note, in 7 out of 25 si*Proc* treated mice the left atrium of the heart

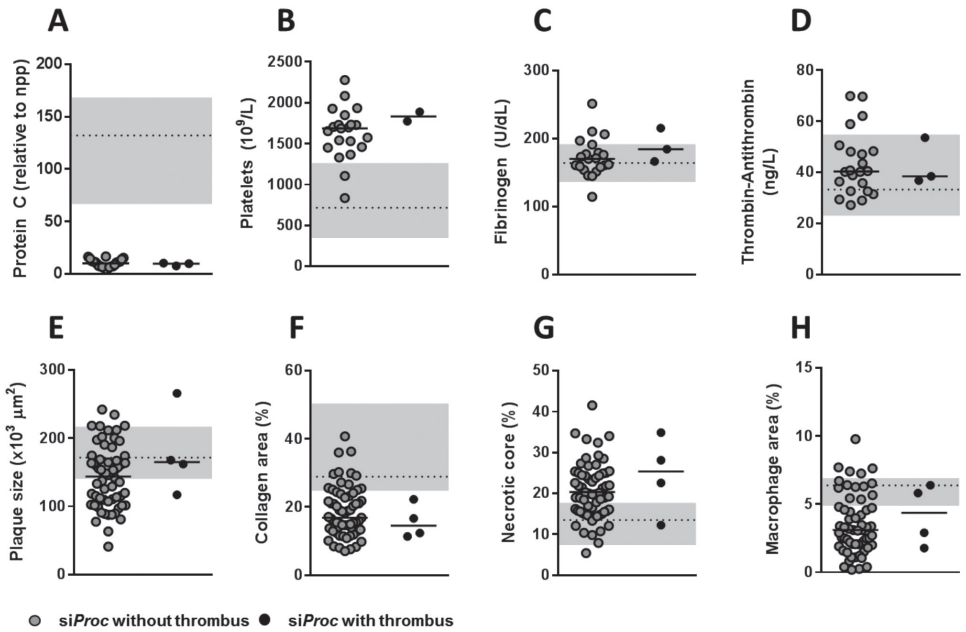

Figure 2 | Blood coagulation parameters and atherosclerotic plaque characteristics of si*Proc*-treated WTD-fed *Apoe*$^{-/-}$ mice without aortic root atherothrombosis are not significantly different compared to mice and plaques with atherothrombosis. (A) Residual plasma protein C levels, (B) Blood platelets, (C) Plasma fibrinogen levels, (D) Plasma thrombin-antithrombin complex levels, (E) Plaque size per aortic valve, (F) Collagen area as a percentage of total plaque size, (G) Necrotic core area as a percentage of total plaque size, (H) Macrophage (CD68-positive) area as a percentage of total plaque size. Lines represent medians. Grey zones in panels represent median and range of 2-9 weeks WTD-fed *Apoe*$^{-/-}$ siNEG mice (panels A-D, n = 9-11) and 9 weeks WTD-fed *Apoe*$^{-/-}$ siNEG mice (panels E-H, n = 4-5). nnp = normal pool plasma.

featured clots (28% with 95% confidence limits of 14.3%-47.6%), composed of fibrin (red) and erythrocytes (green/yellow) without a thrombus-like layered organization (Martius Scarlet staining, supplemental figure 4).

The three mice with an atherosclerotic plaque-associated thrombus had comparable knockdown of plasma protein C compared to si*Proc* mice without such features (10.1% (4.4-16.5) vs. 9.7% (7.6-10.2) of normal pool plasma; p=0.87; figure 2A). In addition, for the mice with and without plaque-associated thrombosis blood platelets numbers, plasma fibrinogen, and thrombin-antithrombin levels were comparable and in the normal range (figure 2B-2D), indicating that the atherothrombotic events in the aortic root did not coincide with, or are part of, a consumptive coagulopathy. Furthermore, plaques with a superimposed thrombus were of similar size and composition (collagen, necrotic core and CD68-positive area, figure 2E-H) as plaques without a thrombus.

DISCUSSION

In the present study we have demonstrated that silencing the natural anticoagulant *Proc* in *Apoe*$^{-/-}$ mice evokes low incidence atherothrombosis in the aortic root. While our brief report was reviewed, data from another independent mouse experiment became available, again showing the unique spontaneous, low incident atherothrombosis phenotype in the aortic root of *Apoe*$^{-/-}$ mice after *Proc* silencing (25%), highlighting that our original observations are reproducible. Our findings concur with other studies in which atherosclerotic plaques in hyperlipidemic mice with a stronger coagulation or an impaired anticoagulation were positive for markers of thrombotic events (5, 6). Altogether, these data indicate that a strong natural anticoagulation system protects mice against atherothrombosis. For now, the low incidence of low protein C-associated atherothrombosis precludes 1) detailing the mechanism why some mice and/or some plaques trigger thrombosis formation and 2) using the current concept as a mouse model for validating anti-atherothrombotic drugs and/or genes. We speculate that, similar as in humans, the plaques that are associated with atherothrombosis in low protein C mice are those that have a thin fibrous cap, demonstrate plaque erosion and/or are highly inflamed. Possible ways to increase the incidence of atherothrombosis may be to study the effects of si*Proc* treatment in mice with even more advanced atherosclerosis, or introduce a "second hit" which is thought to predispose to atherothrombosis (e.g. higher blood pressure, stress, genetic modifications). Future studies should clarify this.

Lowering antithrombin resulted in spontaneous venous thrombosis, which was unexpected given studies in normal C57BL/6 mice (7), but no atherothrombosis. Although it is tempting to speculate on different roles *in vivo* for antithrombin and protein C in atherothrombosis, the extensive venous thrombosis in the *siSerpinc1* group precluded the study of this important anticoagulant in the potential protection against thrombosis in the arterial system, like protein C.

In several si*Proc* treated mice, signs of clotting were observed in the left atrium, a phenotype which has not been reported before in hyperlipidemic or hemostasis mouse models. The absence of clots in major organs and normal levels of blood coagulation parameters in si*Proc* mice indicates that this clot formation is a local cardiac event, and does not represent si*Proc*-mediated disseminated intravascular coagulopathy. Interestingly, Pepler *et al.* reported cardiac clotting, albeit in the ventricle, when providing a procoagulant challenge to endothelial protein C receptor *(Epcr)* mutant mice (9). In addition, lethal perinatal thrombosis in FvQ/Q mice on a 129Sv genetic background has been reported, including features of thrombus formation in the left atrium of the heart (10). Altogether, these data suggest that protein C plays a role in the prevention of cardiac clotting.

In conclusion, our findings indicate that siRNA-mediated silencing of *Proc* in *Apoe*$^{-/-}$ mice creates a condition that allows the formation of spontaneous atherothrombosis, albeit at low incidence. Our unique approach may be of value as a tool to identify factors that increase atherothrombotic complications.

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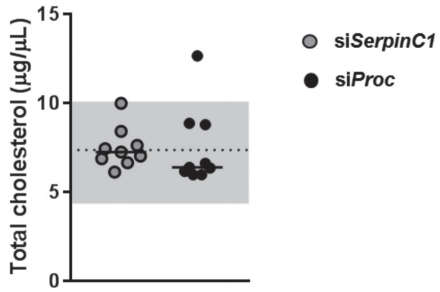
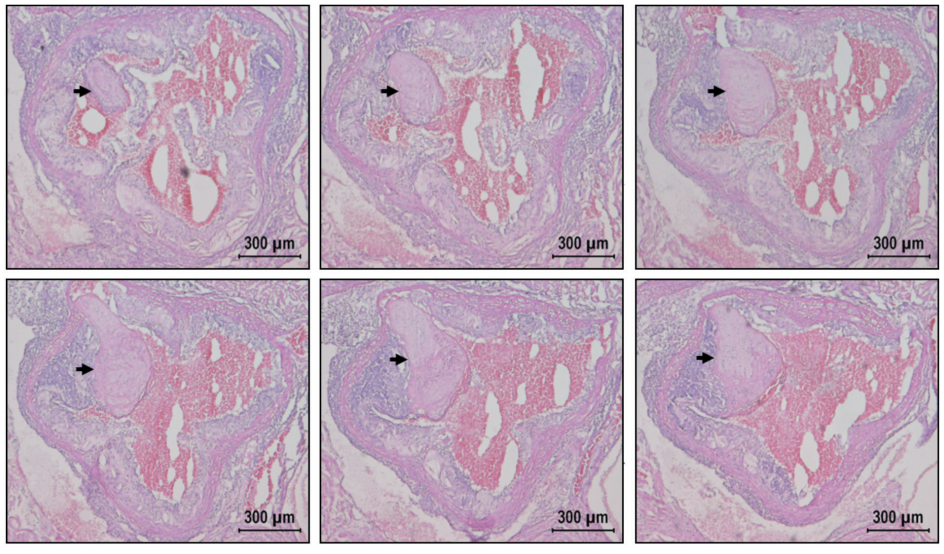
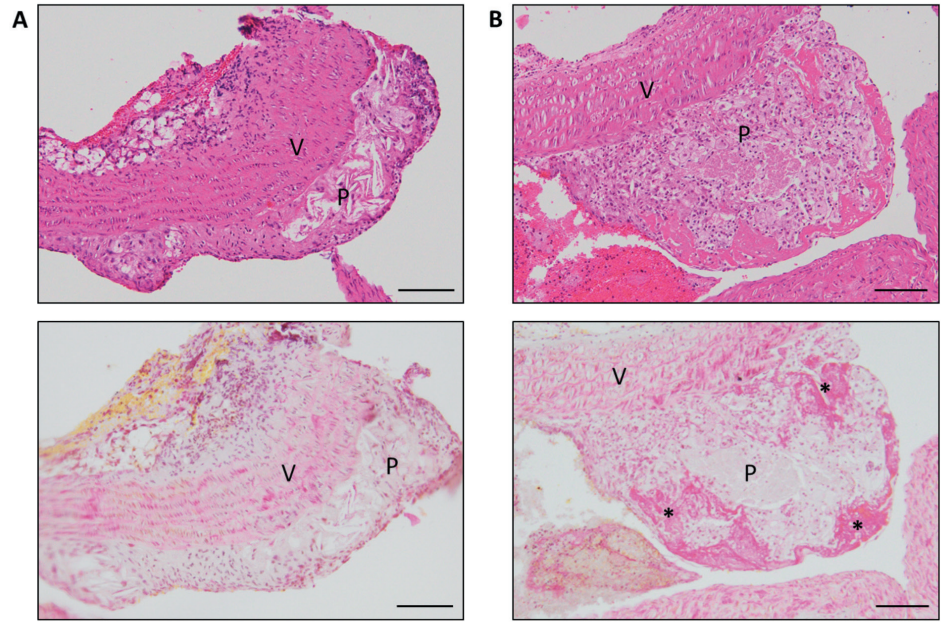


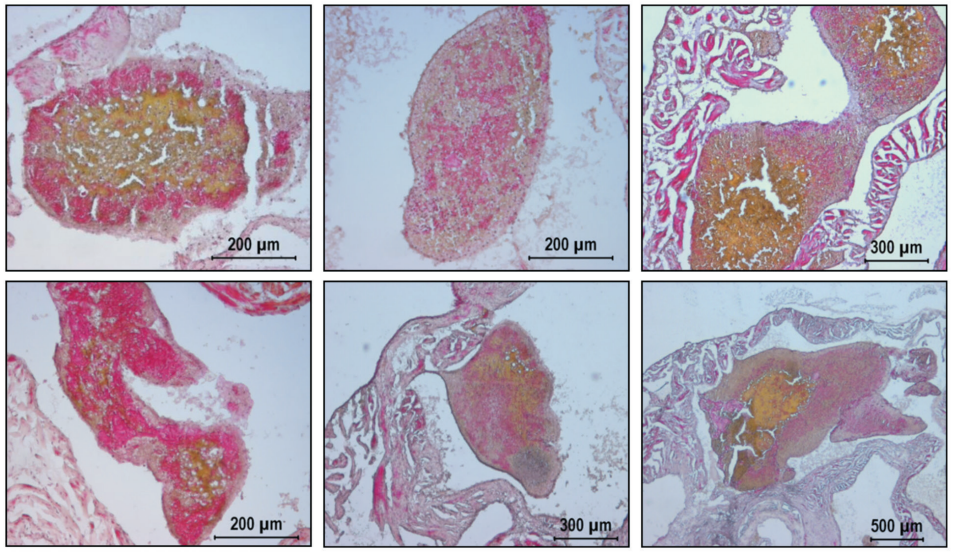
Supplemental figure 1 | Plasma total cholesterol before siRNA treatment of the mice in experiment 1. Lines represent medians. Grey zones in panels represent median and range of total cholesterol prior to siNEG treatment (n = 9).



Supplemental figure 2 | Thrombus formation on top of an atherosclerotic plaque. Hematoxylin and eosin staining of serial sections of a thrombus superimposed on an atherosclerotic plaque in the aortic root of si*Proc*-treated WTD-fed *Apoe*$^{-/-}$ mice (experiment 2), with intervals of 80 µm. The thrombus was superimposed on the plaque for at least 320 µm. Arrows indicate the location of the thrombus.



Supplemental figure 3 | Plaque formation in the aortic arch of si*Proc* treated mice. Upper panels: Hematoxylin-eosin, lower panels: Martius-Scarlett. (A) Representative images of an atherosclerotic plaque in the aortic arch. Plaques stained negative for fibrin (lower panel). (B) Single observation of a plaque in the aortic arch. Within the plaque, fibrin-positive sections were identified in pink (asterisks, lower panel). Of note, this animal also featured aortic root atherothrombosis (figure 1, fourth lane of panels). V: Vessel wall, P: Atherosclerotic plaque. Scale bars represent 100 µm.



Supplemental figure 4 | Si*Proc*-induced clotting in the left atrium. Representative Martius Scarlet stained sections of clotting events in a WTD-fed *Apoe*$^{-/-}$ mice treated with si*Proc* (experiment 2). 1 out of these 7 mice also displayed a thrombus on top of an atherosclerotic plaque. The clots are enriched for erythrocytes (green/yellow) and fibrin (red/pink).