Novel insights in thrombosis pathophysiology using Mice with Impaired anticoagulation

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using Mice with Impaired Anticoagulation

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General Introduction and Outline

VENOUS AND ARTERIAL THROMBOSIS

Thrombosis is the formation of a blood clot inside a blood vessel which can lead to the obstruction of blood flow in the circulatory system, and is a major cause of death worldwide (World Health Organization, www.who.int). Thrombosis can be divided in two categories, either venous or arterial thrombosis, depending on the presenting location (1). Venous thrombosis affects 1-3 per 1000 individuals per year, and mainly occurs in the leg, where it can cause local hypoxia (2-4). When the thrombus detaches and is guided by the bloodstream via the heart towards the lungs, an individual can develop a pulmonary embolism. This complication can be fatal due to right ventricular dysfunction and hypoxia (5, 6). Arterial thrombosis occurs when atherosclerotic lesions (also, plaques) in the arteries rupture, and it is the most common cause of death in the human population (7, 8). Rupture of a plaque leads to the exposure of thrombogenic material from underneath the plaque to the bloodstream, leading to the formation of a thrombus. Arterial thrombi in an artery can disrupt blood flow locally, which causes ischemia in the distal organs. Moreover, thrombi can migrate towards other organs, such as the brain (leading to a cerebrovascular accident), which can result in sudden and fatal hypoxia (9, 10).

The pathophysiology of venous and arterial thrombosis is relatively well-understood, and for several decades multiple therapeutics have been introduced in an attempt to treat the diseases. However, patients receiving these therapeutics show bleeding as a side effect. In order to better understand the diseases and to improve the current generation of therapeutics, an important question remains: what is the exact trigger for a thrombotic event at a certain point in time? The pathophysiology of both diseases can be studied in animal models, which have contributed to new insights in the initiation and progression of the diseases and the development of new and improved medicines. However, the nature of thrombosis in animal models is not spontaneous: complex invasive procedures, such as damaging the endothelial wall or ligating an entire vessel, are required to provoke experimental thrombosis (11-15). In this thesis, we induced spontaneous thrombosis in mice without invasive procedures to study the pathophysiology of the disease. Regarding venous thrombosis, we studied the importance of novel players for spontaneous venous thrombosis in mice. With regard to arterial thrombosis, we assessed the role of natural anticoagulants in mouse atherothrombosis. Remarkably, a transient depletion of natural anticoagulants led to spontaneous atherothrombotic events, a condition which we used to study the influence of several factors associated with the disease.

In the following part of the introduction of this thesis, the coagulation system, which plays a central role in thrombosis research, is briefly outlined. Subsequently, animal models to study venous thrombosis and the novel concept of immunothrombosis are introduced. Following, the role of inherited factors for venous thrombosis and the discovery of a novel risk gene for the

disease are addressed. Finally, the pathogenesis of atherosclerosis and arterial thrombosis and currently used mouse models for this disease are shortly outlined.

THE SYSTEM OF COAGULATION

Hemostasis is the balance between maintaining blood flow throughout the body and protecting against incidences of vascular damage. Blood should be able to flow in closed vessels to fulfill its transport function, and in the event of a damaged vessel the wound must be closed to minimize the risk of blood loss. The complex process of coagulation is involved in creating a protective barrier at the place of injury.

Coagulation (here, the biological process of fibrin formation) can be initiated *ex vivo* in plasma by two coagulation pathways: the extrinsic or intrinsic pathway (1). Although both pathways consist of serine proteases that can consecutively activate one another and converge in the common pathway, the trigger to start coagulation via the extrinsic or intrinsic pathway is different. The extrinsic pathway, or tissue factor (TF) dependent pathway, is initiated when zymogen factor VII (FVII), which is present in the blood, is converted to activated FVII (FVIIa). This process is strongly catalyzed in the presence of TF, which is expressed on the membrane of non-endothelial cells. Upon vascular damage, TF and FVIIa for a complex and convert coagulation factor X (FX) to FXa, which promotes the activation of prothrombin to thrombin. Finally, thrombin converts the soluble blood-dissolved protein fibrinogen to fibrin, which forms a network of long strands. These strands can capture different types of blood cells, thus providing a barrier to prevent blood loss (16).

Coagulation via the intrinsic pathway, also known as the contact-activation pathway, is initiated by the conversion of coagulation factor XII (FXII) to FXIIa. FXII activation is triggered by a wide variety of particles which contain a negative surface, such as DNA, RNA, polyphosphates, and collagen (17, 18). Additionally, it has been suggested that FXII can auto-activate (19). FXIIa can activate coagulation factor XI (FXI) to FXIa, which subsequently activates coagulation factor IX (FIX). Together with activated cofactor VIII (FVIII), FIXa converts FX to FXa as the first component of the common pathway. Eventually, FXa leads to fibrin formation, as described previously.

Activation and propagation of the coagulation pathways are tightly controlled by natural anti-coagulants, which prevent unwanted clotting (20). Natural anticoagulants provide a balance between an overactive coagulation system and unwanted blood loss due to dysfunctional coagulation. Antithrombin predominantly inhibits thrombin, as the name implies, but it can also inhibit serine proteases FVII, FIX, FX, and FXI (16). Protein C is another important natural

anticoagulant, which can inactivate cofactors Va and VIIIa. Protein C is activated by thrombin, a process in which protein S serves as a cofactor (21). A third major anticoagulant is TF pathway inhibitor (TFPI), which can inhibit both FXa and the TF-FVIIa-FXa complex (22).

Interference with the conserved balance of pro- and anticoagulants can lead to pathological situations. Disturbing the pro-coagulants axis can lead to excessive blood loss (bleedings). On the other hand, an overactive coagulation system can cause the formation of a blood clot in undesired situations i.e. thrombosis (1). From a mechanistic point of view, thrombosis can occur when one of the three elements of the triad of Virchow is disturbed (16). This triad was originally described by the German physician Dr. Rudolph Virchow in the 19th century (23, 24). He postulated that thrombosis can occur when 1) blood flow is interrupted (stasis), 2) the vessel wall becomes injured (endothelial damage), or 3) the constituents of the blood are altered (hypercoagulability). Although the exact interpretation of the three specific conditions has changed over time, the paradigm itself is still applicable to how researchers and clinicians approach thrombosis prevention (25).

VENOUS THROMBOSIS AND MOUSE MODELS

Venous thrombosis is ranked as the third cause of cardiovascular death in the world (2). Current therapeutics are effective in combating the disease by shifting the coagulation balance, by means of procoagulant inhibition or anticoagulant stimulation (anticoagulant therapy (26)). An undesired consequence of anticoagulant treatment is bleeding, which remains a clinically relevant issue (27). Since patients treated with the current generation of therapeutic anticoagulants have bleeding episodes, a new generation of anticoagulants with reduced bleeding risk is desired. To gain better insight into the pathophysiology of venous thrombosis and to identify possible novel drug targets, animal models are used to mimic the initiation and progression of the disease (13, 14). Studying venous thrombosis in animals is mainly done in mice, because of their highly conserved coagulation system, low costs of maintenance, access to inbred strains, and possibility for genetic manipulation. Mice have proven value in coagulation research and have contributed to the development of new therapeutics. In preclinical studies, agents which inhibit mouse coagulation factors also inhibit their human counterparts (28-30).

It is striking that mice never develop venous thrombosis without surgical, chemical, or mechanical manipulations; Mice appear to be resistant to the formation of a thrombus. Due to this resistance, venous thrombosis has to be initiated artificially by disturbing one of the elements of the triad of Virchow in the venous vasculature (13, 14). Vascular damage results in exposure of non-endothelial cells to thrombogenic components (e.g. TF and collagen) in the blood, and

the exposure of these components catalyzes a thrombotic phenotype. The notion that vascular damage induces thrombosis in mice is widely used in many different variations, e.g. by chemical, electric, or laser injury (31-36). Although injury-induced experimental venous thrombosis consists of some components which are equivalent to surgery-associated venous thrombosis in humans, it is not applicable to every pathological setting of venous thrombosis in humans. The need for additional preclinical mouse thrombosis models remains.

More recently, it was shown that venous thrombosis can also be induced in mice when another element of Virchow's triad is disturbed: The blood flow (37, 38). Mouse models where stasis is induced have provided additional insights for (mouse) venous thrombosis pathophysiology. Here, platelets, neutrophils, and FXII are key players in the initiation of venous thrombosis (39). Conceptually, these components are thought to be involved in the process of immunothrombosis (40). As the name implies, the concept directly links venous thrombosis to immune cells. This link has been established in mouse venous thrombosis, and there are clues that a similar event occurs in humans (41, 42). In short, a stasis mouse model of venous thrombosis without vascular injury showed that platelets are recruited to the venous vessel wall and are subsequently activated. Activated platelets can serve as a platform for the initiation of thrombus formation via the intrinsic pathway of the coagulation cascade (39). Activated platelets can release polyphosphates, which create a negatively-charged surface suitable for FXII activation to FXIIa. Moreover, activated platelets can attract and activate neutrophils, a subset of leukocytes primarily involved in innate immunity (43). Activated neutrophils can produce neutrophil extracellular traps (NETs) (44). NETs are formed and secreted upon decondensation of the nucleus of the neutrophil, and they consist of several nucleic components such as DNA and histones. Besides their immunological function (NETs contain antimicrobial proteins with a high affinity for DNA), NETs can also form a negatively-charged surface for FXII activation (41, 45, 46).

Platelets, neutrophils, and FXII have been introduced as interesting potential therapeutic targets (47). Platelets are crucially involved in primary hemostasis, which consists of the formation of a platelet plug to quickly close a wound before the coagulation system can become active. Interestingly, neutrophils or FXII are not related to normal *in vivo* hemostasis (41, 48), which suggests that targeting neutrophils or FXII for therapeutic purposes would not result in bleeding. This would make both of them an interesting novel target for venous thrombosis therapy, as compared to the current anticoagulant therapy where bleeding is a major side effect.

The final element in Virchow's triad is the composition of the blood. Mice can be genetically manipulated resulting in the deficiency for genes involved in the coagulation cascade. These mice can serve as animal models for patients with hemophilia or thrombophilia. For instance, comparable to hemophilia A patients, mice deficient for FVIII are prone to bleedings (49).

Regarding thrombophilia, mice deficient in anticoagulant genes (antithrombin, protein C, or TFPI) will die perinatally due to severe coagulopathy (50-52). Mice with a less severe genetic "thrombophilic" modification, such as factor V Leiden or TFPI heterozygosity, will survive birth and will not develop spontaneous venous thrombosis, despite having a subclinical prothrombotic phenotype (52-54). The lack of venous thrombotic events in mice without surgical interventions precludes research on Virchow's triad third element; The blood composition. In the research described in the current thesis, we attempted to overcome this problem by transiently lowering natural anticoagulants to induce a prothrombotic state in mice.

GENETIC RISK FACTORS FOR VENOUS THROMBOSIS

Venous thrombosis can be induced in animal models in order to better understand the pathophysiology of the human disease. Factors which may contribute to venous thrombosis, such as neutrophils and FXII, can be identified and inhibited to investigate their importance in the initiation and progression of the disease. Another approach to obtain a better insight in venous thrombosis is to study the human population. Risk factors which predispose to venous thrombosis can be identified within the diverse human population by comparing individuals with and without the disease (55). The current paradigm for venous thrombosis is that the actual event is an accumulation of different risk factors leading to a certain threshold (56). Identifying these risk factors can aid to understand venous thrombosis pathophysiology, explain thrombotic events, and ideally predict the disease. Roughly, thrombotic risk factors can be divided into two groups: Environmental and genetic. Some examples of the most pronounced risk factors for venous thrombosis are obesity, bed rest, and oral contraceptives (environmental), and antithrombin/protein C/protein S deficiency, and the factor V Leiden and prothrombin G20210A mutation (genetic) (2).

At present the most common risk factors in the human population have been identified and their role in venous thrombosis has been elucidated. Genetic risk factors are directly linked to the coagulation cascade; deficiencies in natural anticoagulants (e.g. antithrombin, protein C) predispose to venous thrombosis, while deficiencies of a procoagulant proteins (e.g. FIX, FVIII) result in bleeding (57). Moreover, a specific gain of function mutation in a procoagulant gene leads to an increased risk of venous thrombosis (e.g. factor V Leiden, which leads to a form of coagulation factor V that cannot be inactivated by anticoagulant protein C (58)).

Identifying novel risk factors using the human population is still of interest. Unidentified factors are likely not useful to target or to screen in a population, due to their low ratio for increased risk for venous thrombosis. However, they can help us to improve our understanding of the

pathophysiology of the disease. For this purpose, large international consortia have been established to compare genomes of venous thrombosis patients with those of healthy controls, to investigate in which genomic loci the two groups differ (59). In two of these large studies, a novel risk gene was identified: *SLC44A2* (60, 61). This gene was not associated with thrombosis before, nor involved in the classical coagulation cascade (16). In this thesis, we investigated the role of SLC44A2 in immunothrombosis.

ATHEROTHROMBOSIS AND ANTICOAGULATION

Diseases resulting from arterial thrombosis (also, atherothrombosis), such as a cerebrovascular accident and myocardial infarction, are the most frequent cause of death in the human population (World Health Organization, www.who.int). A common theme to both arterial and venous thrombosis is the formation of a clot in the bloodstream, however, they are the result of a different pathophysiology (5, 7).

The initiation process leading to atherothrombosis, called atherosclerosis, is characterized by chronic thickening of the arterial vessel wall. In short, activation of endothelium in vessels with increased shear stress causes the retention of low-density lipoproteins (LDL). Increased levels in the circulation of these particles, due to e.g. a diet rich in fat, are associated with an increased risk for arterial thrombosis (62). Activated endothelial cells cause monocytes to migrate through the endothelium towards the lipid particles. Monocytes clear the lipid particles and become "foamy" macrophages, because of their high lipid content. These macrophages get trapped under the endothelium. As a result, smooth muscle cells migrate and proliferate towards the endothelium to restore the damaged tissue, covering the foamy macrophages in the process. The foamy macrophages become necrotic and eventually form a large mass of prothrombotic material underneath the vulnerable endothelial wall. Over time, the atherosclerotic necrotic core causes calcium deposition and hardening of the artery (63). The vulnerable atherosclerotic vessels are prone to atherothrombosis; the rupturing of an atherosclerotic lesion, which leads to the exposure of prothrombotic (necrotic) material to the blood. This results in the rapid formation of a blood clot (thrombus). Within minutes, the thrombus can block the blood supply to vital organs, which causes acute clinical problems and can be fatal (7, 64).

Atherosclerosis, the initial process leading to atherothrombosis, can be studied *in vivo* in transgenic mice with a modified lipoprotein metabolism (65, 66). When these mice are fed a "Western-type diet", a diet containing high levels of cholesterol and/or fat, they develop atherosclerotic lesions (also, plaques) in the arterial vasculature. Preferentially, atherosclerosis occurs in areas with disturbed flow patterns, such as the aortic root and in and around

branches of large vessels (67). Although mouse models mimicking human atherosclerosis have helped us to study the pathophysiology of the disease, mice never develop atherothrombosis spontaneously; a mouse with an unprovoked heart attack has never been described. The lack of atherothrombotic events in mice precludes *in vivo* studies on atherothrombosis. Moreover, the actual value of atherosclerosis mouse models to better understand the processes which occur in the human disease is unclear (68, 69). The reason for the absence of atherothrombotic events in mice is currently unknown; however species differences between mice and humans related to this matter have been described, such as differences in size, metabolism, heart rate, vessel anatomy, plaque composition, and many more (70).

One of the main species differences between human and mice is that mice have a more potent anticoagulant system than humans. When prothrombin is activated in plasma, thrombin activity is inhibited approximately nine times faster in mouse plasma compared to human plasma (71). This implies that the natural anticoagulant potential of mice is significantly higher than in humans, which is likely the result of higher levels of circulating natural anticoagulants. For this reason, we hypothesized that mice with severe atherosclerosis will not give rise to atherothrombosis due to a more potent anticoagulation system, although plaques may be damaged or rupture, of which evidence has even been reported (72-74).

OUTLINE OF THE THESIS

Within this thesis, we aimed to introduce a transient imbalance in the coagulation profile of mice. Introducing a transient imbalance circumvented the use of invasive methods to introduce thrombosis, such as by damaging or inducing stasis of blood vessels. This allowed us to study the pathophysiology of thrombosis in a setting that mimics human scenarios for thrombosis, where an imbalance (e.g. genetic profile or obesity) is a major contributor.

As a tool to introduce an imbalance of the coagulation profile of mice, we used RNA interference (RNAi; also, small interfering RNA; siRNA). siRNA consist of short stretches of RNA, and is used to inhibit the transcription of specific mRNAs. When siRNAs enter a cell, they can interact with their mRNA complementary counterpart. This specific interaction leads to mRNA breakdown and thus decreased protein production (75). In contrast to its use in *in vitro* studies, RNAi as a research tool to study gene function *in vivo* is a relatively new concept (76). Currently, siRNA *in vivo* studies are restricted to genes expressed in the liver by hepatocytes, which happens to be the cell type that produces the majority of the coagulation factors. This means that RNAi thus can be used to study the function of hepatic genes *in vivo*, when genetically modified animals

deficient in a specific gene are not viable. Moreover, inhibition of genes by RNAi can be achieved much faster and cheaper than genetically modifying animals.

RNAi is not only used for research purposes; RNA therapeutics or oligonucleotides (of which siRNA is a subclass) have been introduced to treat a wide variety of diseases (77). In **chapter 2**, the molecular background of oligonucleotides and current applications in several animal models in the context of pharmacotherapy of coagulation (thrombosis and hemophilia) is summarized. Moreover, the perspective for oligonucleotides as therapeutic modalities within the fields of thrombosis and hemophilia is discussed.

In **chapter 3**, we investigated the influence of platelets, neutrophils, and FXII in mouse spontaneous venous thrombosis. Here, venous thrombosis is induced by the acute siRNA-dependent inhibition of natural anticoagulants antithrombin and protein C. We showed that platelets are crucial for the onset of spontaneous venous thrombosis, while neutrophils were not rate-limiting. Remarkably, acute siRNA-mediated depletion of FXII even seemed to exacerbate the thrombotic phenotype. This counter-intuitive observation around FXII function is extensively investigated and discussed in **chapter 4**.

Based on studies in which single nucleotide polymorphisms (SNPs) were investigated, the gene *SLC44A2* was identified as a risk factor for venous thrombosis (60, 61). In **chapter 5**, we investigated whether the SNP most significantly different between venous thrombosis patients and healthy controls (the "top" SNP) could be linked to altered levels of thrombosis plasma biomarkers.

In **chapter 6**, we used siRNA to study the role of the natural anticoagulant protein C in the progression of mouse atherosclerosis towards atherothrombosis. Protein C-low mice spontaneously developed a unique atherothrombotic phenotype; Structured fibrin-rich thrombi on atherosclerotic lesions in the aortic root were identified, albeit at a low frequency. In **chapter 7**, we aimed to reproduce the unique low protein C-induced atherothrombotic phenotype in mice. Moreover, in order to gain more insight in the pathophysiology of atherothrombosis, we attempted to increase the development of atherothrombosis by transiently increasing blood pressure. Also, atherothrombosis was redirected to a predefined vascular site of atherosclerosis.

In **chapter 8,** the thesis is discussed, and **chapter 9** is a Dutch summary of the findings.

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Oligonucleotides Targeting Coagulation Factor mRNAs: Use in Thrombosis and Hemophilia Research and Therapy

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ABSTRACT

Small interfering (si) RNAs and antisense oligonucleotides (ASOs; here for simplicity reasons, both referred to as oligonucleotides) are small synthetic RNA or DNA molecules with a sequence complementary to a (pre)mRNA. Although the basic mechanisms of action between siRNAs and ASO are distinct, a sequence-specific interaction of the both oligonucleotides with the target (pre)mRNA alters the target's fate, which includes highly effective sequence-specific blockade of translation and consequently depletion of the corresponding protein. For a number of years, these oligonucleotides have been used as a tool in biological research to study gene function *in vitro*. More recently, safe and specific delivery of these oligonucleotides to the liver of mammals has been achieved and optimized. This not only allowed their use for *in vivo* gene studies in physiology and disease, but also opened the opportunity for the development of a new generation of RNA-specific drugs for therapeutic purposes. In 2013, the first oligonucleotide product targeting RNA from the hepatic cholesterol pathway was approved. For blood coagulation, a large portion of key proteins are produced in the liver, and thereby siRNAs and ASOs can also be used as appropriate tools to target these proteins *in vivo*. In this review, we describe the first use of oligonucleotides for this purpose from zebrafish to primates. As the use of oligonucleotides allows avoidance of early lethality associated with full deficiency of several coagulation factors, it has proved to be of value for studying these proteins in physiology and disease. Currently, oligonucleotides are tested as therapeutics, with the ultimate goal to beneficially modulate the hemostatic balance in thrombosis and hemophilia patients. We discuss both the preclinical and clinical studies of a number of siRNAs and ASOs with the potential to be introduced as drugs for prophylactic and/or treatment of thrombosis or hemophilia. We conclude that for the coagulation field, oligonucleotides are of value for research purposes, and now the moment has come to fulfill their promise as therapeutics.

INTRODUCTION

Small interfering (si) RNAs and antisense oligonucleotides (ASOs; here for simplicity reasons, both referred to as oligonucleotides) are small synthetic RNA or DNA molecules with a sequence complementary to a (pre)mRNA. Sequence-specific interaction of the siRNA or ASO with the target (pre)mRNA alters the target's fate, which includes sequence-specific blockade of translation and consequently depletion of the corresponding protein. siRNAs and ASOs have potential in research and therapy; design of sequence-specific oligonucleotides and testing their efficacy *in vitro* can be relatively easily achieved, and off-target effects are limited. Moreover, siRNAs and ASOs have a relatively simple chemistry, malleability, and delivery (at least *in vitro*), allowing the oligonucleotides to become a frequently used tool in biological research for studying gene function in a variety of eukaryotic cell types. The major differences between siRNAs and ASOs comprise differences regarding their chemical structure and their mode of action, but considering their primary biological impact i.e. silencing the target (pre)mRNAs target, both are highly effective (1-3).

Already in 1978, it was shown that ASOs proved successful in targeting (pre)mRNAs *in vivo* in experimental animals (4). More recently, in 2003, also siRNAs showed strong *in vivo* potency (5), and delivering siRNAs to the liver proved feasible and successful (6, 7). siRNAs and ASOs both successfully escape degradation in both the blood circulation and intracellular lysosomes, and effectively reach the appropriate cellular compartment to find their complementary (pre)mRNA. Hepatic delivery allowed the use of oligonucleotides for *in vivo* gene function studies in normal (hepatic) physiology or for disease analogues to the *in vitro* approach. Moreover, siRNAs and ASOs allow validation of therapeutic targets in pre-clinical trials forming the stepping stone towards clinical application (8-10). As blood coagulation factors are predominantly expressed in the liver, venous thrombosis and hemophilia are diseases that may particularly benefit from the oligonucleotide approach; both when it comes to use as a research tool in preclinical research as well as an approach for therapy.

In this review, we will discuss the possibilities for using oligonucleotides in venous thrombosis and hemophilia research and therapy. First, we will outline the chemical and cellular differences between siRNAs and ASOs. Next, we will discuss their usefulness to study coagulation physiology and pathophysiology (i.e. thrombosis and hemophilia) in animal models. Finally, we will examine the current status of the exciting first clinical applications of oligonucleotide approach in venous thrombosis and hemophilia, and the potentials that come along with this novel treatment modality.

SIRNA AND ASO BIOLOGY AND DELIVERY

Although the target of siRNAs and ASOs is the same i.e. the (pre)mRNA of the gene of interest, their mechanism of action is different. In short, siRNAs are synthetic RNA duplexes consisting of two unmodified annealed 21-mer oligonucleotides. When siRNAs enter the cytosol, the antisense strand i.e. the strand which specifically targets an mRNA, forms a complex with an endoribonuclease enzyme called Dicer for loading into RNA-induced silencing complex (RISC), which enables their stabilization. A single RISC, with the RNase Argonaute-2 (for which siRNA competes with microRNAs, as discussed in (11)) as its functional unit for accommodating RNA breakdown and the antisense strand for the specificity, can target and break down multiple mRNAs in the cytosol. This process is also known as RNA interference (1, 12). Whereas siRNAs enter the cell as a duplex, ASOs are single stranded antisense nucleic acids (DNA, RNA, or a chemical analogue), typically 8-50 nucleotides long. ASOs are not recognized by Dicer and not built into RISC upon entering the cytosol; depending on their chemical modification, they directly recognize their (pre)mRNA target in the cytosol or nucleus, due to their complementarity (13). ASOs can block, break down (after recruitment of RNase H), or induce exon skipping of the mRNA (2). In contrast to siRNAs, where a single RISC can degrade multiple mRNAs, ASOs typically interact with a single (pre)mRNA.

With the desire to use oligonucleotides *in vivo*, the delivery to the correct cell and cellular compartment without being degraded has been an important issue. Thus far, the delivery of oligonucleotides to the liver in mammals is an area where significant progress has been made: siRNAs can be packaged in liposomes with a high tropism for the liver, and they can be conjugated to specific ligands (the so-called N-acetylgalactosamine (GalNAC) ligands, which allows the uptake via hepatocyte-specific asialoglycoprotein receptors) which mediate receptor-mediated take-up by hepatocytes (14-17). A liposome-based formulation and hepatic delivery has also been described for other oligonucleotides (18). Upon systemic delivery, the biodistribution of ASOs is broad, and the highest concentrations are typically found in the liver and the kidneys (19-21). This delivery is an aspecific process and works via a yet unknown mechanism, however it is likely that the relative amphipathic nature of molecules contribute to this cell-specific distribution. Differences in the delivery of the compound (packaging versus an aspecific process) and the later discovery of (the functioning of) siRNAs are probably the main reasons why ASOs are ahead for entering into (pre)clinical trials. More recently, it has been reported recently that ASOs can also be GalNAc conjugated, and that they are (successfully) tested in (pre)clinical trials (22, 23).

Because the liver appears to be the most successfully targeted organ thus far - although there are interesting reports showing e.g. successful delivery of siRNAs to endothelium and leukocytes (24, 25) - we will focus in this review on targeting mRNA encoded by genes expressed in

hepatocytes. This successful hepatic delivery of oligonucleotides makes an mRNA-targeting approach specifically interesting for studies and eventually therapies where a liver-produced protein is a major contributor to the physiology and/or pathophysiology of a certain disease, such as hepatitis, hypercholesterolemia, hemophilia, and thrombosis (26). For this review, we will focus solely on the use of oligonucleotides for targeting liver-produced coagulation proteins, to study their role in biology and modulate their activity in hemophilia and thrombosis with the ultimate goal to fight or prevent the disease.

SIRNAS AND ASOS TO STUDY COAGULATION GENE FUNCTION IN PHYSIOLOGY AND DISEASE

A conventional approach to study gene function *in vivo* is by generating genetically modified animals. Recently, the developments leading to CRISPR/cas9-mediated genome editing have allowed generation of mutant and/or knockout within a period of months (27). Nevertheless, extensive breeding programs, also for the generation of proper (littermate) controls, remain expensive, and gene deletion may preclude detailed study of gene function due to perinatal lethality or gene redundancy. Regarding coagulation, most genes from the extrinsic, common, or anticoagulant pathways have been deleted in mice, which often resulted in early lethality, mostly as a result of coagulopathy (28, 29).

As an alternative to the genetic modification approach, oligonucleotides can be used to transiently silence a gene of interest. Using oligonucleotides is simple, fast, cheap, and there are no issues with generating the appropriate controls, because a group of identical mice can be injected with a scrambled control sequence, which is structurally comparable. Moreover, because of the acute silencing of oligonucleotides, analyses can be performed before potential compensatory pathways have become active, and results can be easy translated to other species (including human), as long as the genome has been sequenced.

SIRNAS AND ASOS IN ANIMAL MODELS FOR COAGULATION - ZEBRAFISH

A relatively new animal model for the study of blood coagulation and related processes is the zebrafish (*Danio rerio*). These fish possess a conserved and sophisticated coagulation system, which in essence follows the same principles as the system in mammals (30, 31). Moreover, the low cost of maintenance and their low turnover time between subsequent generations make them appealing for (bio)medical research.

The oligonucleotide of choice in this animal are the so-called morpholinos, a subclass of ASOs which got their name because their backbone consists of methylenemorpholine rings (32). These oligonucleotides do not degrade the targeted mRNA, but rather block translation by making the target mRNA inaccessible for ribosomes. The coagulation factors fibrinogen, prothrombin, and factor VII have been silenced *in vivo* using morpholinos.

For fibrinogen, zebrafish produce orthologues for the three chains of the mature protein, and it has an *in vivo* expression pattern comparable to mammals. Moreover, fibrinogen strands are incorporated in the (experimental) thrombus of a zebrafish, and upon morpholino-mediated downregulation of either of the three orthologues, larvae suffer from intracranial and intramuscular hemorrhages (33).

Morpholino-mediated downregulation of prothrombin causes two distinct phenotypes in zebrafish larvae. Some larvae suffer from serious developmental issues causing premature death (even before blood flow has developed), while others develop a bleeding phenotype during a later stage of embryonic development (34). These results recapitulate the hemophilic phenotype that is seen in experimental mammalian animals with deficiencies of these factors. Moreover, a microarray analysis was performed on mRNA from zebrafish treated which were with prothrombin-morpholinos (35). A total of 63 upregulated or downregulated genes were identified, although their exact function remains to be determined.

Using coagulation factor VII-specific morpholinos, it was shown in zebrafish that hepsin plays a crucial role in activating factor VII (36). Besides fibrinogen, prothrombin, and factor VII, more genes involved in hemostasis, such as von Willebrand factor and genes involved in thrombocyte production and function, have been targeted in zebrafish using morpholinos (37-39).

Although translating results from zebrafish to humans remains difficult due to a wide evolutionary gap between both species, the presented data imply that using zebrafish-specific advantages can contribute in unraveling processes of hemostasis, with a clear role for the use of oligonucleotides as a tool.

SIRNAS AND ASOS IN ANIMAL MODELS FOR COAGULATION - MAMMALS

In order to study processes involving coagulation factors, mammalian animal models, such as mice, rats, and rabbits, are genetically closer related to humans. In these mammals it has been shown that siRNAs and ASOs can be delivered to the liver *in vivo* to silence (pre)mRNAs of proteins produced in hepatocytes (26, 40-42). Several genes involved in the extrinsic, common, or anticoagulant pathway of coagulation have been targeted.

Antithrombin, protein C, and protein S are important natural anticoagulants, and individuals with partial deficiencies in either of these factors have an increased risk to develop venous thrombosis, while full deficiency is rare and very severe or incompatible with life (43). Genetically modified mice missing either of these anticoagulants die *in utero* because of thrombotic complications. However, using siRNAs, the anticoagulants antithrombin or protein C can be silenced transiently in adult mice to study them in a less anticoagulant environment. When both natural anticoagulants are silenced simultaneously mice develop spontaneous venous thrombosis, a phenotype which is characterized by the formation of fibrin-rich thrombi in the head and rapid consumption of coagulation factors (44).

This mouse model allowed studying factors involved in (experimental) venous thrombosis pathophysiology. Platelets appeared to be crucial for spontaneous venous thrombosis to occur, whereas neutrophils were not rate limiting, and lowering of plasma coagulation factor XII surprisingly seemed to aggravate rather than rescue the thrombotic phenotype (45).

Oligonucleotides can be used as a tool to study the effect of transiently silencing a certain gene, to bypass early lethality. An interesting example of such an approach has been described for studying the role of (pro)thrombin in Sickle cell disease (SCD) (46). Genetically modified mice missing prothrombin are not viable due to fatal prenatal bleeding complications, so the authors were forced to choose another approach. Using prothrombin-specific designed gapmers (a class of ASOs where the internal sequence block is protected from nuclease degradation by artificially modified ribonucleotide monomers) prothrombin was transiently silenced SCD mice. It was shown that in SCD mice reduced levels of prothrombin lowered inflammation and endothelial cell dysfunction, and improved multiple SCD-associated organ pathologies and overall survival. These data imply that targeting a single coagulation factor i.e. prothrombin can ameliorate SCD pathology.

Using a comparable approach, the transcription factors hepatocyte nuclear factor 4α and CCAAT/enhancer-binding protein α were silenced using siRNAs, to study their regulatory role in the transcription of coagulation genes, demonstrating that by means of siRNAs the role of a gene in a certain process can be simply and rapidly unraveled (47).

OLIGONUCLEOTIDES AS THERAPEUTICS – ANIMAL STUDIES

Besides using oligonucleotides as a tool for the study of gene function, they are also candidate therapeutics in the field of blood coagulation. All key proteins (with the exception of tissue factor and membrane bound receptors such as thrombomodulin, endothelial protein C receptor, and protease-activated receptors) are predominantly expressed in the liver. Moreover, a major advantage of using oligonucleotide drugs over other more conventional therapeutic strategies lies in the drugs' target: (pre)mRNAs. Targeting proteins is complex due to their three-dimensional structure and small conformational differences which can cause complete loss of function of a drug, while (pre)mRNAs just differ in their gene-specific sequence. This means that conventional ("protein-based") drugs have to undergo an elaborate screening process for compounds that inhibit the protein of interest in both experimental animals (during preclinical studies) and humans (actual trials), while for RNA-based drugs solely the oligonucleotide sequence needs to be adjusted. Moreover, oligonucleotide drugs can stably repress protein levels over the course of several weeks. In addition, oligonucleotides are not expected to become subject to an immunogenic response, inhibitors, or resistance during therapy.

The fundamental process of blood coagulation and the disease of venous thrombosis can be effectively inhibited in humans by using agents such as vitamin K antagonists, heparins, and the new generation of direct oral anticoagulants (48, 49). All these therapeutics target components of the common pathway of coagulation. Outside the common pathway, coagulation factor IX might be an interesting candidate to target for the prevention of thrombosis. With an siRNA against this coagulation factor, it has been shown in rats that protein activity can be reduced with 50-99%. This reduced activity prevented experimental thrombosis without causing a bleeding phenotype. Over 99% inhibition of factor IX activity resulted in a bleeding phenotype, similar to factor IX deficient rats (50). This observation makes an "intermediate" dose of siRNAs against factor IX (resulting in 50-99% reduction of activity) potentially interesting for therapeutic purposes.

Although targeting individual components from the coagulation cascade proved effective in preventing thrombosis, bleeding often remains a clinically relevant side effect. To circumvent this problem, coagulation factors XI and XII, both involved in the intrinsic, or contact-activation, pathway of coagulation have been introduced to target for preventing venous thrombosis (51-53). Based on data derived from mouse studies, deficiency or transient lowering of factor XI and XII prevents thrombosis without (severe) bleeding as a side effect, unlike any other traditional coagulation factor. These reports coincide with human data, where individuals with a factor XII deficiency do not have a bleeding tendency, and factor XI deficient patients (although occasionally suffering from mild and injury-related events, hemophilia C) even seem to be

protected from developing thrombotic events (54-56). Moreover, both plasma proteins are exclusively produced in the liver, making them accessible for oligonucleotide therapy.

Coagulation factor XII is a plasma protease which upon activation initiates the intrinsic pathway of coagulation. When rats deficient in factor XII were compared with rats treated with a specific siRNA against factor XII (99% reduction in plasma factor XII), both showed protection in arterial as well as in venous thrombosis models (57). In rabbits, a factor XII-specific ASO prolonged the time to occlusion in a catheter thrombosis model by 2.2 fold (58). Comparable thromboprotective effects were obtained in mouse studies. Specific lowering of factor XII or (pre)kallikrein (an activator of the zymogen factor XII) using an ASO, resulted in reduced thrombus formation models for arterial and venous thrombosis, without a significant effect on normal hemostasis (59).

Coagulation factor XII is not only involved in (experimental) hemostasis, but also in release of bradykinin from high molecular weight kininogen, an inflammatory component mediating the dilation of blood vessels. Specific gain-of-function mutations in factor XII causing increased contact-mediated auto activation, which causes enhanced release of bradykinin and massive swelling, underlies hereditary angioedema type III (60). Interestingly, experimental data in mice show that upon lowering factor XII or (pre)kallikrein using a specific oligonucleotide, certain characteristics associated with this disease can be prevented, which opens perspectives for treatment (61-63).

Coagulation factor XI is activated by factor XII towards activated factor XI. In mice, ASO-mediated specific and dose-dependent reduction of plasma factor XI showed an antithrombotic effect in various arterial and venous thrombosis models (40). In parallel groups, mice were treated with either warfarin or enoxaparin, a commonly used vitamin K antagonist and low molecular weight heparin, respectively. Although the antithrombotic effect was considered equally effective in all groups, factor XI ASO-treated mice performed significantly better in a hepatectomy surgical bleeding model. In rabbits, lowering of factor XI prolonged the time to occlusion in a catheter thrombosis model, in a similar fashion as factor XII (58). After these promising results, the role of factor XI and its therapeutic antithrombotic potential was tested in non-human primates. Again using mRNA-specific ASOs, it was first shown that plasma factor XI can be lowered in a dose-dependent manner without increasing the risk of bleeding (64). In a second study, the antithrombotic potential of FXI-ASO was tested in non-human primates. In an arteriovenous shunt thrombosis model protection from thrombosis was reported, which coincided with a decreased thrombin generation (65). These studies formed solid proof to continue with using an ASO against factor XI in clinical trials. Interestingly, silencing coagulation factors XI and XII also attenuated atherothrombosis in mice (66, 67), which implies that silencing these factors also might be useful in treating atherothrombotic complications.

Besides targeting procoagulant proteins to prevent venous thrombosis, targeting anticoagulants might also be useful in preventing disease, in a situation where the hemostatic balance has shifted towards bleeding i.e. hemophilia. Current therapies to treat hemophilia are based on administering plasma derived or recombinant proteins to replace the missing coagulation factors. The need for developing alternative treatment strategies is high, since a large portion of treated hemophilia patients develops inhibitory antibodies against these administered proteins. A promising approach which likely circumvents this problem is by silencing an anticoagulant using oligonucleotides, which can cause a shift towards a renewed hemostatic equilibrium. This hypothesis was tested by targeting antithrombin (68). When factor VIII-deficient hemophilic mice were treated with an siRNA against antithrombin, the hemophilic phenotype disappeared in a dose-dependent manner without thrombotic complications. In the next animal model of choice, non-human primates (cynomolgus monkeys) with antibody induced FVIII deficiency, a dose-dependent silencing of antithrombin was again observed. Moreover, thrombin generation were restored to normal levels when treating the monkeys with the siRNA, which opened the perspective to test this drug in humans.

OLIGONUCLEOTIDES AS THERAPEUTICS – HUMAN STUDIES

Recently, the first oligonucleotide drug targeting specifically the liver has been approved by the United States' Food and Drug Administration. This therapeutic, an ASO called mipomersen (Kynamro®), directly targets the pre-mRNA of apolipoprotein B, which causes a significant downregulation of the corresponding plasma protein. This specific downregulation allows more release of very low density lipoprotein (VLDL) from the liver to the bloodstream, which will cause a drop of LDL-cholesterol levels in plasma (69, 70). This is in line with the concept of the oligonucleotide drugs, which has been outlined previously. Mipomersen has been approved to treat homozygous familial hypercholesterolemia, and the availability of mipomersen is currently being investigated for more risk groups with high cholesterol levels to protect them from cardiovascular disease (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm337195.htm).

The approval of mipomersen might cause a domino effect for the use of oligonucleotides which target mRNA in the liver for treating other human diseases: The first hurdle of safely delivering oligonucleotides to the liver has been overcome, so by using the same chemical formulation many more hepatic mRNAs can be targeted, which is an appealing prospect. Two oligonucleotide drugs are currently being tested in the field of coagulation, one for venous thrombosis treatment and one for hemophilia.

Coagulation factor XI has been investigated extensively in animal models for its role in coagulation, and whether it can be a therapeutic target to prevent venous thrombosis without introducing bleeding as a side effect (71). Recently, the results of a clinical trial were reported, where an ASO against factor XI (FXI-ASO) was used for the prevention of deep-vein thrombosis after knee arthroplasty (72). Previously, FXI-ASO was considered safe to use in humans since no side effects (including bleeding) were observed in a prior study (73). When comparing a high and low dose FXI-ASO with conventional enoxaparin treatment, the bleeding risk was not significantly different (although less bleeding events were scored for FXI-ASO patients). Interestingly, treatment of patients with the low dose of FXI-ASO appeared safe considering the risk of deep-vein thrombosis, where the high dose was even considered superior to enoxaparin. Currently, a phase 2 clinical trial has been completed in which the efficacy of FXI-ASO in patients with end-stage renal disease was evaluated, with the primary goal to monitor the safety and tolerability of the ASO (ISIS 416858, Ionis pharmaceuticals). Significant and dose-dependent reductions in FXI activity were observed upon treatment (http://ir.ionispharma.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=2217775).

As outlined previously, on the other side of the spectrum hemophilia can be treated by transient lowering of antithrombin, to restore the hemostatic balance. Currently, a human-specific antithrombin siRNA named Fitusiran is being tested in phase 1 clinical trials. This siRNA, amongst others candidate siRNAs which are tested in clinical trials, is chemically modified to improve its systemic stability and is conjugated to a GalNAc-ligand, which allows hepatocyte-specific uptake (17). The phase 1 clinical trial is done in a diverse group of individuals, including healthy volunteers, patients with hemophilia A or B, and with or without inhibitors against supplemented recombinant proteins (74). The goal of this trial is to evaluate the safety and tolerability of multiple doses of subcutaneously administered Fitusiran. Moreover, thrombin generation assays in plasma derived from hemophilia patients are performed to test whether they recover towards levels of healthy individuals. Preliminary data show that hemophilia patients did not develop any serious (thromboembolic) complications because of the drug. Considering functionality of the drug, when antithrombin levels were lowered to >75% thrombin generation peaks were increased in a dose-dependent manner with 289%. Positive interim results of two clinical trials (phase 1 and phase 2) for Fiturisan have been reported late 2016 (ALN-AT3SC, Alnylam pharmaceuticals, http://www.alnylam.com/capella/presentations/fitusiran-data-ash-2016/).

CONCLUSIONS

Hepatic *in vivo* gene silencing using oligonucleotides has shown to be specific and rapidly applicable in multiple animal models. The approach can not only serve as an alternative for using genetically modified animals (although restricted to the liver), it can also help unraveling biological processes which cannot be studied otherwise. Moreover, promising preclinical trials for the development of new oligonucleotide drugs to treat thrombosis and hemophilia have been reported.

Since the introduction of mipomersen in 2013, the first oligonucleotide drugs are slowly finding their way towards the clinic (currently, three ASOs and no siRNAs yet). Together with all the preclinical data, it has been shown that the process of delivering oligonucleotides to the liver can be achieved in a safe way (thus far, no specific toxicities have been observed). Currently, preventing adverse events (at the injection site or from infusions) of oligonucleotide drugs remains an important issue, which means that reducing the dosage of the drugs is crucial. The dosages can be lowered by, for instance, the conjugation of GalNAc-ligands to oligonucleotides to improve cell-specific delivery (22). When these delivery and dosage issues can be solved, more clinical situations will present where using oligonucleotides will be regarded advantageous over using classical therapies.

Thrombosis and hemophilia are two diseases where the current treatment can be improved, and the two drugs which are currently investigated in clinical trials show promise. An important advantage compared to current therapies for hemophilia is that the effect of oligonucleotides remains for a longer time period, in contrast to the low-interval dosing of conventional treatment. This would mean that patients will go from daily to monthly treatment. Moreover, hemophilia patients who develop inhibitors against supplemented factor VIII or IX will benefit from this novel treatment option, since oligonucleotides will likely not provoke this immune response.

Although the mode of action of oligonucleotides in humans compared to current therapies can be regarded as a great benefit, it can be a problem in situations when rapid reversal of the silenced protein is necessary. In some cases, singular treatment with recombinant proteins will be a solution, although correct dosing would be crucial. A more elegant solution would be to use an oligonucleotide antidote. Recently, it was shown in mice that upon prothrombin-specific ASO treatment, complementary sense oligonucleotide antidote (SOAs) reversed the antithrombotic phenotype by specifically binding of ASOs intracellularly (75). Even though these data are promising, therapeutic usefulness has to be proven for individual ASOs. Moreover, it has been shown that reversal of siRNA therapy is feasible using complementary high affinity oligonucleotides as a “synthetic target” or decoy to abrogate silencing activity of antisense-

loaded RISC (ReversirTM, http://www.alnylam.com/web/assets/Reversir_OTS_101315.pdf). Currently, the strategy has been reported to be functional for reversal of the effect of an siRNA against coagulation factor IX. The major disadvantage of this oligonucleotide-specific approach is that it may take too long for the antidote to restore original levels of the initially silenced protein. For this reason, in the case of a sudden event such as a surgical intervention, an oligonucleotide-specific antidote may not be adequate and recombinant proteins or fresh frozen plasma treatment are still necessary.

In conclusion, in fundamental research oligonucleotides targeting coagulation factors in the liver can serve multiple purposes, and can improve our understanding about coagulation biology, in physiology and disease. Considering oligonucleotide-based silencing of coagulation factors to treat thrombosis or hemophilia, for the short term, validation studies focused on the safety and efficacy of the products for clinical applications look promising. For the long term, a whole new class of drugs might enter the market, where oligonucleotides might have the potency to be delivered to other organs than the liver, or allow allele-specific silencing. Moreover, an entire panel of genes could be silenced by including multiple oligonucleotides in a single treatment. Time will tell whether the new generation of RNA-targeting therapeutics can fulfill its promise.

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3

Role of Platelets, Neutrophils, and Factor XII in Spontaneous Venous Thrombosis in Mice

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ABSTRACT

Recently, platelets, neutrophils, and factor XII have been implicated as important players in the pathophysiology of venous thrombosis. Their role became evident in mouse models where surgical handlings were used to provoke thrombosis. Inhibition of anticoagulation in mice using small interfering (si) RNA targeting *Serpinc1* and *Proc* also results in a thrombotic phenotype, which is spontaneous (no additional triggers), and reproducibly results in clots in the large veins of the head and fibrin deposition in the liver. This thrombotic phenotype is fatal, but can be fully rescued by thrombin inhibition. In the present study, this model was used to investigate the role of platelets, neutrophils, and factor XII. After administration of siRNAs targeting *Serpinc1* and *Proc*, antibody-mediated depletion of platelets fully abrogated the clinical features as well as microscopic aspects in the head. This was corroborated by strongly reduced fibrin deposition in the liver. Whereas neutrophils were abundant in siRNA-triggered thrombotic lesions, antibody-mediated depletion of circulating Ly6G-positive neutrophils did not affect onset, severity, or thrombus morphology. In addition, absence of circulating neutrophils did not affect quantitative liver fibrin deposition. Remarkably, siRNA-mediated depletion of plasma factor XII accelerated the onset of the clinical phenotype; mice were more severely affected, with more severe thrombotic lesions. In conclusion, in the present study, onset and severity of the thrombotic phenotype are dependent on the presence of platelets, but not circulating neutrophils. Unexpectedly, factor XII has a protective effect. This study challenges the proposed roles of neutrophils and factor XII in venous thrombosis pathophysiology.

INTRODUCTION

Venous thrombosis (VT) is a complex disease and its pathogenesis is incompletely understood. Recently, it is recognized that cellular components of the blood may contribute to the initiation and propagation of VT (1, 2). Mouse models have been important tools to study the pathogenesis of VT. In models based on flow restriction (stasis), induced by partial ligation of the inferior vena cava, and in the absence of any vascular and/or endothelial damage, it was shown by von Brühl and others that blood leukocytes were actively recruited to the inflamed venous vessel wall, resulting in initiation and propagation of VT (1-3). An interplay between these recruited blood cells (mainly platelets and neutrophils) and coagulation factor XII (FXII) appeared critical for thrombus formation (1).

Although this mouse model for VT proved valuable for identifying potential novel players in VT, the role of blood stasis, hypoxia, and endothelial activation may be overestimated compared to the human situation of VT (1, 3). Particularly in cases where imbalanced coagulation, either as a result of environmental conditions or genetic background, is the driving risk factor for VT (4, 5). It cannot be excluded that in the model used by von Brühl et al. the numerous surgical handlings required to establish stasis in the inferior vena cava contribute to a proinflammatory state. Furthermore, the retrograde formation of thrombi is an important difference between that model and the human situation.

Recently, we described a mouse model with an acute imbalance in coagulation, achieved by strong inhibition of the hepatic expression of antithrombin (*Serpinc1*) and Protein C (*Proc*) using synthetic small interfering (si) RNA (6). Inhibition of these anticoagulants resulted in a highly reproducible, siRNA dose-dependent, and thrombin-dependent thrombotic coagulopathy, which, without interventions, is fatal. Likely due to vascular bed-specific hemostasis and local flow characteristics (7), (fibrin-layered) thrombi were reproducibly formed in large veins in the head (in the masseter and mandibular area). Moreover, fibrin was deposited in the liver and plasma fibrinogen was consumed resulting in prolonged clotting times. As thrombus initiation and propagation in this model required no additional triggers other than inhibition of anticoagulant gene expression (achieved by intravenous siRNA injection), we used this model to further evaluate the role of platelets, neutrophils, and FXII in the initiation and propagation of VT.

METHODS

Animal experiments

C57BL/6J female mice (18-20 g) were purchased from Charles River (Maastricht, the Netherlands). siRNAs targeting mouse antithrombin (si*Serpinc1, cat*. #S62673, Ambion, Life Technologies, Carlsbad (CA), USA), protein C (si*Proc*, cat. #S72192) and a control siNEG (cat. #4404020) were designed and/or used as described previously (6). For siRNA-mediated silencing of coagulation factor XII *(F12)*, RNA-strand sequences were; sense: 5'-CCACAAAUGCAUCCACAAAtt-3' and antisense: 5'-UUUGUGGAUGCAUUUGUGGtg-3' (cat. #S81735). For *in vivo* use, siRNAs were complexed with Invivofectamine® 2.0 (Invitrogen, Life Technologies) and injected intravenously (tail vein) at a dose of 5.75 mg/siRNA/kg body weight. This dose reproducibly results in spontaneous macrovascular VT limited to the head (in the masseter and mandibular area), irrespective of sex and strain. siRNA-complexes targeting *F12* were injected 24 hours before si*Serpinc1*/si*Proc* treatment.

For platelet depletion a rat monoclonal antibody against mouse GP1b (#R300, Emfret, Würzburg, Germany) was used. Depletion of neutrophils was achieved using a rat monoclonal antibody targeting mouse Ly6G (clone 1A8, Biolegend, San Diego, USA), and a rat isotype control IgG (clone RTK2758, Biolegend) as a control. Antibodies were injected intravenously (5 mg/kg body weight) 6 hours after si*Serpinc1*/si*Proc* injection, unless indicated differently.

Animals were sacrificed, and citrated blood and liver were collected as described (8, 9). Mouse heads were fixated in 4% formaldehyde. All experimental procedures were approved by the institutional animal welfare committee.

Liver and blood analyses

Liver transcript levels of *Serpinc1*, *Proc*, and *F12* were determined using qPCR, with *Actb* as a house-keeping gene (8, 9). siRNA-mediated hepatic silencing of *Serpinc1* and *Proc* silencing was routinely confirmed[6]. Liver fibrin deposition was determined by immunoblotting using the monoclonal antibody 59D8 (10).

Blood neutrophil numbers were measured using flow cytometry (LSR II, BD Biosciences, San José, USA) using αLy6G-phycoerythrobilin (clone 1A8, BD Biosciences). Platelet and neutrophil numbers were determined with a hematology analyzer (Sysmex XE-2100). *Ex vivo* platelet activity (with and without stimulus) was determined as described (11). Plasma FXII activity was determined using an activated partial thromboplastin time-based assay using FXII-deficient human plasma, using C57BL/6J mouse pool plasma for calibration (9). Plasma nucleosome levels and thrombin generation (tissue factor and ellagic acid-induced) were determined as described (12, 13).

Phenotype assessment

The spontaneous thrombotic phenotype following si*Serpinc1*/si*Proc* injection has been described extensively before (6), and developed in all mice 2 to 3 days after siRNA injection. Because of the severe nature of the clinical symptoms that accompanied the thrombotic phenotype, animals were sacrificed 72 hours after si*Serpinc1*/si*Proc* injection, unless indicated differently.

After sacrifice (not including animal perfusion), formalin-fixed heads were decalcified (in 20% formic acid), dehydrated, paraffin-embedded, and sectioned. After analysis of coronal serial sections of the head and neck, sections (4 µm) were made starting directly caudal of the eyes, since this area was most clearly and reproducibly affected and thrombi in large veins were found here (in si*Serpinc1*/si*Proc* injected animals). Selected sections were stained using hematoxylin and eosin or according to Carstairs' methodology (14). Severity of the phenotype at the microscopic level was scored based on the presence and extent of thrombotic lesions, subcutaneous and intramuscular bleeding, and subcutaneous edema in the entire section (figure S4). Incidence and appearance of thrombotic lesions in the selected sections was categorized and scored (see figure S6 for elaborate explanation).

Immunohistochemistry

Paraffin embedded coronal sections of the head area (i.e. serial sections of those described above) were stained with a rat monoclonal anti-mouse Ly6G (clone A8, Biolegend). For detection, a horseradish peroxidase (HRP) labelled rabbit anti-rat IgG antibody was used (Dako, Agilent Technologies, Glostrup, Denmark). HRP activity was detected using diaminobenzidine (Dako).

RESULTS

Platelets are crucial for spontaneous thrombosis

In si*Serpinc1*/si*Proc*-treated animals, in thrombi of the mandibular area of the head, platelets (Carstairs' light blue positive) appeared abundantly present in thrombi and also co-localized both with leukocytes (dark blue/purple) and fibrin (bright red/pink, see figure S1A). Additionally, a reduction in blood platelet counts coincided with increased liver fibrin deposition and development of the clinical features of the thrombotic coagulopathy (figures S1B). Flow cytometry analysis showed that the circulating platelets did not display a significant increase of surface activation markers before onset of the thrombotic phenotype (figure S1C). To investigate the role of platelets during spontaneous thrombotic coagulopathy following silencing of anticoagulant genes *Serpinc1* and *Proc*, platelets were depleted using an antibody targeting mouse GP1b, 6 hours after si*Serpinc1*/si*Proc* injection. Successful platelet depletion (no platelets detectable in whole blood) was confirmed in a dedicated pilot study (data not shown) and in a parallel group

that did not receive si*Serpinc1*/si*Proc* (figure 1A; median: 616x10^9 platelets/L (range: 554, 642 minimum and maximum, respectively) vs. 0x10^9 platelets/L (0, 7), *P*=0.036).

Fully in line with previous observations (6), animals treated with siRNAs targeting *Serpinc1* and *Proc* and subsequently injected with saline (designated as -αGP1b in figure 1) developed the typical clinical features of the thrombotic coagulopathy within three days: Mice developed unilateral lesions around the eye and swellings in the head. Moreover, they became lethargic, unresponsive to stimuli, hypothermic, and showed a significant loss in body weight. One of the affected mice died before it could be included for further analysis. In contrast, si*Serpinc1*/si*Proc* +αGP1b mice (+αGP1b in figure 1) appeared fully healthy (figure 1B), and did not experience weight loss compared to the si*Serpinc1*/si*Proc* -αGP1b group, which emphasizes their retained health (figure S2A; -2.05g (-3.05, 0.15) vs. 0.13g (-0.99, 0.77), *P*=0.043). Strikingly, platelet depletion at a later time point (si*Serpinc1*/si*Proc*-treated mice received αGP1b when the first two mice presented the clinical features of the thrombotic phenotype) also fully rescued mice from thrombotic coagulopathy. All mice in the reference group became affected as expected (9/9 vs. 2/9, -αGP1b and +αGP1b, respectively. *P*=0.002).

On a microscopic level, in the si*Serpinc1*/si*Proc* -αGP1b group, thrombi were found in all mice in the larger and smaller veins of the selected coronal sections of the head (figure 1C and S3). Moreover, extensive multifocal red blood cells extravasations (hemorrhages) were present especially in the masseter and mandibular area, with associated subcutaneous edema (7 of 7 mice). In contrast, in the si*Serpinc1*/si*Proc* +αGP1b mice, neither thrombi nor notable injuries were observed (figure 1D, S3, and S5A). When the presence of thrombi was scored, two types of thrombi were defined based on their composition and structure (figure 1E and figure S6). Interestingly, one mouse that was not clinically affected (yet) did appear to have thrombi in a vessel in the investigated coronal section of the head (figure 1B, -αGP1b). In contrast, no mice in the si*Serpinc1*/si*Proc* +αGP1b group appeared to have any thrombi. In line with these observations, liver fibrin deposition was strongly and significantly reduced in the si*Serpinc1*/si*Proc* +αGP1b group (figure 1F; 75.7 ng/mg (21.40, 143.9) vs. 9.46 ng/mg (6.34, 21.69), *P*=0.001), although liver fibrin levels were above background level observed in control animals solely injected with control siRNA (siNEG, figure 1F; 4.50 ng/mg (3.13, 5.72), *P*<0.001).

Circulating neutrophils are not a major mediator of spontaneous thrombosis

Mice were depleted of neutrophils 6 hours after si*Serpinc1*/si*Proc* injection, using an antibody targeting the neutrophil-specific Ly6G membrane protein (+αLy6G in figure 2). In this experimental setup, an isotype IgG antibody was used in the group not depleted of neutrophils (-αLy6G in figure 2). Flow cytometry analysis confirmed in a dedicated experiment that the Ly6G-positive i.e. neutrophil population was fully absent in the circulation up to 4 days after antibody

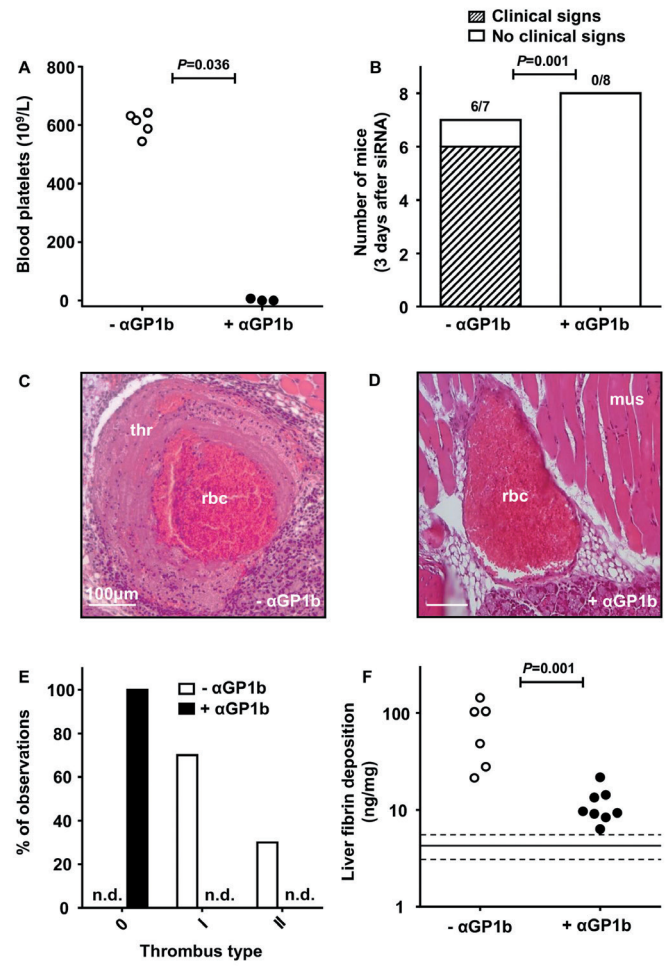

Figure 1 | Depletion of platelets prevents thrombotic coagulopathy following siRNA-mediated hepatic knockdown of *Serpinc1* and *Proc*. A, Blood platelets numbers in mice from a parallel group not receiving siRNA 3 days after injection with saline (open circles) or with a rat monoclonal antibody targeting mouse GP1b (black circles). *P*=0.036, Mann Whitney Rank-sum test. B, Scoring of the clinical phenotype in mice treated with siRNAs targeting *Serpinc1* and *Proc*. Animals showing characteristic clinical coagulopathy (hatched bar) and animals unaffected (open bars) 3 days after siRNA treatment (end of experiment). One of the mice from the -αGP1b-group died because of the thrombotic coagulopathy. *P*=0.001, Fisher-exact test. C/D, Representative thrombus identified in a vein in the control group (-αGP1b, panel C), and a representative vein in the platelet depleted group (+αGP1b, panel D) in H&E stained sections. thr: Thrombus with typical fibrin layers, rbc: postmortem clotted blood rich in red blood cells, mus: Striated muscle tissue. White bars indicate 100µm. E, Scoring for the presence of thrombi. 0: no thrombi found, I + II: thrombi categories based on structure and layering (see method section and figure S4). Open bars: -αGP1b (n=10), black bar: +αGP1b (n=16), n.d. not detected. F, Levels of fibrin deposition in the liver of the platelet depleted group (+αGP1b) and the control group (-αGP1b). *P*=0.001, Mann Whitney Rank-sum test. Solid and dashed lines indicate fibrin levels found in solely siNEG injected C57BL/6J female mice (median and range, resp. 4.50 ng/mg (3.13, 5.72)). Data are presented as the median with the range (minimum and maximum, respectively).

injection (data not shown). Using the same approach, we again did not detect neutrophils in the circulation in the si*Serpinc1*/si*Proc* +αLy6G group one day after antibody injection and one day before onset of the phenotype (figure S7). Additionally, after sacrifice the absence of neutrophils in the si*Serpinc1*/si*Proc* +αLy6G treated group was confirmed (figure 2A; 720 neutrophils/μL (320, 1120) vs. 0 neutrophils/μL (0, 280), *P*<0.001).

si*Serpinc1*/si*Proc* treated mice in both the neutrophil-depleted (si*Serpinc1*/si*Proc* +αLy6G) and in the isotype IgG treated group (si*Serpinc1*/si*Proc* -αLy6G) developed the typical clinical signs of the thrombotic coagulopathy (figure 2B). Moreover, body weight was lowered for mice in both groups to a comparable extent (figure S2B; -0.85g (-3.24, 0.13) vs. -2.48g (-3.61, -0.31), *P*=0.132). On a microscopic level, coronal sections of the head were analyzed and in both groups thrombi were found in large veins, as well as hemorrhages and edema. Severity scoring yielded no differences in severity (figure S5B). In the si*Serpinc1*/si*Proc* -αLy6G group, Ly6G-positive cells were abundantly present in thrombi and followed the alignment of structures identified as fibrin and adhered to the thrombotic venous vessel wall (figure 2C). Ly6G-positive cells in thrombi were absent in the si*Serpinc1*/si*Proc* +αLy6G group (although in some animals strongly reduced Ly6G-positive signal was found in some cells in the thrombus, see figure 2D). Generally, thrombus leukocyte density was affected after neutrophil depletion (compare in figures 2C, 2D, and S8). Depletion of neutrophils did not significantly affect the organizational structure and lining of the thrombi (figure 2E and S6). In line, increased liver fibrin deposition compared to an siNEG control group of mice was evident, but no differences between both si*Serpinc1*/si*Proc* treated groups were observed (figure 2F; 67.87 ng/mg (9.28, 587.5) vs. 51.92 ng/mg (10.94, 3126), *P*=0.931).

To investigate whether the thrombotic coagulopathy following si*Serpinc1*/si*Proc* injection coincided with the formation of neutrophil extracellular traps (NETs), and whether neutrophil depletion affects NET formation, we determined plasma levels of extracellular nucleosomes as a NET biomarker (12). Plasma nucleosome levels were moderately but significantly increased (*P*=0.003 and *P*<0.001, respectively si*Serpinc1*/si*Proc* -αLy6G and si*Serpinc1*/si*Proc* +αLy6G) in mice with the thrombotic coagulopathy when compared to untreated mice (figure S9). However, no differences were found in plasma from mice with or without detectable neutrophils in the circulation (si*Serpinc1*/si*Proc* -αLy6G: 46 U/mL (7, 756), si*Serpinc1*/si*Proc* +αLy6G: 59 U/mL (10, 771), *P*=0.448), suggesting extracellular nucleosomes detected in plasma of thrombotic animals were not derived from neutrophils.

Reduced FXII propagates development of the spontaneous venous thrombosis

The role of FXII was studied using an siRNA approach (si*F12*). For mice treated with si*F12* only (without si*Serpinc1*/si*Proc* injections), FXII plasma activity was decreased (72 after siRNA injection) by 87.3% (93.2-81.4, *P*=0.029) as compared to pool plasma, while FXII levels of the siNEG treated

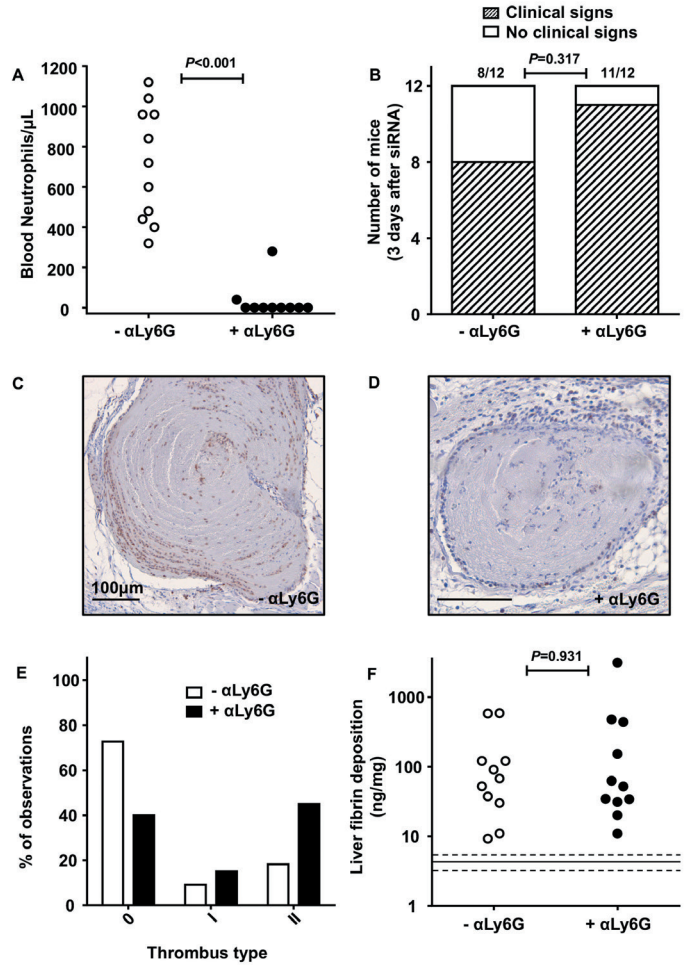

Figure 2 | Depletion of neutrophils does not influence progression of thrombotic coagulopathy following siRNA-mediated hepatic knockdown of *Serpinc1* and *Proc*. A, Blood neutrophils numbers in mice 3 days after injection with a rat monoclonal antibody targeting mouse Ly6G (black circles) or a rat IgG control (open circles). *P*<0.001, Mann Whitney Rank-sum test. B, Scoring of the clinical phenotype in mice treated with siRNAs targeting *Serpinc1* and *Proc*. Animals showing characteristic clinical signs (hatched bar) and animals unaffected (open bars) 3 days after siRNA treatment (end of experiment). One of the mice in both groups (-αLy6G and +αLy6G) died because of the thrombotic coagulopathy. *P*=0.317, Fisher-exact test. C/D, Ly6G staining of thrombi found in sections of the head, in the -αLy6G (panel C) and +αLy6G (panel D). Hematoxylin was used for counterstaining, black lines indicate 100 μm. E, Scoring for the presence of thrombi. 0: no thrombi found, I + II: thrombi categories based on structure and layering. Open bars: -αLy6G (n=22), black bars: +αLy6G (n=20). F, Levels of fibrin deposition in the liver of the neutrophil depleted group (+αLy6G) and the control group (-αLy6G). *P*=0.931, Mann Whitney Rank-sum test. Solid and dashed lines indicate fibrin levels found in solely siNEG injected C57BL/6J female mice (median and range, resp. 4.50 ng/mg (3.13, 5.72)). Data are presented as the median with the range (minimum and maximum, respectively).

group were in the normal range (figure 3A; 110.9% (101.0, 118.9) of pool plasma). Moreover, groups treated with si*Serpinc1*/si*Proc* in combination with si*F12* or siNEG (designated +si*F12* and -si*F12* in figure 3, respectively) were analyzed for hepatic *F12* transcript levels (after sacrifice). A reduction in hepatic *F12* transcript of 86.0% (87.9-84.9, *P*<0.001) was observed in the si*Serpinc1*/si*Proc* +si*F12* group.

To investigate whether thrombin generation (TG) was altered in mice with lower levels of FXII, we performed a TG assay on plasma from mice 48 hours after si*F12* treatment i.e. 24 hours after si*Serpinc1*/si*Proc* injection, with ellagic acid (contact-activation initiated) or tissue factor as triggers for TG. At this time point, animals did not show any clinical singularities and were completely healthy. Endogenous thrombin potential values did not differ between si*Serpinc1*/si*Proc* -si*F12* and si*Serpinc1*/si*Proc* +si*F12* groups in both measurements (ellagic acid: 1019 nM.min (518, 1810) vs. 1488 nM.min (552, 1689), -si*F12* and +si*F12*, resp. *P*=0.151; tissue factor: 919 nM.min (670, 2073) vs. 807 nM.min (613, 949), -si*F12* and +si*F12*, resp. *P*=0.193), and no other differences were observed in TG curves (data not shown). In line, low FXII did not significantly affect APTT (as determined in siNEG and siF12-only animals; 38.7s (32.5-38.9) vs. 39.4s (38.3-45.9), *P*=0.40, 1:1 diluted plasma).

Remarkably, with lower plasma levels of FXII, mice treated with siRNAs against *Serpinc1* and *Proc* (si*Serpinc1*/si*Proc* +si*F12*) developed earlier and more severely clinical symptoms of thrombosis compared to the siNEG treated group (si*Serpinc1*/si*Proc* -si*F12*, figure 3B, 4/10 vs. 9/9, *P*=0.011). Because the onset of the clinical symptoms was premature (within 48 hours after si*Serpinc1*/si*Proc* treatment) compared to previous experiments, we stopped the experiment and sacrificed animals 2 days after si*Serpinc1*/si*Proc* treatment. To emphasize the difference between both groups, FXII-lowered mice experienced significantly more body weight loss during the experiment (figure S2C; -0.05 g (-1.60, 0.60) vs. -2.30 g (-3.10, -1.60), *P*<0.001). This surprising outcome was confirmed in a second independent experiment with an identical setup, where again in large majority of mice in the si*Serpinc1*/si*Proc* +si*F12* group onset of the typical coagulopathy was evident 2 days after si*Serpinc1*/si*Proc* injection, while animals in the si*Serpinc1*/si*Proc* -si*F12* group were healthy at this early time point (0/8 and 7/8, *P*=0.001, body weight gain: -0.35 g (-0.70, 0.50) vs. -0.95 g (-1.90, -0.20), *P*=0.004).

On a microscopic level, thrombus formation, hemorrhages, and edema were more severe in the si*Serpinc1*/si*Proc* +si*F12* group compared to the si*Serpinc1*/si*Proc* -si*F12* group (figure 3C and 3D as an example, figure S5C for severity scoring). Blinded analysis of the coronal sections yielded thrombotic lesions characterized by structured fibrin layering were present in the si*Serpinc1*/si*Proc* -si*F12* group, but absent in the si*Serpinc1*/si*Proc* +si*F12* group (figure 3E). Instead, in the si*Serpinc1*/si*Proc* +si*F12* group lesions were observed mostly in disrupted veins consisting of

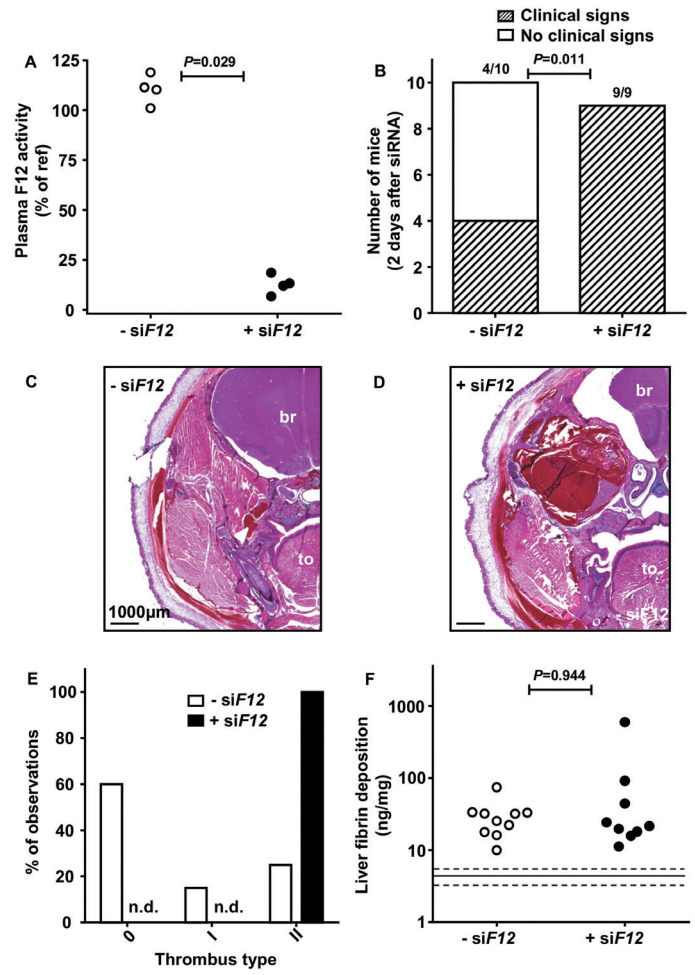

Figure 3 | siRNA-mediated hepatic knockdown of *F12* worsens thrombotic coagulopathy induced by knockdown of *Serpinc1* and *Proc*. A, Plasma FXII activity in mice 3 days after injection with an siRNA targeting *F12* (black circles) or siNEG (open circles). *P*=0.029, Mann Whitney Rank-sum test. Normal pool plasma was used as an internal reference. B, Clinical phenotype in mice treated with siRNAs targeting *Serpinc1* and *Proc*. Animals showing characteristic clinical signs (hatched bar) and animals unaffected (open bars) 2 days after siRNA treatment (end of experiment). *P*=0.011, Fisher-exact test. C/D, Representative H&E stained coronal head sections from the –si*F12* group (panel C) and the +si*F12* group (panel D). For orientation of the selected section, br: Brain, to: Tongue, arrowheads indicate extravasated red blood cells. Black lines indicate 1000µm. E, Scoring for the presence of thrombi. 0: no thrombi found, I + II: thrombi categories based on structure and layering. Open bars: -si*F12* (n=20), black bars: +si*F12* (n=18), n.d. not detected. F, Levels of fibrin deposition in the liver of the FXII deficient group (+si*F12*) and the control group (-si*F12*). *P*=0.944, Mann Whitney Rank-sum test. Solid and dashed lines indicate fibrin levels found in solely siNEG injected C57BL/6J female mice (median and range, resp. 7.13 ng/mg (6.10, 7.96)). Data are presented as the median with the range (minimum and maximum, respectively).

mixtures of red blood cells and structures identified as eosin-positive fibrin, lacking a typical layered structure (type II thrombotic lesions). However, detailed histological analysis of the thrombi did not yield clues that low FXII levels uniquely impacts clot structure. Despite the more severe clinical phenotype and more severe lesions observed microscopically in the si*Serpinc1*/si*Proc* +si*F12* group, liver fibrin deposition levels were comparable for si*Serpinc1*/si*Proc* treated groups (figure 3F; 28.71 ng/mg (9.95, 74.57) and 21.55 ng/mg (11.24, 596.1), –si*F12* and +si*F12*, respectively, *P*=0.944).

DISCUSSION

In the present study, we investigated the role of platelets, neutrophils, and factor XII in the pathophysiology of VT using a mouse model that features spontaneous onset of VT i.e. in a thrombosis model that does not require additional triggers or handlings other than silencing expression of two liver-derived anticoagulants through simple intravenous injection of synthetic siRNAs. In this model, platelets, but not neutrophils, were found to be rate-limiting in thrombus formation, while low plasma FXII was found to aggravate spontaneous thrombosis. The results of this study therefore challenge the proposed roles of neutrophils and FXII in VT pathophysiology.

Platelets, recently identified as major players in experimental VT in a ligation model, are recruited early after flow restriction and are involved in stabilization and accumulation of innate immune cells (1). Thrombus formation does not take place in the absence of platelets. Also in the present study, rescue from thrombotic coagulopathy after platelet depletion was complete. These observations provide further evidence for an important role of platelets during experimental VT, and is in line with previous studies (1, 15).

In the present model, the thrombotic coagulopathy rapidly progresses with a transition within hours from a condition where animals have no thrombotic lesions detectable in the head and minimal liver fibrin deposition, towards a condition where veins in the head are occluded with thrombi and liver fibrin deposition is evident. We now suggest that platelets are particularly important for the burst of fibrin and thrombus formation, but not for initial fibrin formation. This is supported by 1) thrombi were not detected in platelet-depleted mice, while liver fibrin deposition was found to be increased compared to baseline level (figure 1F). Also 2) late platelet depletion just before the expected onset of the thrombotic phenotype fully rescues. Finally 3), von Willebrand factor (VWF), a protein important for platelet adherence but not for massive fibrin formation, is not involved in si*Serpinc1*/si*Proc* induced thrombosis. VWF-deficient animals did not respond differently upon silencing of anticoagulation as compared to animals that express VWF (4 out of 6 *Vwf-/-* mice vs 5 out of 6 *Vwf+/+* control mice macroscopically and microscopically

affected within 72 hours after si*Serpinc1*/si*Proc* injection). Overall, the present study confirms an important but different role for platelets as posed previous in VT pathophysiology, and thereby further evidence to investigate antiplatelet therapy as prophylactic treatment against (recurrent) VT (16, 17).

Neutrophils have been linked in recent years to play a role in VT, via a specialized cell death program where NETs are released (18, 19). During thrombus formation in a mouse model of experimental VT, NETs released upon neutrophil recruitment to the (proinflammatory) vessel wall are indispensable (1). The role of neutrophils became evident using an antibody-mediated depletion strategy identical to the method used in this study (depletion of Ly6G-positive cells). Also for the electrolytic inferior vena cava model of VT, where the vena cava is directly activated by an electric current causing endothelial damage, it was demonstrated vein wall neutrophils were the most common cell type present in acute VT (20). Moreover, neutrophils and NETs have also been identified in human specimens of VT (21, 22).

When VT follows silencing of anticoagulant genes, Ly6G-positive neutrophils were abundantly present within the thrombi, seemingly recruited and aligned to the fibrin layers, which is consistent with previous observations (1, 2). However, in our model, neutrophils were not rate-limiting in thrombus formation. We were unable to detect any phenotypically relevant impact of neutrophil depletion, which is in strong contrast with previous observations. Hence, the proposed role of neutrophils in the thrombosis pathophysiology does not hold true for conditions where endothelial activation and/or vessel wall inflammation are considered absent (i.e. not triggered by surgical handlings). Therefore, we expect a less vital role for neutrophils in humans when manifestation of VT is clearly associated with thrombophilia.

The abundance and specific alignment of neutrophils in the spontaneously formed thrombi in the head (figure 2C) suggest that neutrophils are not innocent bystanders during thrombosis. Neutrophils may have an active role in the inflammatory process associated with thrombosis, possibly once the thrombus is formed. The finding of occasional Ly6G+ cells in thrombi in the si*Serpinc1*/si*Proc* +αLy6G group (while undetectable in the circulation) suggests that the formed thrombi are strong triggers for neutrophil recruitment, possibly from sources other than the circulation. Overall, our data indicate that the role of neutrophils in thrombosis may depend on the trigger, and encourage studies on the role of neutrophils after thrombus formation.

FXII has been a therapeutic candidate for treatment of VT since it may be involved in thrombus formation, but not essentially in haemostasis (2, 23, 24). In our model of spontaneous VT, strong reduction in plasma FXII was achieved through silencing the hepatic transcript, resulting in reduced plasma protein activity 10-20% of control. At these levels, plasma performs normally in

thrombin generation assays following intrinsic and extrinsic stimulation and produces normal aPTTs. Surprisingly, we observed an effect of low plasma FXII on thrombus formation: We observed a faster onset and more severe thrombotic coagulopathy in mice with low levels of FXII.

We are puzzled by this observation, and considered three potential mechanisms for FXII. First, we considered low FXII-enhanced secondary bleeding tendency as a mechanism underlying the observed more severe coagulopathy. In the experiment that reproduced the exacerbating effect of low FXII in spontaneous thrombosis, microscopic analysis of coronal sections of the head area showed that animals of the *siSerpinc1/siProc* -si*F12* group did not all develop the thrombotic phenotype (yet) after 2 days, while all si*Serpinc1*/si*Proc* +si*F12* animals did. In other words, lowering FXII levels seemed to accelerate thrombosis onset, which argues against an effect of low FXII through enhancing the secondary bleedings in the head.

Secondly, we considered changes in the fibrinolytic pathway as a mechanism by which low FXII modulates spontaneous VT. FXII also contributes to clot structure and fibrinolysis (25-30). FXII can interact with pro-fibrinolytic factors (25) and deficiency of FXII causes less dense/stiff clots (26, 27). In line, activation of FXII with polyphosphates in normal whole blood, but not FXII-deficient plasma, increased clot firmness (28, 29). Detailed histological analysis of the thrombi formed during our experiments however did not yield clues whether low FXII indeed uniquely impacts clot structure. Of note, the present thrombosis model was not particularly sensitive to alterations in fibrinolysis, as the antifibrinolytic agent tranexamic acid did not affect the onset or progression of si*Serpinc1*/si*Proc*-mediated thrombosis (at a dose 2.5g tranexamic acid/kg/day starting before siRNA injections, data not shown). This argues against an impact of low FXII levels on thrombosis onset and severity through altered clot formation and/or fibrinolysis.

Thirdly, we considered changes in the kinin/kallikrein pathway as a mechanism by which low FXII may modulates spontaneous VT (31). We investigated the impact of low FXII on edema formation. Edema in the masseter and mandibular area is a clear macroscopically visible feature of spontaneous thrombosis. We hypothesized that low FXII reduces kallikrein formation and consequently the release of bradykinin, which hinders formation of edema (opposite to hereditary angioedema type III, where a gain of function in FXII induces edema formation[32]. In thrombotic mice with low FXII impaired edema formation would worsen the phenotypic response, through an inability to deal with intravenous pressure following thrombotic occlusion. Quantitation of edema in the head (measured by the thickness of the edemic dermis in coronal sections of the head) did not yield clues that low FXII altered edema formation (data not shown). As antibodies and/or chromogenic substrates for detection of (activity) mouse components of the kallikrein/kinin pathway are lacking, we are not able to provide additional data further exploring kinin/kallikrein pathway as a mechanism of low FXII in spontaneous thrombosis.

We conclude that in the present VT model based on lowering anticoagulation factors, FXII inhibition does not rescue the thrombotic phenotype. Further studies are needed to clarify the mechanisms of thrombus formation and the role of FXII and related proteins (like coagulation factor XI) in thrombosis following impaired anticoagulation.

In this report we challenge the proposed essential roles for platelets, neutrophils, and FXII in VT pathogenesis. Mouse models where thrombosis is induced by stenosis-induced stasis, leading to hypoxia and endothelial activation, demonstrate a crucial role for these factors, but our data imply neutrophils and FXII are not essential in thrombus formation when impaired anticoagulation is the driving force. Hence, targeting neutrophils or FXII for therapeutic purposes is an interesting thought, but might not be applicable for treatment of every case of VT. On the other hand, our study provides further rationale for antiplatelet therapy as prophylactic treatment for (recurrent) VT.

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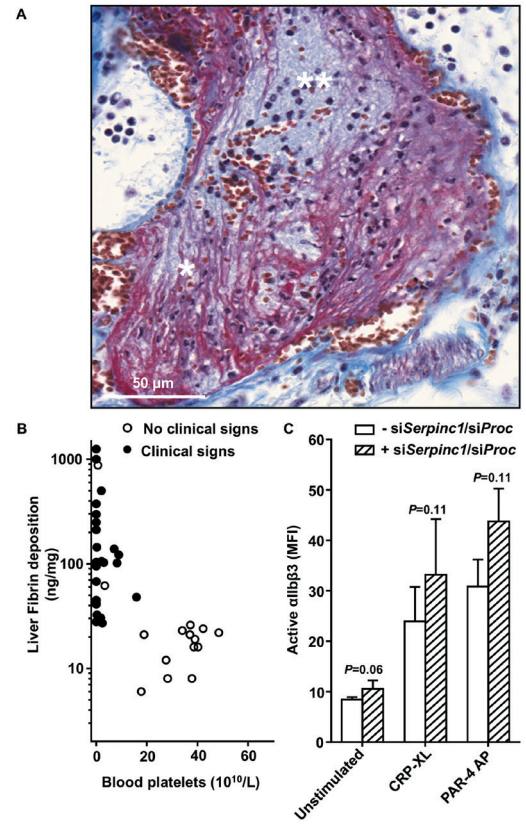
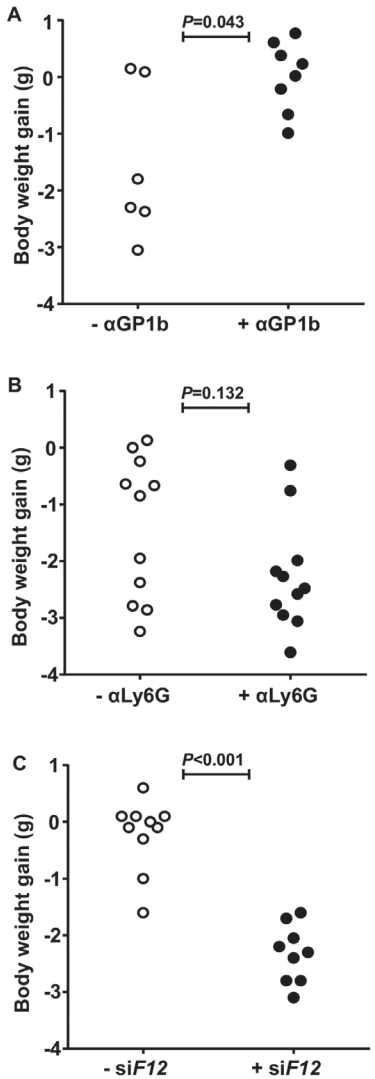
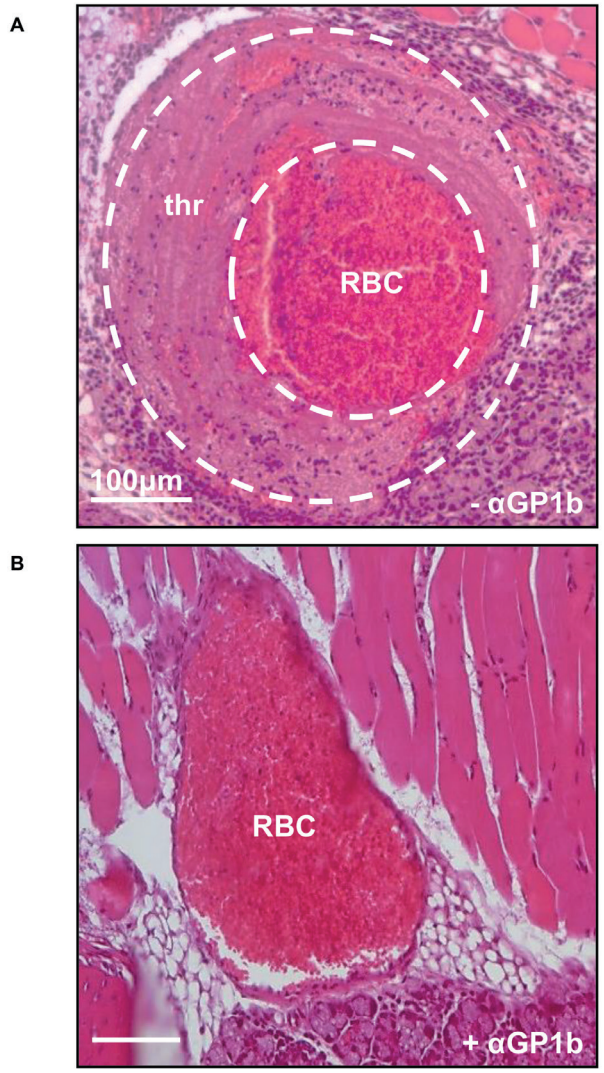


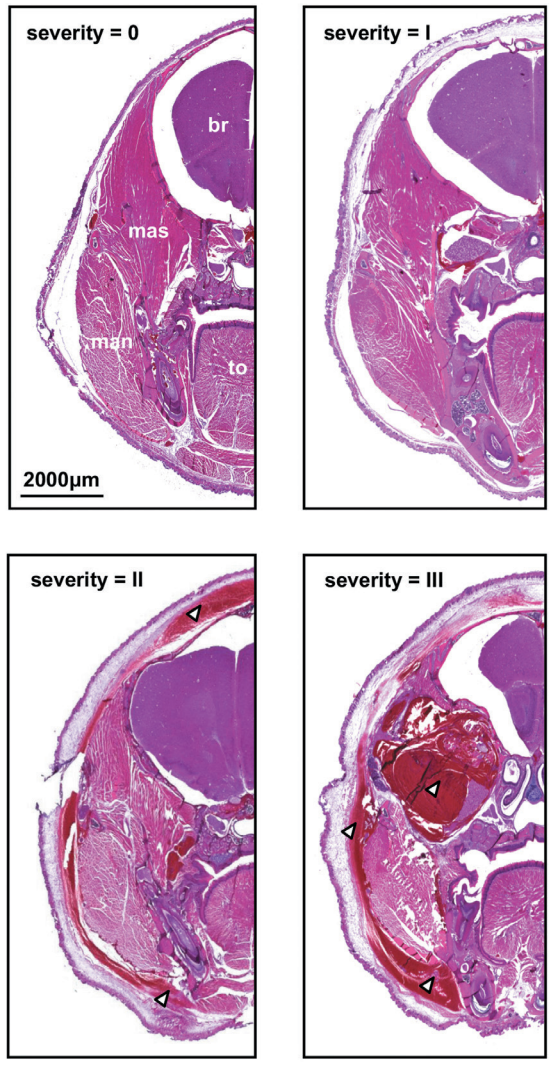
Supplementary figure S1 | Platelets in spontaneous venous thrombosis in mice. A, Venous thrombus in the head (masseter and mandibular area) of a si*Serpinc1*/si*Proc* injected mouse for which paraffin-embedded section was stained according to the Carstairs' method. This methods stains fibrin bright red/pink, platelets navy blue, erythrocytes orange/red, collagen clear blue, and nuclei dark blue/purple. Platelets are abundantly present in the thrombus and localize between the alignment of structures identified as fibrin (single white asterisk) and also co-localize with leukocytes (double white asterisk). White bar indicates 50µm. B, Blood platelets numbers related to liver fibrin deposition for animals treated with si*Serpinc1*/si*Proc* featuring spontaneous venous thrombosis (filled circles) or not (yet) (open circles). Data are pooled from three different experiments (total 38 animals). For reference, normal C57Black/6J female mice (injected solely with siNEG; n=6) have no or low liver fibrin deposition i.e. 4.5 ng/mg (3.1, 5.7) and blood platelet levels numbers of 61x1010/L (54x1010, 64x1010). C, Blood platelet activation before onset of the thrombotic coagulopathy i.e. 48 hours after si*Serpinc1*/si*Proc* treatment (+si*Serpinc1*/si*Proc*, n=5 animals), compared to a non-treated control group of mice (PBS injection, -si*Serpinc1*/si*Proc*, n=4 animals). Flow cytometry analysis was performed to determine the expression (median fluorescence intensity, MFI) of the platelet surface marker active integrin αIIbβII in absence or presence of a (ex vivo) stimulus i.e. cross-linked collagen-related peptide (CRP-XL, 0.25µg/mL) or protease-activated receptor-4 activation peptide (PAR-4 AP, 0.1mM). Expression (MFI) of P-selectin, another platelet surface marker, yielded comparable results (data not shown). *P*-values indicate differences in expression observed for – and +si*Serpinc1*/si*Proc* animals (Mann Whitney Rank-sum test).



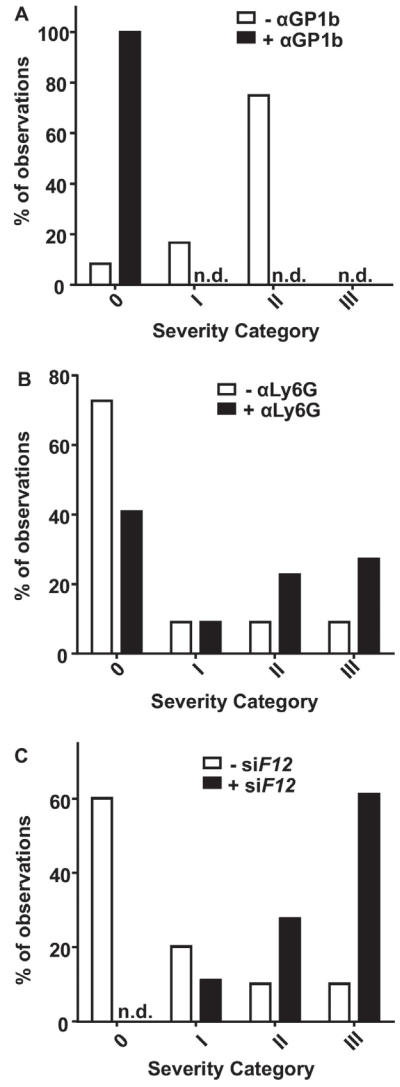
Supplementary figure S2 | Net mice body weight gain after si*Serpinc1* and si*Proc* injection. A, Body weight gain 3 days after si*Serpinc1*/si*Proc* treatment. One of the mice from the -αGP1b-group died because of the thrombotic coagulopathy. -αGP1b (si*Serpinc1*/si*Proc* -αGP1b, open circles): -2.05g (-3.05, 0.11), +αGP1b (si*Serpinc1*/si*Proc* +αGP1b, black circles): 0.13g (-0.99, 0.77). *P*=0.043, Mann Whitney Rank-sum test. B, Body weight gain 3 days after si*Serpinc1*/si*Proc* treatment. One of the mice from both groups (-αLy6G and +αLy6G) died because of the thrombotic coagulopathy. –αLy6G (si*Serpinc1*/si*Proc* -αLy6G, open circles): -0.85g (-3.24, 0.13), +αLy6G (si*Serpinc1*/si*Proc* +αLy6G, black circles): -2.48g (-3.61, -0.31). *P*=0.132, Mann Whitney Rank-sum test. C, Body weight gain 2 days after si*Serpinc1*/si*Proc* treatment. -si*F12* (si*Serpinc1*/si*Proc* -si*F12*, open circles): -0.05g (-1.60, 0.60), +si*F12* (si*Serpinc1*/si*Proc* +si*F12*, black circles): -2.30g (-3.10, -1.60). *P*<0.001, Mann Whitney Rank-sum test. Data are presented as the median with the range (minimum and maximum, respectively).



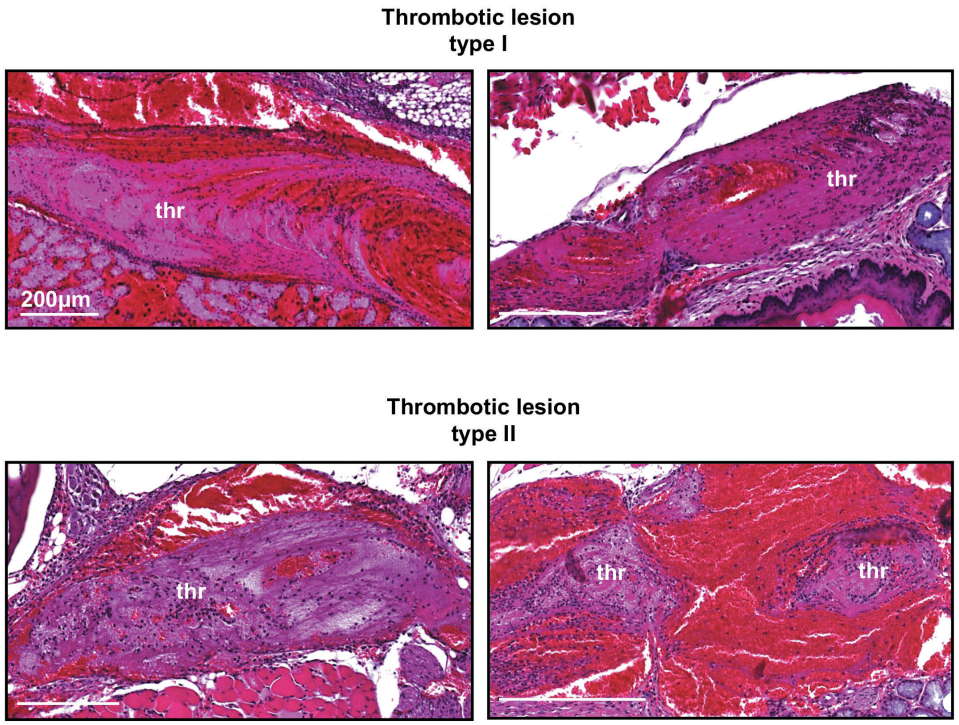
Supplementary figure S3 | Blood vessels in si*Serpinc1*/si*Proc* -αGP1b and si*Serpinc1*/si*Proc* + αGP1b mice. Representative thrombus identified in a vein in the control group (-αGP1b, panel C), and a representative vein in the platelet depleted group (+αGP1b, panel D) in H&E stained sections. In panel A, the white scattered lines marks the area identified as a thrombus. rbc: postmortem clotted blood rich in red blood cells, thr: thrombus. White lines indicates 100 µm. Please note, panels represent the same sections as figures 1C and 1D.



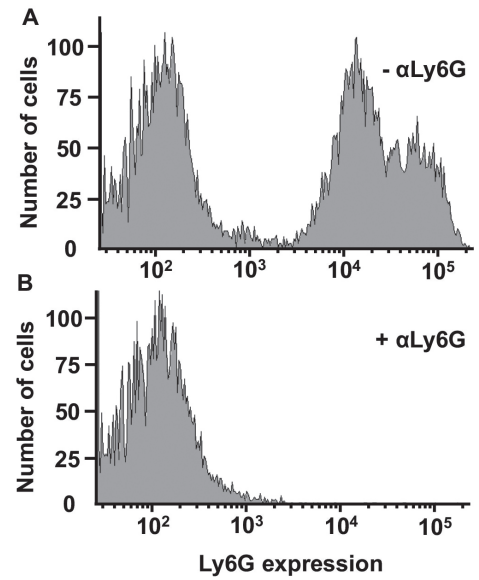
Supplementary figure S4 | Coronal sections of mice heads, representative for four different severity categories. Category 0: Unaffected, category I-III: increasing scores are based on hemorrhages, thrombotic lesions, and edema. For orientation of the selected section (left upper panel), br: Brain, to: Tongue, mas: Masseter muscle, man: Mandibular maxilaris. Black line indicates 2000 µm. Black/white arrowheads indicate bleedings. Please note, lower panels represent the same sections as figures 3C and 3D.

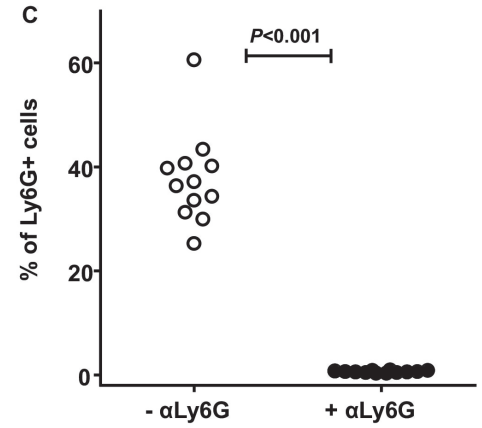


Supplementary figure S5 | Severity scoring for individual mice, divided in four categories, 0-III, see figure S2. A, Severity scores of head sections of mice from the - αGP1b group (si*Serpinc1*/si*Proc* -αGP1b, open circles, n=10) and the +αGP1b group (si*Serpinc1*/si*Proc* +αGP1b, black circles, n=16). n.d. not detected. B, Severity scores of head sections of mice from the –αLy6G group (si*Serpinc1*/si*Proc* -αLy6G, open circles, n=22) and the +αLy6G group (si*Serpinc1*/si*Proc* +αLy6G, black circles, n=20). C, Severity scores of head sections of mice from the –si*F12* group (si*Serpinc1*/si*Proc* -si*F12*, open circles, n=20) and the +si*F12* group (si*Serpinc1*/si*Proc* +si*F12*, black circles, n=18). n.d. not detected. Because of the mostly unilateral nature of lesions, two semi-quantitative scores were granted per mouse during both scoring systems (one left and one right in the head).

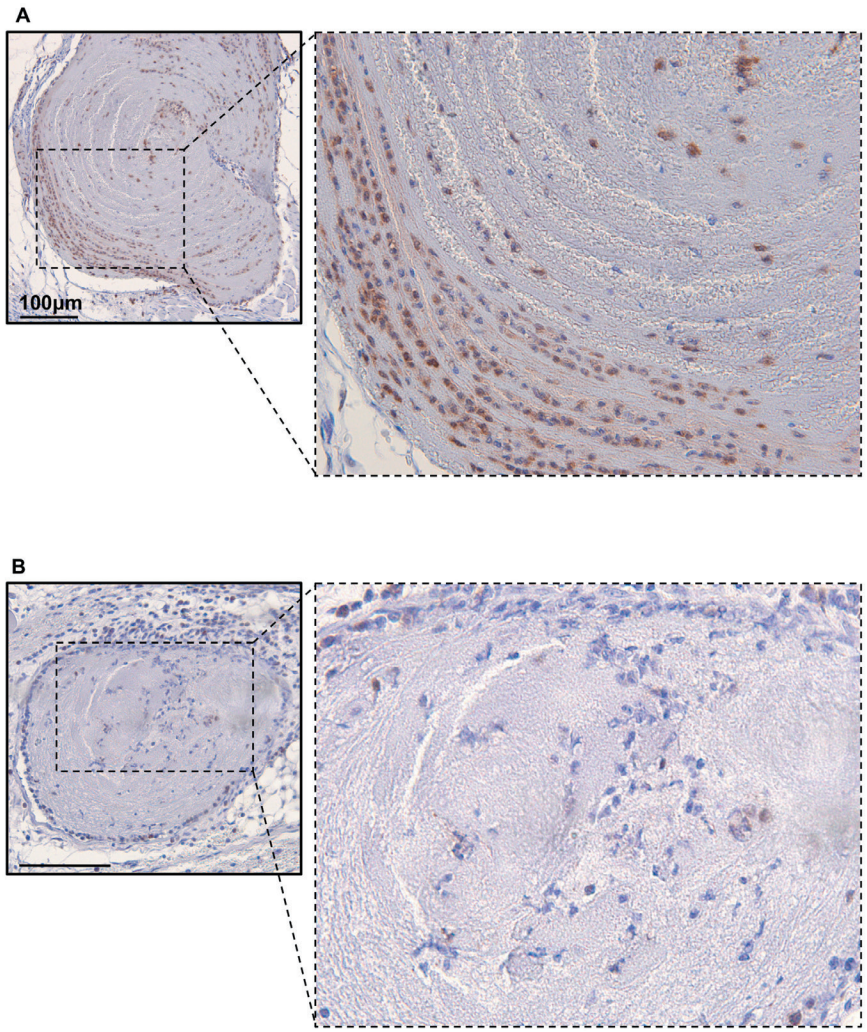


Supplementary figure S6 | Representative sections of mice heads for two different thrombus categories. Thrombi were divided into two different types (blinded analyses, two observers). Thrombus categories were distinguished based on the presence of (I, upper panels) typical organized thrombi within a (mostly) intact venous vessel with layered structures microscopically identified as eosin-positive fibrin, and (II, lower panels) clear structures identified as fibrin (thrombi) embedded or surrounded in red blood cells lacking a typical layered structure. In the latter, no clear vessel structures were identified due to possible vein rupture. The veins around the cranial part of the trachea and the masseter and mandibula maxillaris muscle area were analyzed to score thrombus types, since thrombotic lesions and bleedings were reproducibly found here. thr: Fibrin-rich thrombus. White bars indicate 200 µm.

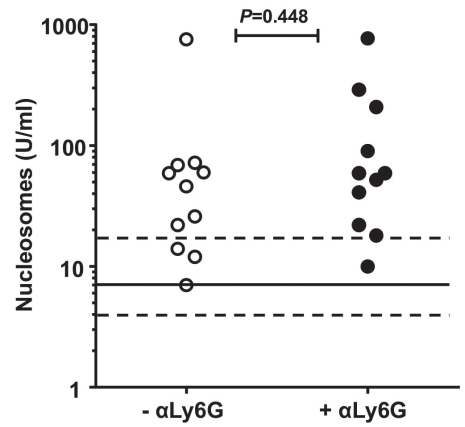




Supplementary figure S7 | Analysis of Ly6G-positive cells in whole blood 1 day after antibody injection using flow cytometry. A/B, Representative analysis of Ly6G expression in viable leukocytes of samples from groups treated with an isotype IgG control antibody (si*Serpinc1*/si*Proc* -αLy6G, panel A) and an Ly6G-depleting antibody (si*Serpinc1*/si*Proc* +αLy6G, panel B). C, Percentage of Ly6G-positive cells from the viable leukocyte population, –αLy6G (si*Serpinc1*/si*Proc* -αLy6G, open circles): 36.80 % Ly6G+ cells (25.30, 60.60), +αLy6G (si*Serpinc1*/si*Proc* +αLy6G, black circles): 0.65% Ly6G+ cells (0.40, 1.00). *P*<0.001, Mann Whitney Rank-sum test. Data are presented as the median with the range (minimum and maximum, respectively).



Supplementary figure S8 | Ly6G staining of thrombi found in sections of the head. In the -αLy6G (si*Serpinc1*/si*Proc* -αLy6G, panel A) and +αLy6G (si*Serpinc1*/si*Proc* +αLy6G, panel B) treated mice. Hematoxylin was used for counterstaining, black lines indicate 100 µm. Please note, panels represent the same sections as figures 2C and 2D.



Supplementary figure S9 | Levels of nucleosomes in plasma. Samples were obtained upon sacrifice. – αLy6G (si*Serpinc1*/si*Proc* -αLy6G, open circles): 46 U/ml (7, 756), +αLy6G (si*Serpinc1*/si*Proc* +αLy6G, black circles): 59 U/ml (10, 771), *P*=0.448, Mann Whitney Rank-sum test. Solid and dashed lines indicate nucleosome levels found in uninjected C57BL/6J female mice (median and range, resp. 7 U/ml (4, 17)). Data are presented as the median with the range (minimum and maximum, respectively).

4

Coagulation Factor XII Does Not Contribute to Mouse Venous Thrombosis Induced By Silencing of Antithrombin and Protein C

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Coagulation factor XII (FXII) has been proposed as a safe therapeutic drug target for venous and arterial thrombosis. Numerous experimental animal studies showed that inhibition of FXII interferes with thrombus formation, without affecting normal hemostasis (1-4). Conversely, we recently reported that inhibition of FXII by means of RNA interference (RNAi) does not confer protection in experimental VT but even augmented vascular occlusions. In a mouse model where VT follows spontaneously shortly (2-3 days) after reduction of natural anticoagulants antithrombin and protein C by RNAi (using synthetic lipid complexed siRNAs i.e. si*Serpinc1*/si*Proc*, see (5)), additional silencing of FXII (si*F12*) resulted in VT to occur even faster with a more severe thrombotic coagulopathy (figure 1A and (6)).

To dig deeper into the conflicting and unexpected finding observed upon silencing of FXII in our RNAi-based model, mice deficient for FXII (*F12*$^{-/-}$, see (2)) were subjected to the si*Serpinc1*/si*Proc* induced spontaneous VT model. Onset and incidence of the clinical phenotype that coincide with the spontaneous VT phenotype were similar in wild type (WT) and *F12*$^{-/-}$ mice (number of mice affected: WT: 9/11, *F12*$^{-/-}$: 9/11, *P*=1.0, figure 1B and figure S1A; Body weight loss: WT: -13.3% (-18.6;-8.7), *F12*$^{-/-}$: -11.7% (-15.8;-5.1), *P*=0.15, figure 1B). In addition, thrombotic coagulopathy and thrombi similarly formed in the large veins in the mandibular area of the head in WT and *F12*$^{-/-}$ mice (figure S1B and C). These observations indicate that genomic deletion of FXII (*F12*$^{-/-}$), like inhibition of *F12* by siRNA, does not confer protection in the mouse spontaneous VT model.

To clarify why siRNA-mediated *F12* silencing, in contrast to full *F12* genomic deficiency, accelerated and exacerbated the spontaneous thrombotic coagulopathy, normal female C57Black/6J mice were treated solely with siRNA against *F12* (the same si*F12* as used in (6), targeting exon 3 of the *F12* mRNA; here, si*F12*-A). As expected, si*F12*-A injected mice (without si*Serpinc1*/si*Proc* treatment) showed strong inhibition of hepatic *F12* transcript (compared to negative control siRNA i.e. siNEG injected mice; si*F12*-A: 11.3% (8.0-20.7), *P*<0.001, figure 1C). Surprisingly, plasma coagulation analysis revealed that for tissue factor (TF)-induced thrombin generation (TG) the peak height for si*F12*-A injected mice was significantly increased, compared to siNEG (siNEG; 68.9nM (65.9;74.7) and si*F12*-A: 86.8nM (82.3;95.0), *P*<0.001, figure 1D and S2A). This observation was reproduced in multiple independent experiments, with an increase of TF-induced TG peak height of approximately 20%, although it has been reported that TF-induced TG is independent on FXII activity (2).

The si*F12*-A related increase in TF-induced TG peak height was not observed for *F12*$^{-/-}$ mice, compared to WT controls (WT: 68.1nM (43.3;76.2) and *F12*$^{-/-}$: 63.3nM (62.0;66.7), *P*=0.08, figure 1D and S2A). As expected, ellagic acid (EA)-induced TG was clearly defective of plasma from *F12*$^{-/-}$ mice (figure 1E and S2B), while EA-induced TG of plasma from si*F12*-A mice was not altered despite FXII was reduced to 11% (figure 1C). Hence, the differential response for si*F12*-A treatment

and genetic *F12* deficiency in the spontaneous VT model (figure 1A and 1B) is paralleled by a differential response in plasma thrombin generation (Figure 1D and 1E), with si*F12*-A displaying an unexpected prothrombotic shift in both TG assays.

To exclude that the si*F12*-A related impact on TG is the result of si*F12*-A effects other than FXII-lowering i.e. off-target FXII-independent effects of the siRNA, two additional control siRNAs were designed. We obtained an siRNA similar to si*F12*-A, except for nucleotides 9-11 of the siRNA seed sequence which are replaced with their complementary base pairs: si*F12*-A$^{C9/11}$ (table S1). For the si*F12*-A$^{C9/11}$ mismatch control, false-positive si*F12*-A off-target effects will likely maintain their activity, whereas true positive si*F12*-A on-target effects will lose impact[7]. Moreover, an additional siRNA targeting *F12* mRNA was obtained (here, si*F12*-B), which targets exon 9 of the *F12* mRNA and inhibits *F12* mRNA more potent than si*F12*-A (figure S3). Both siRNAs were designed not to target other mRNA transcripts, which was confirmed by BLAST.

Next, mice were treated with siNEG, si*F12*-A, si*F12*-A$^{C9/11}$, or si*F12*-B (1:2 diluted with siNEG to match si*F12*-A inhibition of *F12*), and sacrificed after 3 days. Liver transcript analysis confirmed that si*F12*-A and (diluted) si*F12*-B treated mice had equal and strongly reduced *F12* hepatic transcript levels, while for control si*F12*-A$^{C9/11}$ injected mice *F12* transcript was not affected (compared to siNEG, si*F12*-A: 10.8% (3.9;19.2), si*F12*-A$^{C9/11}$: 132.8% (58.1;208.4), si*F12*-B: 10.0% (5.5;16.6), *P*<0.001, figure 1F). Remarkably, plasma from both si*F12*-A and si*F12*-A$^{C9/11}$ injected mice showed the typical prothrombotic shift in TG peak height upon TF activation (siNEG: 52.0nM (37.9;62.4), si*F12*-A: 63.3nM (51.1;74.0), si*F12*-A$^{C9/11}$: 61.0nM (47.3;68.8), *P*=0.012), figure 1G and S2C). Plasma from si*F12*-A$^{C9/11}$ injected animals responded aberrant when TG was initiated by EA, while despite low levels of FXII plasma from si*F12*-A treated mice was again not different from siNEG injected animals in TG peak height (siNEG: 47.5nM (31.3;68.6), si*F12*-A: 42.0nM (24.5;64.1), si*F12*-A$^{C9/11}$: 67.8nM (28.2;86.2), *P*=0.002, figure 1H and S2D). In contrast to si*F12*-A and similar to FXII-deficient plasma, si*F12*-B treatment did not influence the peak height in TF-induced plasma TG (siNEG: 52.0nM (37.9;62.4), si*F12*-B: 48.3nM (40.9;66.1), *P*=0.22, figure 1I and S2D), while EA-induced TG was strongly decreased in si*F12*-B plasma (siNEG: 47.5nM (31.3;68.6), si*F12*-B: 20.7nM (10.1;35.9), *P*<0.001, figure 1I and S2D). These results indicate that the observed prothrombotic shift in plasma TG upon si*F12*-A treatment cannot be attributed to FXII and appears a false-positive FXII-independent off-target effect.

The observed prolonged time to tail in the curves of TF-induced TG in si*F12*-A and si*F12*-A$^{C9/11}$ plasma (figure S2C) suggested that the plasma's ability to inhibit thrombin is affected by these siRNAs. In line with this, antithrombin levels were decreased for si*F12*-A and si*F12*-A$^{C9/11}$ mice only (compared to siNEG, si*F12*-A: 70.7% (21.7;85.4), si*F12*-A$^{C9/11}$: 74.4% (61.1;85.1), si*F12*-B: 101.9% (87.2;118.3), *P*<0.001, figure S4). Of note, in these mice a selection of liver-produced plasma

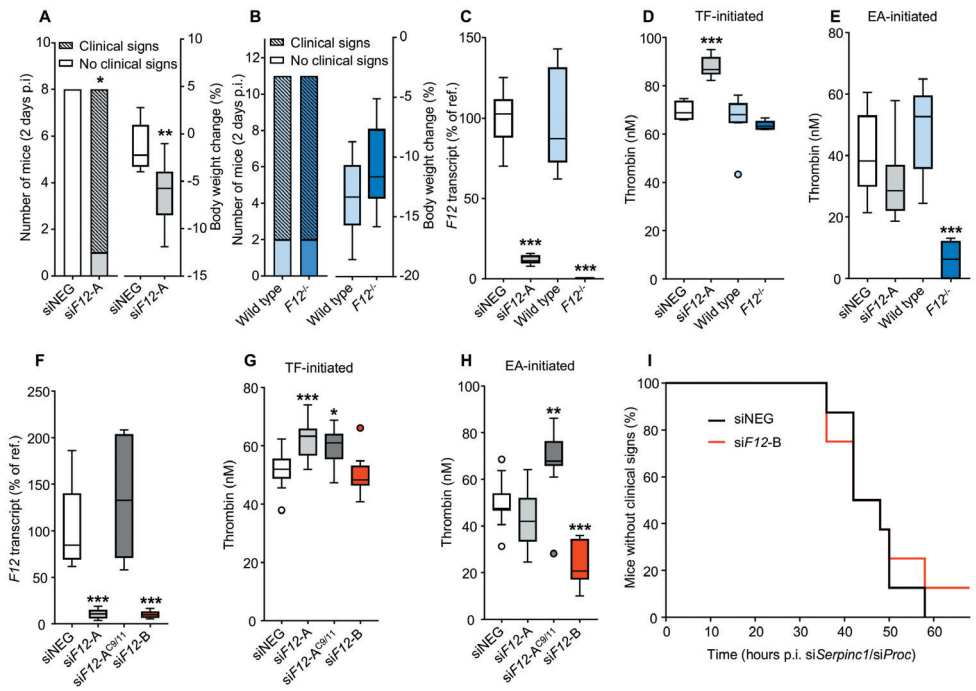

Figure 1 | Spontaneous venous thrombosis and plasma thrombin generation following silencing or deletion of factor XII. (A) Upon silencing of antithrombin and protein C (si*Serpinc1*/si*Proc* (si*Serpinc1*: cat. #S62673, si*Proc*: cat. #S72192; Ambion, Thermo Scientific, Carlsbad (CA), USA) 5.75 mg/siRNA/mouse, hepatocyte-delivered, using invivofectamine® 2.0 reagent (Thermo Scientific)), pretreatment of female C57Black/6J mice with si*F12*-A (5.75 mg siRNA/mouse, n=8, gray bars) resulted in earlier onset of spontaneous venous thrombosis, as compared to siNEG treatment (5.75 mg siRNA/mouse, n=8, white bars). This was represented as scoring of the clinical signs (left), and loss in body weight (right) 48 hours after si*Serpinc1*/si*Proc* injection (for complete methodology, see 6) (B). Upon silencing of antithrombin and protein C (si*Serpinc1*/si*Proc*, 5.75mg/siRNA/mouse), deficiency for FXII (female *F12*$^{-/-}$ mice, n=11, dark blue bars) did not affect onset (and severity, supplemental figure 2) of spontaneous venous thrombosis, as compared to wild type female C57Black/6J mice. (n=11, light blue bars). Both groups of mice had a similar (onset of) presentation of the clinical signs (left) and loss in body weight (right) 48 hours after si*Serpinc1*/si*Proc* injection (invivofectamine® 2.0 reagent, 5.75 mg/siRNA/mouse). As determined after sacrifice, silencing of hepatic *Serpinc1* and *Proc* was similar for wild type and *F12*$^{-/-}$ mice (*Serpinc1* transcript (normalized for wild type): *F12*$^{-/-}$ (median (range)): 1.13 (0.34;3.08), *P*=0.66, *Proc* transcript: *F12*$^{-/-}$: 0.90 (0.53;2.15), *P*=0.07). (C) C57Black/6J female mice were treated solely with siNEG (invivofectamine® 3.0 reagent, 1.2 mg siRNA/mouse, n=11, white bar) or si*F12*-A (1.2 mg siRNA/mouse, n=11, gray blue), or untreated and wild type (n=11, light blue bar) or deficient for *F12* (*F12*$^{-/-}$, n=12, dark blue bar). Subsequently, hepatic *F12* transcript was determined (forward primer: AATCCGTGCCTTAATGGGGG, reverse primer: TCATAGCAGGTCGCCCAAAG) with *Actb* as a house keeping gene8. (D) Plasma thrombin generation analysis was performed and thrombin peak heights determined (for thrombin generation curves, see supplemental figure 2A). Thrombin generation was initiated using 1pM tissue factor (final concentration) in 1:3 diluted mouse plasma (for a more detailed methodology, see 9). (E) Peak heights for plasma thrombin generation, initiated using 20 µg/ml ellagic acid (final concentration, for thrombin generation curves, see supplemental figure 2B). (F) Female C57Black/6J mice were treated solely

Figure 1 | Continued

with siNEG (n=11, white bars), si*F12*-A (n=11, grey bars), si*F12*-A$^{C9/11}$ (n=11, dark grey bars) and si*F12*-B (n=11, red bars, diluted 2:1 with siNEG) at a final dose of 1.2 mg siRNA/mouse, invivofectamine® 3.0 reagent). Mice were sacrificed 3 days after siRNA treatment and hepatic *F12* transcript levels were determined (*Actb* as a house keeping gene). (G and H) plasma thrombin generation were determined and peak heights displayed, initiated using (G) 1pM tissue factor (for thrombin generation curves, see supplemental figure 2C) and (H) 20µg/ml ellagic acid (for thrombin generation curves, see supplemental figure 2D. (I) Female C57Black/6J mice were injected with siNEG (n=8, black line) or si*F12*-B (n=8, red line) at a dose of 1.2 mg siRNA/mouse. One day after injection, mice were injected with siRNAs targeting antithrombin and protein C ((si*Serpinc1*/si*Proc*, 0.6 mg/siRNA/mouse, using invivofectamine 3.0). Mice were monitored and scored for clinical signs characteristic to the spontaneous VT. Once affected, mice were sacrificed. Liver *F12* transcript analysis revealed that hepatic *F12* transcript levels for the si*F12*-B mice was 9.8% (7.0;22.0) of siNEG. This lowering is comparable to residual *F12* transcript as previously observed for si*F12*-A (11.5% (6.9;20.9) of siNEG, experiment from panel A). Data are displayed in Whisker-Boxplots. *: *P*<0.05, **: *P*<0.01, ***: *P*<0.001 (compared to reference), based on Kruskal-Wallis 1 ANOVA test or Mann-Whitney U test.

protein levels (FXII-related coagulation factor XI and prekallikrein; inflammation-related proteins fibrinogen, serum amyloid A, and (the absence of) several liver enzymes) were considered normal and comparable, independent of siRNA treatment (data not shown).

In a setting of spontaneous VT, we found that si*F12*-B pre-treatment of mice did not rescue or exacerbate the thrombotic response (number of mice affected: siNEG: 8/8 and si*F12*-B: 7/8, *P*=1.0, figure 1I). This confirmed that the incidence and severity of spontaneous VT is not influenced by FXII; neither upon transient inhibition by siRNA (figure 1I) nor in permanent genetic deficiency (figure 1B).

We conclude that treatment of mice with the siRNA targeting exon 3 of the *F12* mRNA (used in (6), here si*F12*-A) induces unexpected additional changes in the plasma coagulant response, which are unrelated with FXII inhibition. The use of si*F12*-A$^{C9/11}$ let us conclude that this coagulant response of si*F12*-A is likely caused by the siRNA sequence outside the seed region. Although no indications, we cannot exclude that si*F12*-A false-positive effects go beyond coagulation. For siRNA studies, we now recommend to include C9/11 mismatch control siRNA. Although siNEG use controls for the procedure, delivery vehicles, chemistry, and activation of the RISC machinery, it appears to have insufficient ability to distinguish between true and false positives. Notwithstanding the findings, the data obtained with *F12*$^{-/-}$ and si*F12*-B injected mice let us conclude that FXII, in contrast to what has been reported for several experimental thrombosis models (2-4), does not contribute to venous thrombosis that follows spontaneously upon silencing of antithrombin and protein C in mice.

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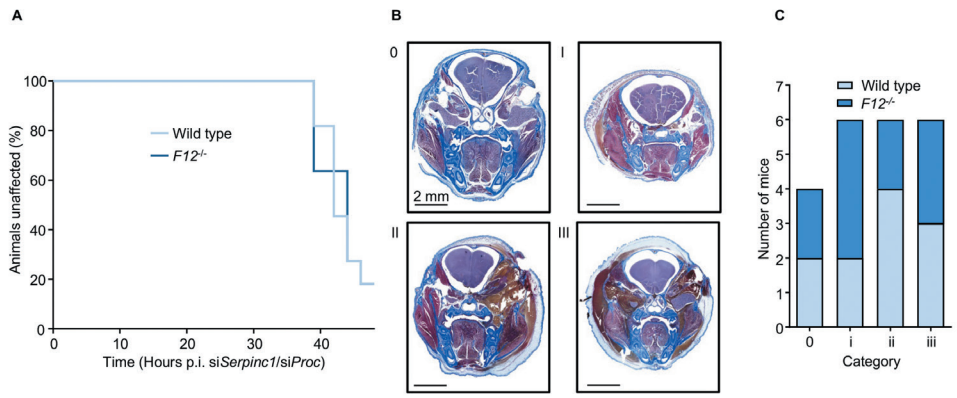
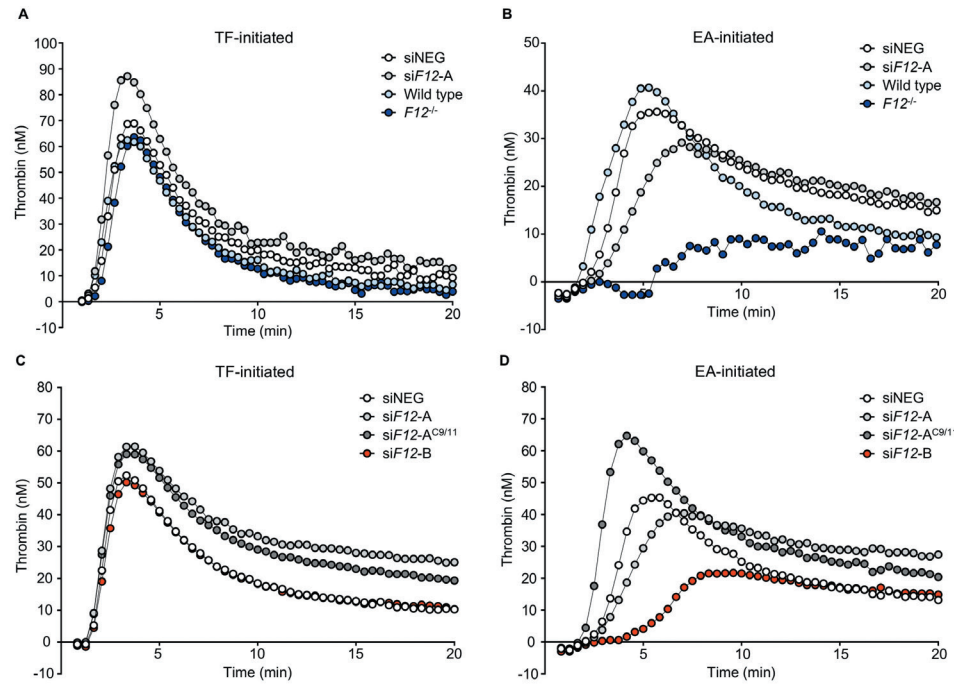
Supplemental table 1: siRNA sequences of si*F12*-A, si*F12*-A$^{C9/11}$, and si*F12*-B. Silencer® select siRNAs were purchased at Thermo Scientific (Waltham (MA), USA). An *in vitro* screen in mouse primary hepatocytes showed that si*F12*-A and si*F12*-B were both capable of lowering *F12* transcript equally (data not shown). Capital letters indicate complementary base pairs of the siRNA, while low case letters indicate the 3' overhang of the siRNA. For the sense-strand, two thymines are added. The seed region of all siRNA sequences are in bold. For the antisense strand, the two base pairs added are complementary with the target mRNA. The sequence of siNEG (catalog number 4390844) is not available.

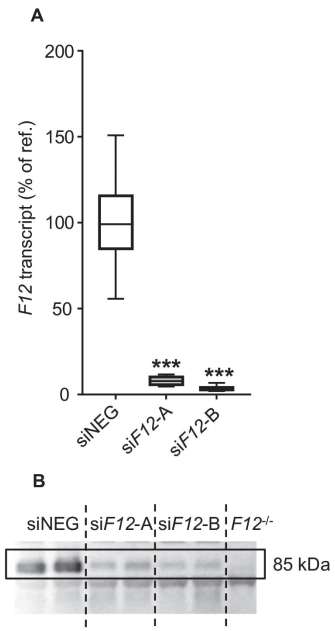
siRNA	siRNA ID	Sense (5'-3')	Antisense (5'-3')
si*F12*-A	s81735	CCACAAAU**GCA**UCCACAAAtt	UUUGUGGAU**GCA**UUUGUGGtg
si*F12*-A$^{C9/11}$	s535456	CCACAAAU**CGU**UCCACAAAtt	UUUGUGGAA**CGA**UUUGUGGtg
si*F12*-B	s81736	CACCUCUA**GUU**GUCCCUGAtt	UCAGGGACA**ACU**AGAGGUGca



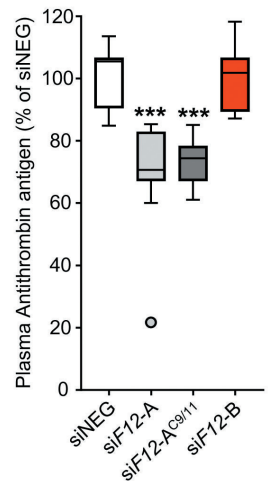
Supplemental figure 1 | Spontaneous VT progression is similar in wild type and *F12*$^{-/-}$ mice. (A) Female wild type (n=11) or *F12*$^{-/-}$ (n=11) mice were treated with siRNAs against antithrombin and protein C (si*Serpinc1*/si*Proc*, 5.75 mg/siRNA/mouse, invivofectamine® 2.0 reagent). From siRNA injection (t=0 hours), mice were inspected regularly for the presence of clinical signs of spontaneous VT. (B and C) Upon sacrifice and dissection, all mouse heads were formalin, fixed, decalcified, and embedded in paraffin. Coronal sections were made, similar as described in (6). Sections of all mice were stained using the Carstairs' methodology, and based on their thrombotic coagulopathy, severity of the phenotype was scored and subdivided in four categories (panel B): 0: no thrombotic coagulopathy, I: thrombotic coagulopathy in large veins, but no clear signs of vein rupture. Limited edema and/or bleeding, II: unilateral, thrombotic coagulopathy is clearly visible, with large thrombi associated with ruptured veins. Clear signs of edema and bleedings, III: Bilateral thrombotic coagulopathy, accompanied by edema and bleedings. (C) No significant differences in severity scores were found for the wild type and the *F12*$^{-/-}$ groups. Black bars represent 2 mm. Moreover, thrombi were scored based on structure and organization, but no differences were found. Edema, which is typical to the spontaneous VT phenotype, was quantified (measured by the thickness of the edemic dermis in coronal sections of the head, similar to (6)), but no significant differences were found. In addition, sections of mouse heads were stained for fibrin, but no differences in thrombus fibrin were observed.



Supplemental figure 2 | Plasma thrombin generation curves of mice treated with siRNA. (A and B) Plasma thrombin generation was induced with tissue factor (end concentration of 1pM, panel A and C) or ellagic acid (20µg/ml, panel B and D). Panel A: siNEG (n=11), si*F12*-A (n=11), wild type (n=8), and *F12*$^{-/-}$ (n=6). Panel B: siNEG (n=11), si*F12*-A (n=11), wild type (n=8) *F12*$^{-/-}$ (n=8). Plasma from siNEG and si*F12*-A treated mice were collected at a different time point than plasmas from wild type and *F12*$^{-/-}$ mice. (C and D) Plasma thrombin generation was induced with tissue factor (end concentration of 1pM, panel C) or ellagic acid (20µg/ml, panel D). Panel C and D: all groups, n=11. Values below 0 are the result of an apparent incorrect calibration of the thrombinoscope, but were included in the measurement.



Supplemental figure 3 | si*F12*-B is more potent than si*F12*-A in inhibiting *F12* liver transcript and FXII plasma protein. (A) *F12* liver transcript upon treatment with siNEG, si*F12*-A, or si*F12*-B (all, n=8). *F12* levels were corrected for the mean of the reference group (siNEG). Data are displayed in Whisker-Boxplots. ***: *P*<0.001 (compared to siNEG), based on Mann-Whitney U-test. (B) Western Blot for FXII of representative mouse plasma samples, treated with siNEG, si*F12*-A, and si*F12*-B. The black lined square highlights the 85kDa band representing the FXII protein. FXII was detected with the 3F7 antibody. As a negative control, *F12*$^{-/-}$ mouse plasma was included.



Supplemental figure 4 | si*F12*-A and si*F12*-A$^{C9/11}$ treated mice display decreased levels of plasma antithrombin. Antithrombin plasma levels were detected using the Antithrombin III murine ELISA kit (Stago, Leiden, The Netherlands). Antithrombin antigen levels were corrected for the mean of the reference group (siNEG). Data are displayed in Whisker-Boxplots. ***: *P*<0.001 (compared to siNEG), based on Mann-Whitney U-test.

5

Circulating Nucleosomes and Elastase α1-Antitrypsin Complexes and the Novel Thrombosis Susceptibility Locus *SLC44A2*

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Venous Thromboembolism (VTE) is the third most common cardiovascular cause of death, and genetic and environmental risk factors are involved in its pathophysiology (1, 2). Thus far, all established genetic VTE risk factors appeared to be directly related to blood coagulation (3). However, a recent meta-analysis of twelve genome wide association studies (GWASs), in which 7,507 VTE-affected individuals and 52,632 control subjects were included, discovered novel loci associated with VTE that cannot be related (yet) to the hemostatic system (4). One of these loci harbors the Solute carrier 44a2 (*SLC44A2*) gene, which encodes a choline transporter. The exonic SNP with the highest risk identified on *SLC44A2* was rs2288904, with the major allele (adenine (A) over guanine (G)) increasing the risk of VTE. Within the GWAS meta-analysis the odds ratio (OR) of this SNP for disease was 1.21 (P=2.75 $\times 10^{-15}$), and its association was confirmed in three separate replication studies (combined: 3,009 VTE-affected individuals and 2,586 control subjects). Interestingly, the association between *SLC44A2* and thrombosis was recently confirmed in a separate study (5).

The non-synonymous lead SNP rs2288904 at the *SLC44A2* locus has been causally related with transfusion-related acute lung injury (TRALI) (6). *SLC44A2*/rs2288904 (A or G) produces an amino acid substitution in the extracellular domain of the *SLC44A2* protein (Arg154Gln). This substitution can trigger (allo-)antibody formation in carriers of the minor (A) allele (during pregnancy and exposure to the major (G) allele variant). Subsequently, upon plasma transfusion these antibodies can trigger TRALI. Although the exact sequence of events is not entirely clear, the relation between rs2288904 and TRALI is well-established. Several clinical studies demonstrated that neutrophils play a key role in TRALI formation (7-9). Moreover, in experimental studies it has been shown that during TRALI neutrophils are activated and neutrophil extracellular traps (NETs) are formed, which mediate the inflammatory response. These TRALI symptoms can be treated with specific agents targeting NET components (10, 11).

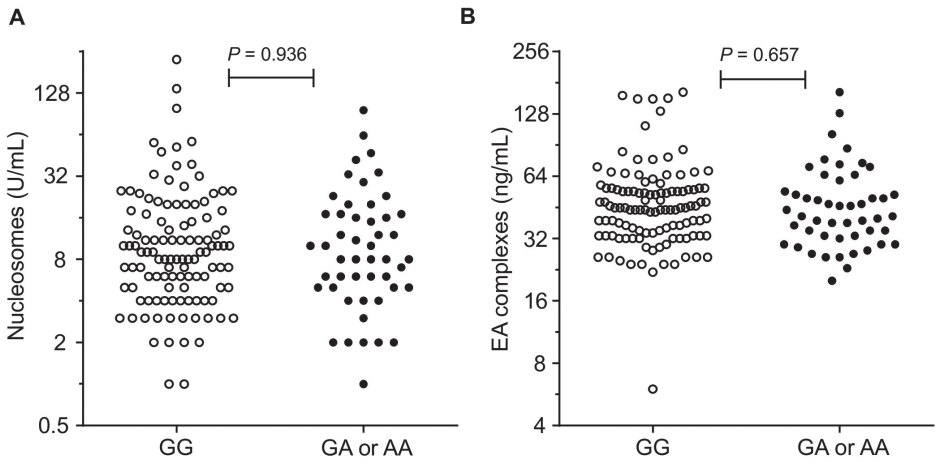
Interestingly, neutrophil activation and NET formation are also linked to VTE development in mouse models, with NET inhibition reducing thrombus formation (12). Moreover, there are claims NET markers are elevated in human VTE patients (13, 14). In the present study, the relation between neutrophil activation and NET formation in VTE and *SLC44A2*/rs2288904 is investigated. Systemic neutrophil activation was evidenced by the presence of circulating elastase α1-antitrypsin (EA) complexes (13). Nucleosome levels in plasma have been reported to be a suitable marker for NET formation in plasma in humans (15).

Because of the association of *SLC44A2*/rs2288904 with VTE and TRALI, and considering the involvement of NETs in both diseases, we hypothesized that *SLC44A2*/rs2288904 genotype modifies neutrophil activation and NET formation. Reduced neutrophil activation and NET formation might consequently be the cause of a protective effect of rs2288904-A (the minor allele) in

its association with VTE. To test this hypothesis, individuals from a previously characterized VTE study population, in whom levels of circulating nucleosomes and EA complexes have been determined, were genotyped for rs2288904 (13). In this cohort it was demonstrated that circulating nucleosomes and EA complexes were increased in deep vein thrombosis (DVT) patients compared to individuals with a suspicion of DVT in whom the diagnosis was ruled out (13). Of note, nucleosomes and EA complexes were measured in plasma obtained from blood without any additional (neutrophil) stimulants. Because *SLC44A2*/rs2288904-A was found to be protective for VTE, we assumed that either one or two copies of this allele are required to decrease the VTE risk. Therefore, we tested for a dominant effect of allele A on the plasma levels of nucleosomes and EA complexes.

We successfully genotyped 162 control subjects and 128 VTE patients from a total of 307 available DNA samples. Genotyping was performed using the Taqman SNP genotyping Assay (Life Technologies, Carlsbad (CA), USA), according to the manufacturers protocol. In the control population (no VTE upon examination), median nucleosomes and EA complex levels of the GG population were 9 U/mL (1-244 U/mL) and 45 ng/mL (6-163 ng/mL), respectively (figure 1A and B). In the combined GA and AA population, levels of circulating nucleosomes and EA complexes were not significantly increased (median nucleosomes: 8 U/mL (1-96 U/mL), P=0.936, and median EA complexes 41 ng/mL (20-163 ng/mL), P=0.657). Moreover, within the VTE patient population or in the two populations combined (290 individuals) no differences were found between the two genotypes (figure 1C: P=0.716 and P=0.413, nucleosomes and EA complexes, within VTE patients, respectively. P=0.575 and P=0.714, nucleosomes and EA complexes, respectively, within all individuals). There were no differences found in circulating nucleosomes and EA complexes between GA and AA individuals in all three groups i.e. the control population, the VTE patient population, and the two populations combined (see legends of figure 1). In conclusion, these results indicate that nucleosome and EA complex levels are not depending on *SLC44A2*/rs2288904 genotype.

Germain et al. demonstrated an overrepresentation of rs2288904 G over A in VTE individuals, both in the meta-analysis (OR: 1.19, (Confidence interval (CI): 1.12-1.26, α=0.05), P=1.07 x10^{-9}) as well as in replication studies (OR: 1.28, (CI: 1.16-1.40, α=0.05), P=2.64 x10^{-7}), illustrating the robustness of the observation (4). However, for the study group used here we were unable to reproduce this observation (OR: 0.86, (CI: 0.47-1.56, α=0.05), P=0.623). Whether this is due to the small samples size in the present study (5,595 vs. 307 individuals, in the replication study and our study population, respectively) or differences between the study populations (healthy controls vs. controls suspected of VTE, but in whom the diagnosis was ruled out, in the replication study and our study population, respectively) is subject to speculation. However, despite the lack of



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	Nucleosomes (U/mL)			EA complexes (ng/mL)		
Population (n)	GG	GA or AA	*P*-value	GG	GA or AA	*P*-value
Control (162)	9 (1-244)	8 (1-96)	0.936	45.0 (6-163)	41.0 (20-163)	0.657
VTE patients (128)	17 (2-323)	17 (1-190)	0.716	51.0 (2-220)	57.0 (25-259)	0.413
Total (290)	11 (1-323)	11.5 (1-190)	0.575	48.0 (2-220)	47.5 (20-259)	0.714

Figure 1 | Nucleosome and elastase α1-antitrypsin complex levels in plasma are not altered in individuals with a different rs2288904 allele. A) Plasma nucleosome levels in either GG carrying individuals (open circles, 113 individuals) or GA or AA carrying individuals (filled dots, 49 individuals). G: guanine, A: adenine. Nucleosome levels were measures as described (13). B) Plasma elastase α1-antitrypsin (EA) complex levels in plasma in either GG carrying individuals (major allele, open circles, 113 individuals) or GA or AA carrying individuals (minor allele, filled dots, 49 individuals). EA complex levels were measures as described (13). C) Overview of nucleosome and EA complex levels in plasma in the control population, the VTE patient population, and the total population (the previous two populations combined). No significant differences were found between GA and AA individuals. Control group (nucleosomes and EA complexes, respectively): 9 U/mL (1-96 U/mL) and 8 U\mL (5-63 U/mL), and 42.5 ng/mL (20-163 ng/mL) and 38 ng/mL (33-71 ng/mL), *P*=0.917). VTE patient population: 16.5 U/mL (1-190 U/mL) and 21 U\mL (3-57 U/mL), and 56 ng/mL (25-259 ng/mL) and 70 ng/mL (37-156 ng/mL). Two populations combined: 11 U/mL (1-190 U/mL) and 16.5 U\mL (3-63 U/mL), and 47.5 ng/mL (20-259 ng/mL) and 50 ng/mL (33-156 ng/mL). Number of individuals per group are as follows: Control; 162 individuals in total, 113 GG and 49 GA or AA (allele frequency G (A): 82.7% (17.3 %)). VTE patients; 128 individuals in total, 85 GG and 43 GA or AA (allele frequency G (A): 80.5% (19.5%)). Total; 290 individuals in total, 198 GG and 92 GA or AA (allele frequency G (A): 81.7% (18.3%)). For statistical analyses a Mann Whitney Rank-sum test was used. Data are presented as the median with the range (minimum and maximum, respectively).

association of *SLC44A2*/rs2288904 with VTE in the present study, our observation remains that nucleosome and EA complex levels are not influenced by rs2288904 genotype.

The range of nucleosomes and EA complexes is large within this specific cohort, both in controls (individuals with a suspicion of DVT in whom the diagnosis was ruled out) and in cases. This was previously attributed to comorbidities and other health conditions (e.g. malignancy or recent surgery), which were present in both groups (13). Here, we considered that variation in *SLC44A2* (on rs2288904) partly explains the observed the variation nucleosomes and EA complexes, however, that was not true for the present cohort.

To avoid selection bias, conclusions regarding the association of circulating nucleosomes and EA complexes and rs2288904 should only be drawn from observations in the control study population only. However, also for the total and the VTE patient population only, no effect of rs2288904 genotype on plasma levels of nucleosomes and EA complexes was found.

Although circulating nucleosomes and EA complexes are at present the best characterized biomarkers for NET formation and neutrophil activation, their lack of association with rs2288904 genotype does not exclude a role for *SLC44A2* in neutrophil activation (and possibly NET formation) in VTE. Studying ex vivo activation of isolated neutrophils from carriers of each genotype is an alternative strategy to study the link between *SLC44A2*/rs2288904, VTE, and neutrophil activation. Such studies can include impact of different genotypes on NET formation, interaction of neutrophils with endothelium, and other functional aspects of neutrophils. Alternative hypotheses explaining the association of *SLC44A2*/rs2288904 with VTE, apart from neutrophil activation, may involve von Willebrand Factor (VWF) or its choline transporter function. It has recently been described SLC44A2 directly interacts with VWF (16), a protein essential for hemostasis. Differences in SLC44A2, for instance due to variation at rs2288904, might alter the interaction between VWF and SLC44A2, possibly impacting VWF function and thereby VTE risk. Moreover, it has been shown SLC44A2 is expressed on endothelium, where it expresses an isoform involved in choline transport (17). Possibly, altered choline homeostasis affects the composition of the endothelial cell membrane and consequently the endothelium's (anti)coagulant surface. In conclusion, despite the negative outcome of the present study, the association between *SLC44A2* and VTE remains of interest for future studies.

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6

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).

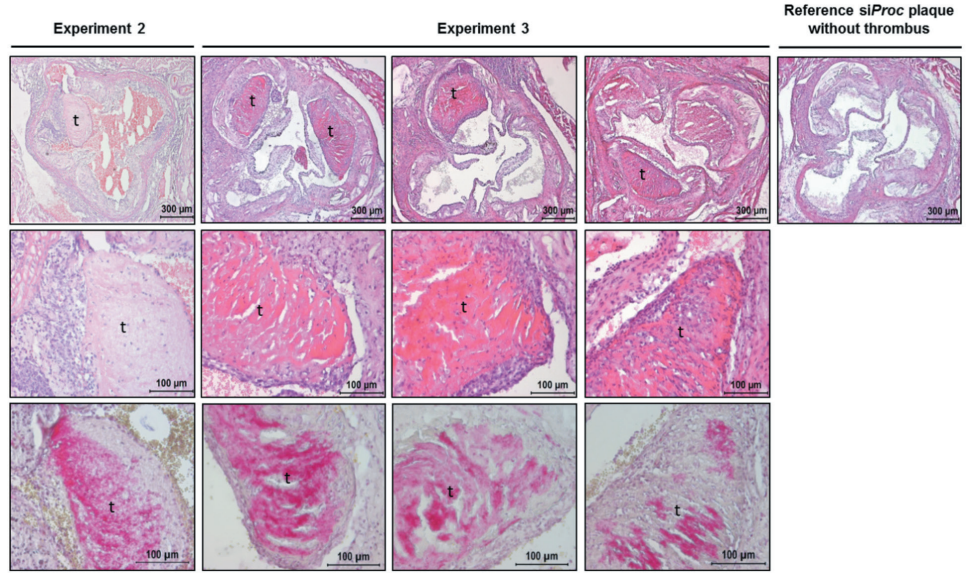

Figure 1 | siRNA-mediated inhibition of plasma protein C causes atherothrombosis in the aortic root in *Apoe*$^{-/-}$ mice. Microhistological images of aortic root atherothrombosis in *Apoe*$^{-/-}$ mice fed WTD for 8 weeks (1/4 mice; experiment 2) and 9 weeks (3/25 mice; experiment 3). Reference plaque 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

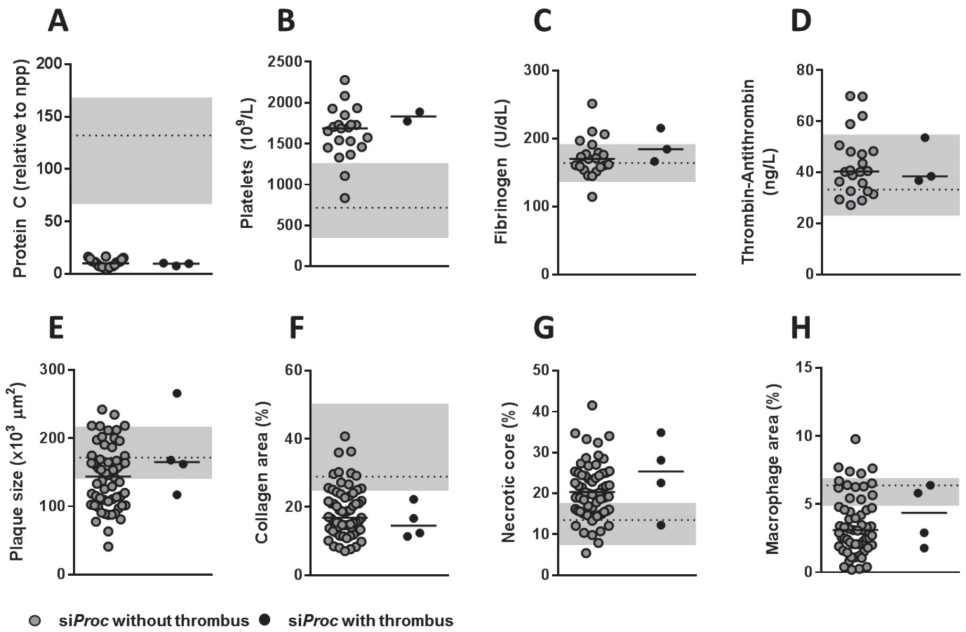

Figure 2 | Blood coagulation parameters and atherosclerotic plaque characteristics of si*Proc*-treated WTD-fed *Apoe*$^{-/-}$ mice without aortic root atherothrombosis are not significantly different compared to mice and plaques with atherothrombosis. (A) Residual plasma protein C levels, (B) Blood platelets, (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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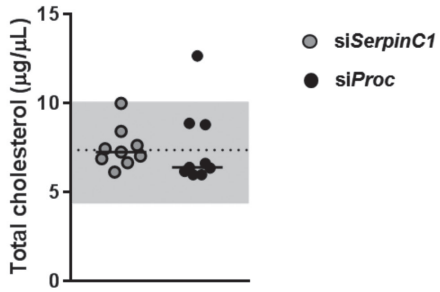
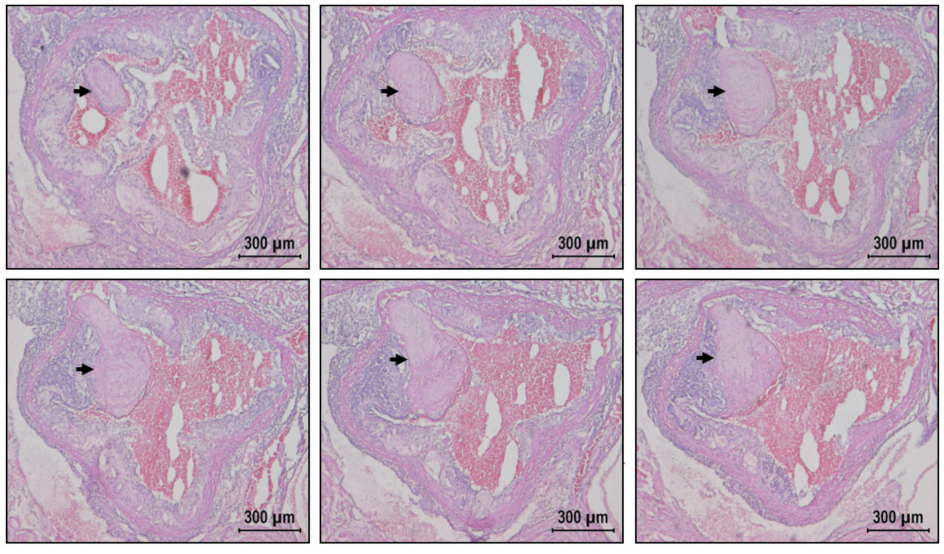
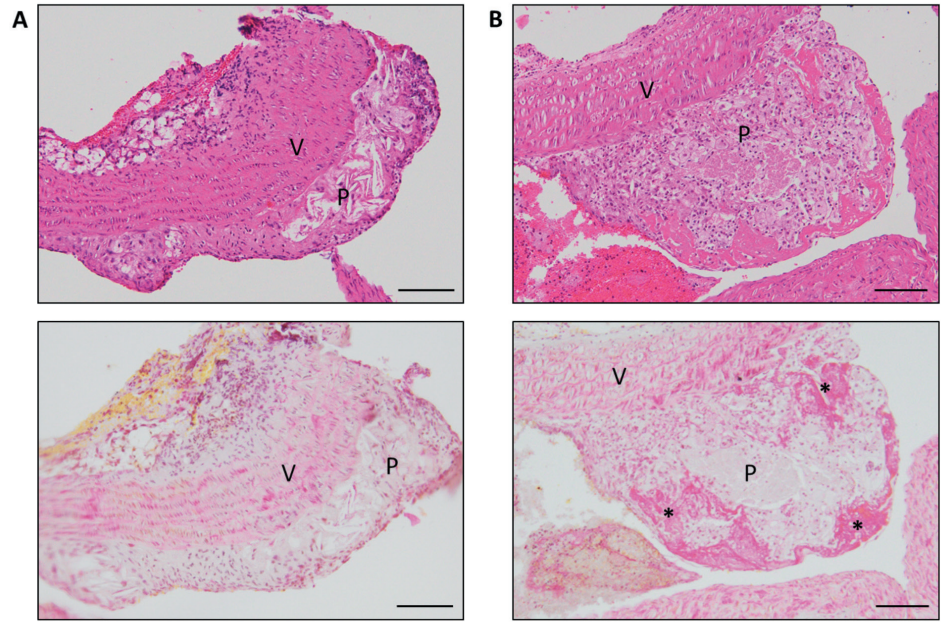


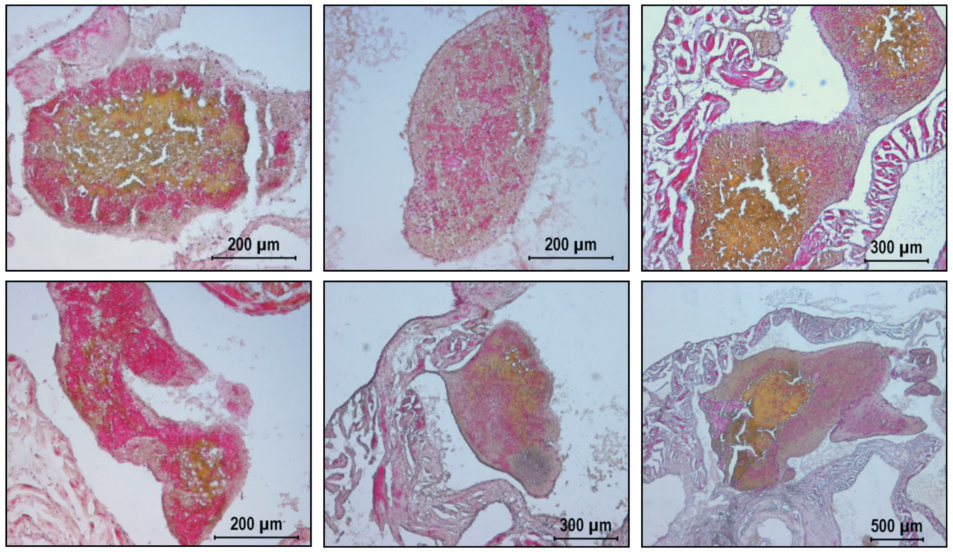
Supplemental figure 1 | Plasma total cholesterol before siRNA treatment of the mice in experiment 1. Lines represent medians. Grey zones in panels represent median and range of total cholesterol prior to siNEG treatment (n = 9).



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).

7

Predilection of Low Protein C-induced Spontaneous Atherothrombosis for the Right Coronary Sinus in Apolipoprotein E deficient mice

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Manuscript submitted for publication

ABSTRACT

Silencing of anticoagulant protein C using RNA interference (si*Proc*) evokes low incident but spontaneous atherothrombosis in the aortic root of apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice. The aims of the current study were 1) to analyze if plaque characteristics or circulating factors could be linked to atherothrombosis susceptibility, 2) to increase the incidence of atherothrombosis by transiently increasing blood pressure, and 3) to direct atherothrombosis to an additional predefined vascular site by applying a semi-constrictive collar around the carotid artery.

In the current study, si*Proc*-driven spontaneous atherothrombosis in the aortic root of *Apoe*$^{-/-}$ mice was reproduced and occurred at an incidence of 23% (9 out of 39 mice), while the incidence of collar-induced atherothrombosis in the carotid artery was 2.6% (1 out of 39 mice). Treatment with phenylephrine, to transiently increase blood pressure, did not increase atherothrombosis in the aortic root of the *Apoe*$^{-/-}$ mice nor in the carotid arteries with collars. Plaques in the aortic root with an associated thrombus were lower in collagen and macrophage content, and mice with atherothrombosis had significantly more circulating platelets. Plasma protein C, white blood cell counts, total cholesterol, fibrinogen, and serum amyloid A were not different amongst si*Proc* treated mice with or without thrombosis. Remarkably, our data revealed that thrombus formation preferably occurred on plaques in the right coronary sinus of the aortic root.

In conclusion, there is a predilection of low protein C-induced spontaneous atherothrombosis in *Apoe*$^{-/-}$ mice for the right coronary sinus, a process that is associated with an increase in platelets and plaques lower in collagen and macrophage content.

INTRODUCTION

Atherothrombosis, characterized by superimposed thrombus formation overlying a ruptured or eroded atherosclerotic lesion, is the cause of death for more than 14 million individuals per year worldwide (in 2015, World Health organization, www.who.int). Atherogenesis, which can eventually lead to atherothrombosis, has been studied extensively in genetically modified mouse models: When mice deficient for genes involved in cholesterol metabolism, such as apolipoprotein E knockout (*Apoe*$^{-/-}$) mice or low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice, are fed a cholesterol-rich diet, they rapidly develop atherosclerotic plaques (1-3). These mouse models have proven to be valuable for unravelling the pathophysiology of atherosclerosis (4, 5). However, unlike in humans, atherothrombosis does not occur spontaneously in mice, although some signs of rupture or erosion and intraplaque hemorrhage have been recorded (6-8).

Recently, we showed that transient (7 days) siRNA mediated lowering of the natural anticoagulant protein C (si*Proc*) in atherosclerotic *Apoe*$^{-/-}$ mice induced superimposed thrombus formation on atherosclerotic plaques in the aortic root (9). Although the incidence of this unique phenotype was low (1 out of 4 and 3 out of 25 in two independent experiments, cumulative incidence of 14%), our novel mouse model might be of use to better understand the pathophysiology of atherothrombosis. Moreover, a mouse model of atherothrombosis will be instrumental for the development of novel strategies for the treatment and prevention of atherothrombosis in humans.

Currently, however, the factors which determine when and where a thrombus develops on top of an atherosclerotic plaque are still largely unknown. We hypothesized that the incidence of atherothrombosis may increase when an additional risk factor for atherothrombosis is introduced on top of the impaired anticoagulant activity by knockdown of protein C. An important risk factor is for the development of atherothrombosis and consequent cardiovascular hospitalization is pulse pressure (10). A transient increase in blood pressure can be achieved in animal models by the administration of phenylephrine (PE), a selective α1-adrenergic receptor agonist. This will increase the strain on atherosclerotic plaques thereby stimulating the risk of plaque rupture and subsequent atherothrombosis. In line, PE administration was associated with increased rupture of p53-treated plaques in *Apoe*$^{-/-}$ mice (11). In the current study we therefore also applied PE treatment to increase the strain on the atherosclerotic plaques and stimulate atherothrombosis incidence in the si*Proc* treated *Apoe*$^{-/-}$ mice. Moreover, we attempted to direct atherothrombosis to the carotid artery by placing perivascular collars, a procedure which induces rapid atherogenesis proximal of the collars (12). This allowed us to investigate the impact of low protein C on plaques at an additional predefined vascular site and of a different origin than the plaques in the aortic root.

MATERIALS AND METHODS

Animals

Female C57BL/6 *Apoe*$^{-/-}$ mice (4-7 weeks old, n=57) were fed a Western-type diet (WTD containing 0.15% cholesterol; Special Diet Services, Sussex, UK). After 6 weeks on WTD, mice received silicone collars around both common carotid arteries, as previously described (11). After 10 weeks on WTD, synthetic siRNA (5 mg/kg; Ambion, Life Technologies, Carlsbad (CA), USA) targeting protein C (si*Proc*, cat. #S72192; n=40), or a control siRNA without a known target in mice (siNEG, cat. #4404020; n=17) was injected intravenously (IV). siRNA was complexed using invivofectamine 3.0, and each mouse received 2.5 nmol siRNA. Prior to siRNA injection, mouse groups were randomized based on weight and age (supplemental table 1). siNEG- and si*Proc*-treated mice received two intravenous injections of either phenylephrine (PE, 8 µg/kg IV, Sigma-Aldrich cat. #P6126-10G, Zwijndrecht, The Netherlands; si*Proc*: n=20, siNEG: n=8) or Phosphate-Buffered Saline (PBS; si*Proc*: n=20, siNEG: n=9) as control. PE or PBS injections were performed 4 and 6 days after the siRNA injection. In both the siNEG and the si*Proc* group, one mouse was removed from the experiment due to procedural errors, and thus not represented in the analyses. For an overview of the experimental procedure, see supplemental figure 1. The animal experimental protocol was in agreement with the national guidelines for animal experimentation and was approved by the Ethics Committee for Animal Experiments (Leiden University, Leiden, The Netherlands).

Tissue harvesting and preparation

Seven days after siRNA injection, mice were anesthetized with a subcutaneous injection of a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg). Citrated blood (300 µL) was drawn from the inferior vena cava, and plasma was collected as described previously (13). For whole blood cell and platelet analysis, an EDTA blood sample (approximately 50 µL) was collected through retro-orbital bleeding, upon blood withdrawal from the vena cava. Subsequently, mice were further exsanguinated and perfused *in situ* with PBS, after which organs were harvested. Tissues were either snap frozen (liver and spleen, stored at -80°C), or fixed for 24 hours in 3.7% neutral-buffered formalin (heart and carotid arteries, Formal-Fixx, Shandon Scientific Ltd, UK) and stored at room temperature for analysis.

Gene expression evaluation

Transcript of *Proc* was assessed by routinely quantitative PCR of hepatic tissue, with *Actb* as a reference housekeeping gene (14).

Histology

Hearts (aortic roots) and carotid arteries were formalin fixed and embedded in OCT compound (Optimum Cutting Temperature; Sakura Finetek Europe B.V., Alphen aan de Rijn, The Netherlands) before sectioning. Cryosections (10mm, 70mm interval) of the aortic root were prepared and mounted in a parallel series on 1% gelatin coated slides. Thrombi and clot structures in the aortic root were identified and scored blindly by two independent researchers on hematoxylin and eosin-stained sections. Plaque size per valve was determined after staining of the cyrosections for neutral lipids using Oil-Red-O and hematoxylin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Corresponding sections were stained using the Masson's Trichrome method (Sigma-Aldrich) for further morphometric analysis of the atherosclerotic plaques, including the percentage of collagen, necrotic core, and macrophage foam cells. All images were analyzed by blinded computer aided morphometric analysis using the Leica DM-RE microscope and LeicaQwin software (Leica Ltd, Cambridge, UK). Transverse serial cryosections (10mm; 80mm interval) of the carotid arteries (and the collars) were made. Sections were routinely stained with hematoxylin and eosin for a general assessment of histology, and were analyzed blindly by two independent researchers.

Blood and plasma analysis

Whole blood cell and platelet analysis were performed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Plasma cholesterol levels were measured by an enzymatic colorimetric assay (Roche diagnostics, Almere, The Netherlands). Protein C plasma levels were determined using an ELISA with a sheep anti-murine protein C polyclonal antibody (Haematologic Technologies Inc. Essex Junction (VT), USA), conjugated with Horseradish peroxidase with the EZ-Link Plus Activated Peroxidase Kit (Thermo Scientific, #31489, Waltham (MA), USA), as described previously (14). Fibrinogen and Serum Amyloid A plasma levels were determined using commercial ELISA kits (Affinity Biologicals, Ancaster (ON), Canada and R&D systems, Minneapolis (MN), USA, respectively).

Statistics

Statistical analysis was performed in Graphpad Instat (GraphPad Software, La Jolla (CA), USA). Data are presented with median and range. Statistical differences were assessed using the Mann-Whitney U test. A *P*-value <0.05 was considered significant. 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). A Chi-Square test was performed for the valve-incidence was done in SPSS (IBM SPSS Statistics for Windows, Version 23.0, Armonk (NY), USA).

RESULTS

At the time of sacrifice, all *Apoe*$^{-/-}$ mice treated with PBS or with phenylephrine (PE) and siRNA (both siNEG and si*Proc*) appeared healthy and did not show any abnormalities. Histological analysis of the aortic root of 10 weeks WTD-fed si*Proc* treated mice demonstrated the presence of atherosclerotic plaque-associated thrombi, with a similar size and composition as previously reported (9). In total, 9 out of 39 si*Proc* treated *Apoe*$^{-/-}$ mice (23.0%, CI (95%): 16.7-47.9) were categorized as atherothrombosis-positive in the aortic root (figure 1A-D and supplemental figure 2). In addition to the 9 mice with typical plaque-associated thrombi, 2 mice developed atypical clots in the aortic root. One atypical clot, although fibrin-positive and clearly associated with an atherosclerotic plaque, did not have the layered structure and did not contain leukocytes as the typical thrombi. These are histological arguments for being a fresh thrombus (supplemental figure 3A). The second atypical thrombus was associated with a valve, although present in the aortic sinus. Despite its unusual location, the thrombus was similar to the typical thrombi found on top of the atherosclerotic plaques. The valve with which it was associated contained a high number of leukocytes, but this was not unique for this specific heart valve (supplemental figure 3B). Both thrombi were not included in further analyses of the thrombus-positive group.

To investigate if an additional predefined site allowing atherothrombosis development could be created, perivascular collars were placed around both the carotid arteries of the *Apoe*$^{-/-}$ mice, 4 weeks prior to siRNA treatment. In line with previous studies (12, 15), histological analysis of the carotid arteries showed that collar placement resulted in maximum atherosclerotic plaque formation proximal to the collar. Although gross visual inspection revealed a large variation in plaque size and composition within groups, these were not different at the site of maximal stenosis between mice in the different groups (supplemental figure 4). In total, 1 out of 39 si*Proc* mice presented a structure identified as a thrombus in one of the carotid arteries (figure 1E-F). The thrombus was associated with the plaque, was rich in erythrocytes, and had a layered structure, comparable to the thrombi found in the aortic root. siNEG treated mice did not show events or structures identified as thrombus, neither in the aortic root and nor in the carotid arteries. It should however be noted that in our long term experience with collar placement around the carotid arteries of *Apoe*$^{-/-}$ mice, the occurrence of thrombosis as a complication of the procedure is not a rare event. Silencing of *Proc* induced atherothrombosis does seems to be restricted to the aortic root.

Livers and blood/plasma were analyzed to determine whether specific markers could be correlated to atherothrombosis susceptibility. As expected, si*Proc* treatment strongly decreased hepatic *Proc* transcript levels, compared to siNEG treated mice (remaining *Proc* transcript: mice without thrombi (-THR): 32.0% (18.0-63.7), mice with thrombi (+THR): 25.8% (18.8-40.0), $P<0.001$,

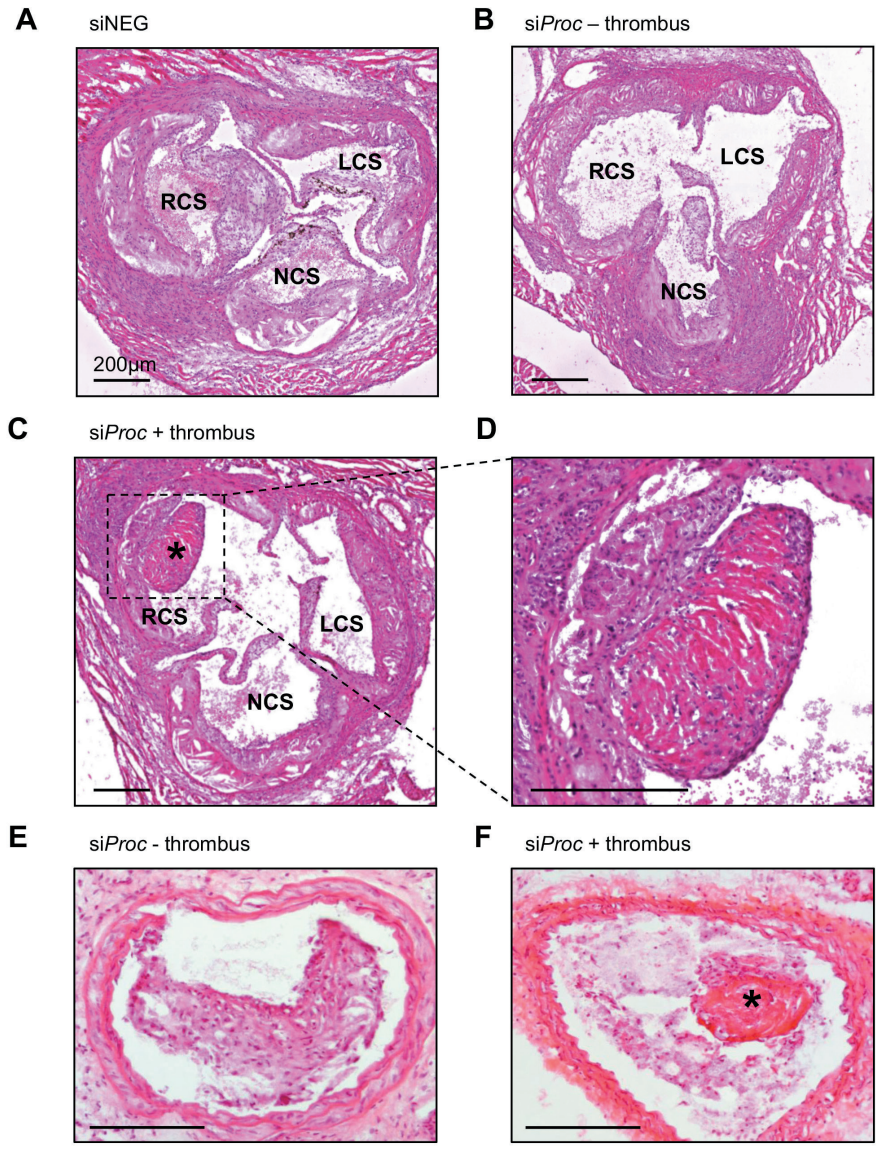


Figure 1 | Atherothrombosis in the aortic root of si*Proc* treated *Apoe*$^{-/-}$ mice. (A) Representative section of the aortic root of a mouse treated with siNEG. (B) Representative section of the aortic root of a mouse treated with si*Proc*, without a thrombus. (C and D) Representative section of the aortic root of a mouse treated with si*Proc*, with a thrombus associated with an atherosclerotic plaque. (E) Representative section of the common carotid artery containing an atherosclerotic lesion upon collar placement, derived from an si*Proc* treated mouse. (F) A thrombus-like structure associated with an atherosclerotic lesion in the common carotid artery. All sections are hematoxylin and eosin (HE) stained. NCS: Non-coronary sinus, RCS: Right coronary sinus, LCS: Left coronary sinus. *: Thrombus. Black bars represent 200 µm.
si*Proc* mice treated with PE had a similar incidence of atherothrombosis compared to PBS-treated si*Proc* mice (4 out of 19 vs. 5 out of 20 mice, respectively, supplemental figure 2).

figure 2A). Interestingly, the *Proc* transcript in si*Proc* treated mice was significantly lower in mice that presented with atherothrombosis, compared to the mice without atherothrombosis (*P*=0.013, figure 2A). Protein C plasma levels were also reduced in si*Proc* treated mice, as compared to the siNEG treated mice (remaining plasma protein C: -THR: 49.4% (18.4-118.7), +THR: 40.5% (33.2-50.3), *P*<0.001, figure 2B). However, in contrast to the *Proc* transcript data, protein C plasma levels did not reach statistical significance between si*Proc* treated mice with and without a thrombus (*P*=0.11, figure 2B).

Blood cell counts (total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, and red blood cells) were comparable between siNEG and si*Proc* treated mice with or without atherothrombosis (supplemental figure 6). Interestingly, platelet levels were significantly higher in si*Proc* treated mice compared to siNEG treated mice (-THR: 1280x10^9/L (728-1632), +THR: 1512x10^9/L (1004-1696), *P*<0.001, figure 2C). Within the si*Proc* treated groups, mice with atherothrombosis had even more platelets (*P*=0.043, figure 2C). To follow-up on this observation, we determined mRNA levels in the spleen of genes associated with platelet production. In agreement with the increased platelet counts, upon si*Proc* treatment, expression of some of these genes (*Gata1*, *Itga2b*, *Nfe2*, and *Fog1*) were increased as compared to siNEG treated mice (data not shown). si*Proc* treated mice with a thrombus showed an increased expression for *Fog1*, while for the other spleen-expressed genes expression levels were not different between mice with and without a thrombus.

Plasma analysis showed that total cholesterol levels were not significantly different between mice treated with siNEG or si*Proc,* with or without atherothrombosis (siNEG: 10.1 mg/mL (5.4-11.9), si*Proc* -THR: 9.5 mg/mL (6.4-12.8), si*Proc* +THR: 10.1 mg/mL (9.1-13.9), *P*=0.43, figure 2D). Besides total cholesterol, fibrinogen and serum amyloid A (SAA, an acute-phase mouse protein associated with inflammation (16)) were determined as possible systemic markers for atherothrombosis susceptibility. In line with fibrinogen being a cardiovascular risk factor in epidemiological studies (17), plasma fibrinogen levels in all groups of *Apoe*$^{-/-}$ mice were significantly higher than levels measured in normal pool plasma (NPP, pool of C57BL/6 mouse plasma). However, among siRNA treated *Apoe*$^{-/-}$ mice no differences were observed (siNEG: 363% (83-598), si*Proc* -THR: 394% (35-562), si*Proc* +THR: 463% (2.6-564), *P*=0.74, figure 2E). SAA levels were slightly increased in plasma of the *Apoe*$^{-/-}$ mice compared to NPP (<0.5 ng/mL), but no differences among the *Apoe*$^{-/-}$ groups were found (siNEG: 0.90 ng/mL (0.40-3.04), si*Proc* -THR: 1.29 ng/mL (0.41-3.12), si*Proc* +THR: 0.80 ng/mL (0.51-3.78), *P*=0.33, figure 2F). In line with the previous results, PE treatment did not influence plasma levels of any of the measured parameters (supplemental figure 7).

In our previous study, we found no significant correlation between plaque size and phenotype and the susceptibility to aortic root atherothrombosis, but with only 4 mice displaying athero-

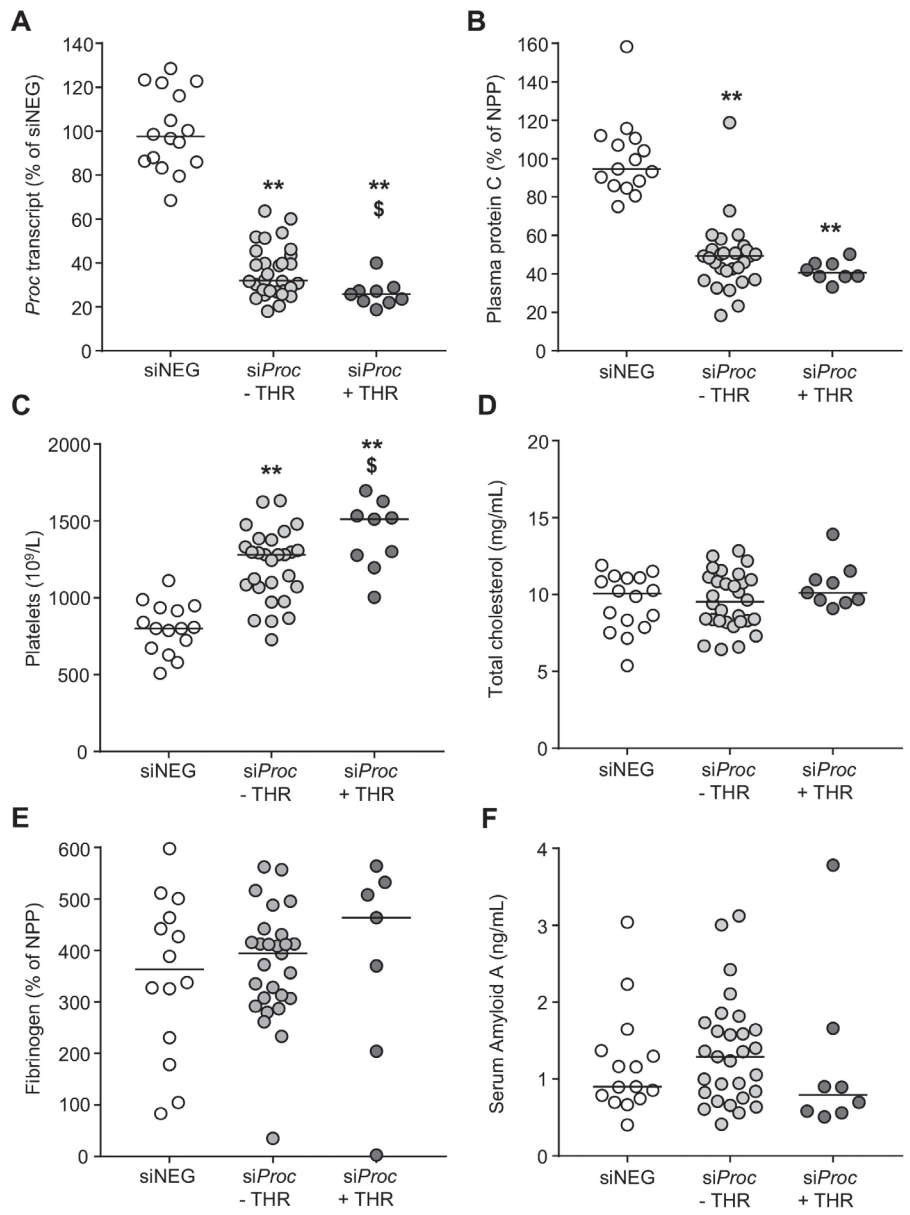


Figure 2 | Liver, blood, and plasma analysis of siNEG and si*Proc* treated *Apoe*$^{-/-}$ mice. (A) *Proc* transcript in the liver upon sacrifice (7 days after si*Proc* treatment) compared to the mean value of siNEG treated mice (100%), (B) Plasma protein C levels, measured by ELISA, compared to the mean value of siNEG treated (100%), (C) Total blood platelet levels, (D) Plasma total cholesterol levels, (E) Plasma fibrinogen levels, measured by ELISA, and expressed as % of normal pool plasma. We did not have any reason to exclude outliers with a low value (e.g. due to coagulation upon blood withdrawal), (F) Serum Amyloid A levels. Black bars indicate the median. *: *P*<0.05, **: *P*<0.01 for siNEG vs. si*Proc*. $: *P*<0.05 for si*Proc* –THR vs. si*Proc* +THR.

thrombosis the power in that study was low (9). Therefore, in the current study the atherosclerotic plaques in the aortic root were also morphometrically analyzed to assess whether plaque size and characteristics could be linked to the presence of atherothrombosis (for examples of plaque stainings, see figures 3A and 3B). Plaque analysis revealed that PE treatment, similar to the atherothrombosis incidence, did not influence any of the plaque characteristics (supplemental figure 5). For this reason, data of the mice with and without PE treatment were pooled to increase statistical power. In the si*Proc* groups, the plaque area (without thrombus (-THR): 191x10^3 µm^2 (55.0-888), +THR: 191x10^3 µm^2 (112-343), *P*=0.59, figure 3C), were not different. Interestingly, collagen and macrophage content were significantly lower in plaques with an associated thrombus (-THR: 13.4% (2.1-29.6), +THR: 8.3% (6.4-15.3), *P*=0.031, figure 3D, and -THR: 9.1% (0.8-34.9), +THR: 6.7% (4.5-13.7), *P*=0.028, figure 3E, respectively), unlike the necrotic core (-THR: 19.5% (4.3-80.2), +THR: 15.3% (7.8-67.0), *P*=0.30, figure 3F). No evidence was found for the occurrence of intraplaque hemorrhages or other abnormalities in the atherosclerotic plaques (with or without superimposed thrombi).

Since in our studies thrombi were exclusively found in the aortic root, we hypothesized that local hemodynamics, which can influence endothelial integrity, plays a role in atherothrombosis formation. For this reason, we re-examined the individual plaques formed in the different cusps within the aortic root (sinuses) for the presence of thrombi, by dividing the aortic root into the left coronary sinus (LCS), right coronary sinus (RCS), and non-coronary sinus (NCS, see (18-20) and figure 4A). We noticed that thrombi had a preference for the RCS, over the LCS and the NCS (figure 4B, *P*=0.018). When combining these data with the data obtained from our previous study, where we detected 4 atherothrombosis events for in total 29 si*Proc* injected *Apoe*$^{-/-}$ (9), the preference for the RCS was even more pronounced (*P*=0.008). To exclude that the predilection for the RCS was the consequence of differences in plaque size and composition, the plaque parameters were reanalyzed for the LCS, NCS, and RCS, but no significant differences among plaques were found (supplemental figure 8).

DISCUSSION

Transgenic mice with an impaired lipoprotein metabolism, such as *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, which are fed a cholesterol-rich diet rapidly develop advanced atherosclerosis (1, 2). However, in contrast to humans, these mice only develop atherothrombotic events upon additional (invasive) plaque damaging interventions (21). The cause for the absence of atherothrombosis is unknown, but it is likely that multiple species-specific factors such as hemodynamics, plaque composition, metabolism, and life span are involved (22). Recently, our group showed that transient lowering of the natural anticoagulant protein C in *Apoe*$^{-/-}$ mice resulted in spontaneous atherothrombosis

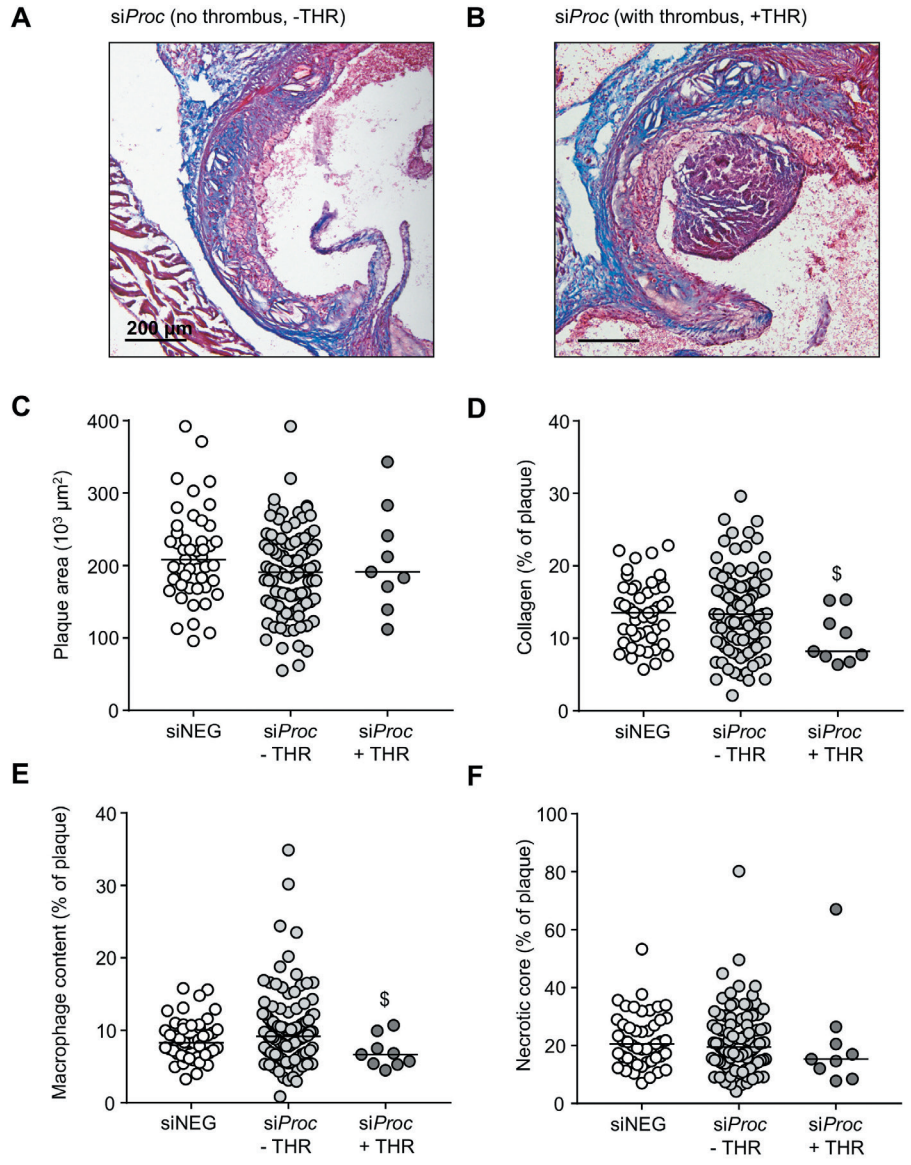
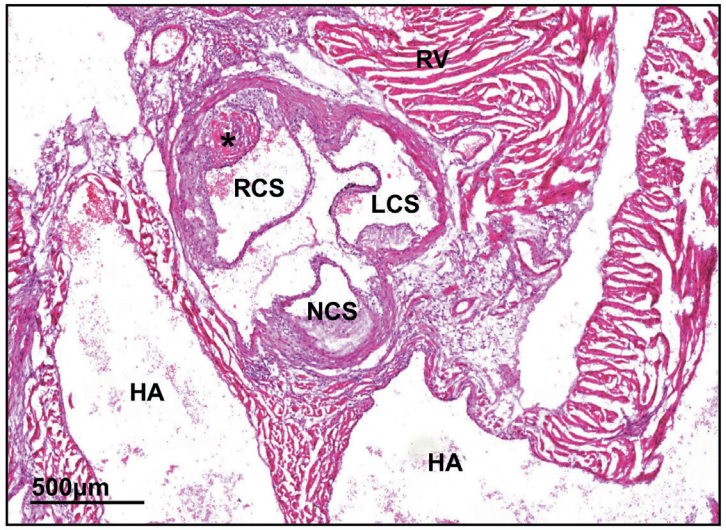


Figure 3 | Plaque composition of siNEG and si*Proc* treated *Apoe*$^{-/-}$ mice. (A-B) Representative photo-micrograph of a Masson's Trichrome stained plaque in the aortic root (RCS) of mice treated with si*Proc*, without (A) and with (B) a thrombus associated to a plaque from the aortic root. Blue areas were assessed as collagen-positive, non-stained areas as necrotic core, and pink nucleated areas as macrophage content/ foam cells. Black bars represent 200 µm. (C-F) Individual plaques from the si*Proc* treated group are divided in plaques without a thrombus (si*Proc* –THR) and plaques containing a thrombus (si*Proc* +THR). (A) Total plaque area, (B) Collagen, (C) Cellular content, (D) Necrotic core. For all panels, the indicated values represent an average measurement of three sections. Black bars indicate the median. $: *P*<0.05 for si*Proc* –THR vs. si*Proc* +THR.

A



B

Study	LCS	NCS	RCS
Ouweneel et al.	0	2	3
	LCS	NCS	RCS
Current	1	1	7*
	LCS	NCS	RCS
Overall	1	3	10**

Figure 4 | si*Proc* induced atherothrombosis in the different sinuses of the aortic root of *Apoe*$^{-/-}$ mice. (A) HE stained section of the aortic root and surrounding tissues. The non-coronary sinus (NCS) of the aortic root is covered by the two atria of the heart (atria are indicated with HA). Left and right of the NCS the left and right coronary sinuses (LCS and RCS, respectively) are present, which are both partly covered by a muscle-rich area, from which the largest part is the right ventricle (indicated with RV). *: thrombus. Black bar represents 500 µm. (B) Overview of three independent studies, where atherothrombosis in the aortic root occurred upon si*Proc* treatment. *: $P<0.05$, **: $P<0.01$.

at plaques formed in the sinuses of the aortic root (9). This implies that potent anticoagulation (co)contributes to absence of atherothrombosis in mice. In the current study we aimed to increase the insight into the factors which associate and possibly determine when and where a thrombus develops on top of an atherosclerotic plaque. For the first time we provide evidence that atherosclerotic plaques located in the right coronary sinus of the aortic root (RCS) are more prone to the development of atherothrombosis, compared to the other two sinuses (left and non-coronary sinuses, LCS and NCS, respectively). Interestingly, plaques with a thrombus were modestly but significantly lower in collagen and macrophage content. Therefore, we conclude that low protein C-induced atherothrombosis in mice is a low incident but reproducible phenomenon, and that it is determined by the location of the plaque, possibly in combination with differences in plaque composition.

Since Leonardo da Vinci in the beginning of the XVI century, scientists have been intrigued by the sophisticated and sustainable tissue and milieu of the aortic valves and root (23-25). The mouse aortic sinuses (in humans, sinuses of Valsalva), play a crucial role in regulating the closing mechanism of the valve leaflets (26). The oscillating shear stress due to the opening and closing of the valves render the cusps susceptible for the development of atherosclerotic plaques. A limited number of studies have distinguished between the three different sinuses and their plaques during their analyses. It has been reported that plaque size is reduced specifically in the RCS upon treatment with two different liver X receptor agonists (19). This means that differences in local anatomy of the aortic root do not only cause the flow to be different, but also can induce different expression patterns of proteins, possibly linked to coagulation. Moreover, Bentzon et al. suggested a causal relation between lesion formation and expansive remodeling of the aortic root sinuses, which was different when comparing plaques in the NCS, LCS, and RCS (27). The observed sinus preference for the development of atherothrombosis suggests that in mice local hemodynamics and wall shear stress are not only involved in atherogenesis (28, 29), but also in the development of atherothrombosis, as has been proposed for the human disease (30, 31). The identification of factors that drive the predilection for the RCS may provide novel clues on factors that drive atherothrombosis in mice and humans.

Plaques in the aortic root which were associated with a thrombus appeared to be lower in macrophage and collagen content. Although the measured values fell within the normal range, the difference was considered statistically different. Macrophages (foam cells) are crucially involved in atherogenesis and a high content of this cell population within a plaque has been associated with instability (32, 33). These observations make our findings in the current study, where in atherothrombotic plaques relatively fewer macrophages were found, unexpected. However, it should be noted that the plaques were advanced and that as a result the macrophage foam cell content was low. It has been described that lower collagen levels in atherosclerotic plaques

are associated with instability and risk of rupture (34). However, because of the current study setup, we cannot conclude whether the observed difference is a cause or consequence of the formation of a thrombus.

In the current study, si*Proc* treatment of *Apoe*$^{-/-}$ mice increased platelet levels, likely due to increased platelet production. A similar increase in platelet numbers was observed in the absence of an atherosclerotic phenotype in an independent experiment in which wild type female C57BL/6 mice on a normal chow diet were treated with an siRNA against *Proc* (data not shown). These findings suggest that the anticoagulant protein C directly or indirectly influences platelet production (and possibly consumption and/or clearance), a phenomenon that, to our knowledge, has not been described before. In human studies, it has been suggested that increased platelet levels are a risk factor for atherothrombotic events, making it tempting to speculate that the higher platelet levels upon si*Proc*-treatment contribute to the development of atherothrombosis in the aortic root (35, 36). For now, it is however not clear whether the increased platelets predict aortic root atherothrombosis susceptibility or whether they are a consequence of the event.

In conclusion, atherosclerotic plaques in the RCS are a predilection site for low protein C-induced spontaneous atherothrombosis in *Apoe*$^{-/-}$ mice, a process associated with vulnerable plaques displaying a lower macrophage and collagen content and vulnerable blood with augmented platelet counts.

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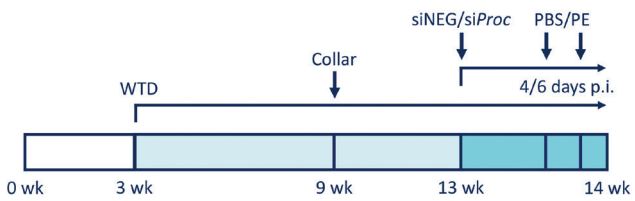
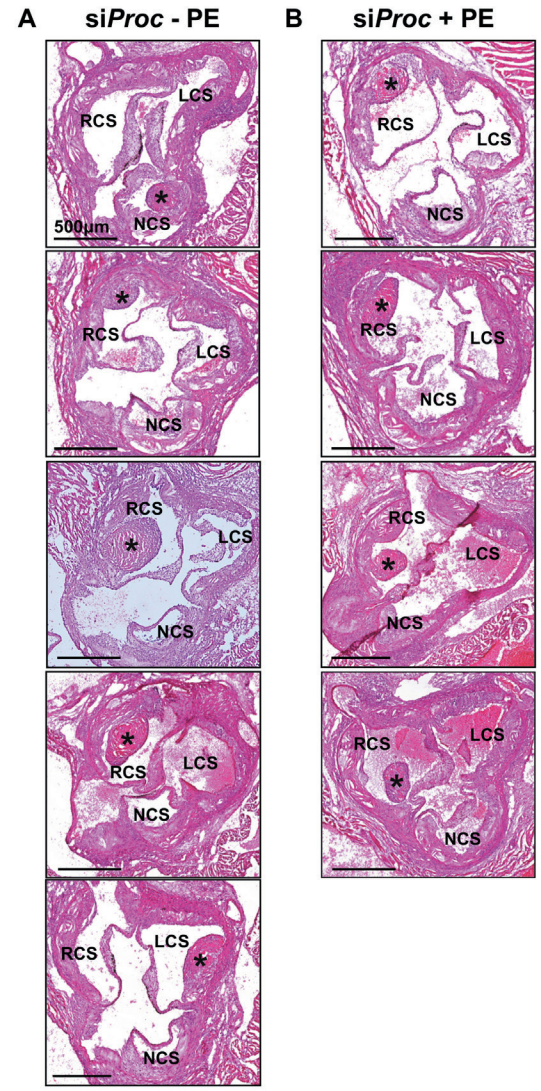
Supplemental table 1 | Group sizes, age, and body weight for all *Apoe*$^{-/-}$ mice. For age and body weight, the median and range is displayed of both parameters before siRNA injection. There were no significant differences between groups.

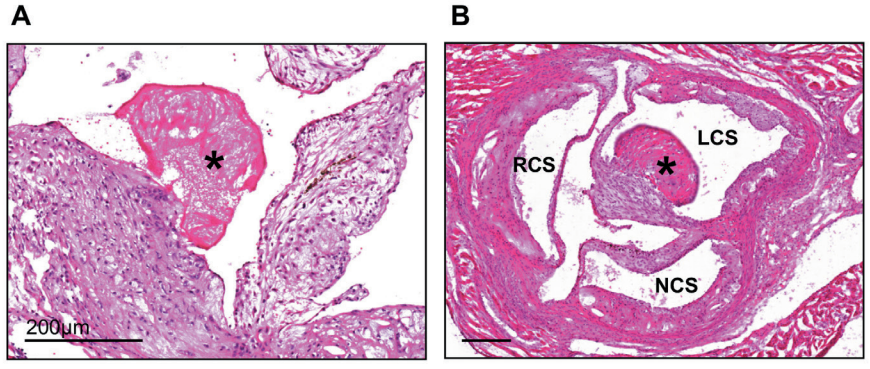
Group	Group size (n)	Age (weeks)	Body weight (g)
siNEG	17	14 (13-16)	22.5 (20.9-27.9)
si*Proc*	40	14 (13-16)	22.7 (20.1-25.7)
siNEG + PBS	9	14 (13-16)	22.4 (20.9-27.9)
siNEG + PE	8	15 (13-15)	22.9 (22.1-24.2)
si*Proc* + PBS	20	14 (13-16)	22.6 (20.3-24.9)
si*Proc* + PE	20	14 (13-16)	22.8 (20.1-25.7)



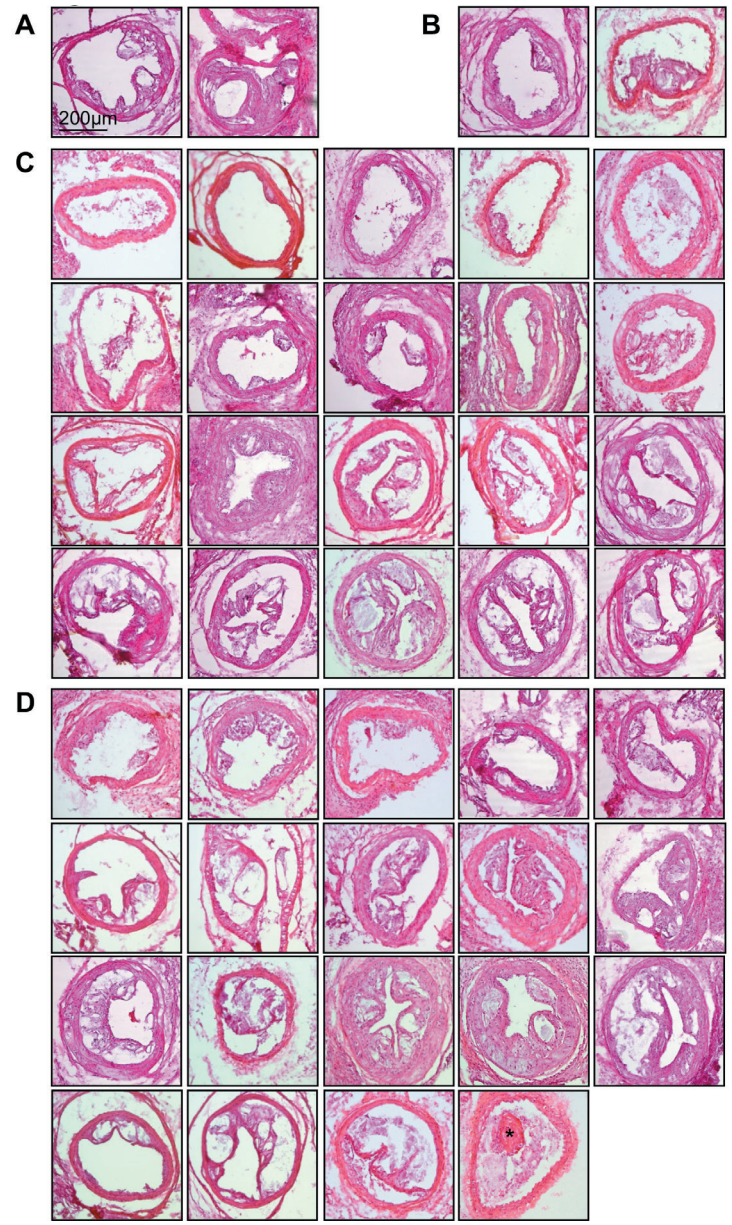
Supplemental figure 1 | Experimental setup. 4-7 weeks old female *Apoe*$^{-/-}$ mice were fed a Western type diet (WTD) for 10 weeks. After 6 weeks of WTD, perivascular collars were placed around the common carotid arteries (left and right). After 10 weeks of WTD, mice were treated with either siNEG or si*Proc*. 4 and 6 days after siRNA treatment (post-injection, p.i.), mice were injected with PBS control or phenylephrine (PE). One week after siRNA treatment (age: 15-18 weeks), mice were sacrificed.



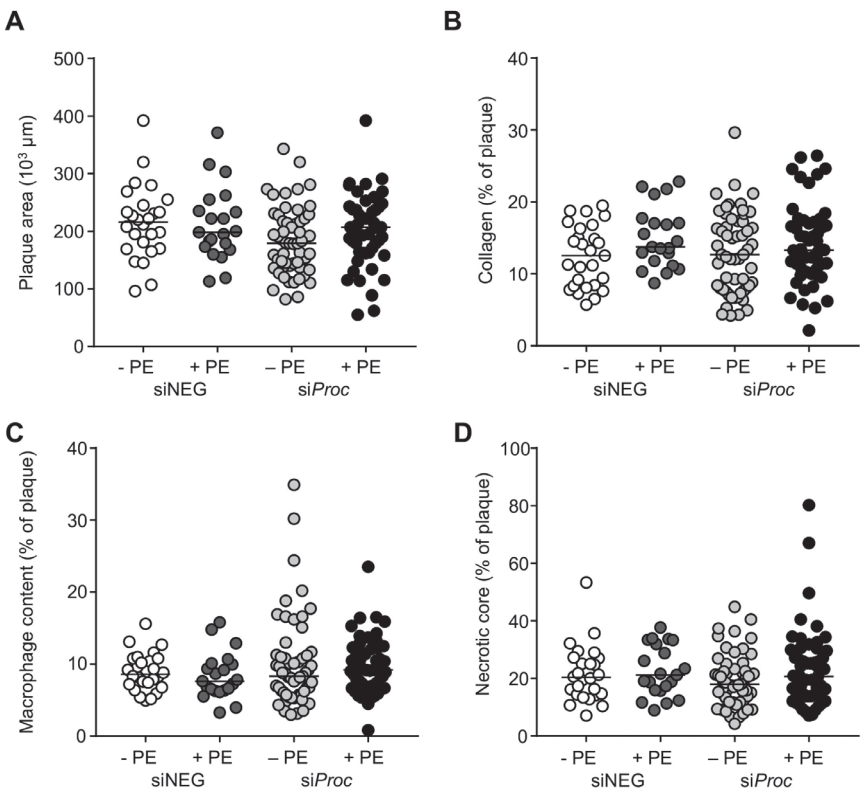
Supplemental figure 2 | Overview of all si*Proc* associated atherothrombotic events in the aortic root of *Apoe*$^{-/-}$ mice. (A) In 5 mice in the si*Proc* group without phenylephrine (- PE) treatment atherothrombosis in the aortic root was observed. (B) In 4 mice in the si*Proc* group with phenylephrine (- PE) treatment atherothrombosis in the aortic root was observed. All sections were HE stained. Black bars represent 500 µm. NCS: Non-coronary sinus, RCS: Right coronary sinus, LCS: Left coronary sinus. *: Thrombus.



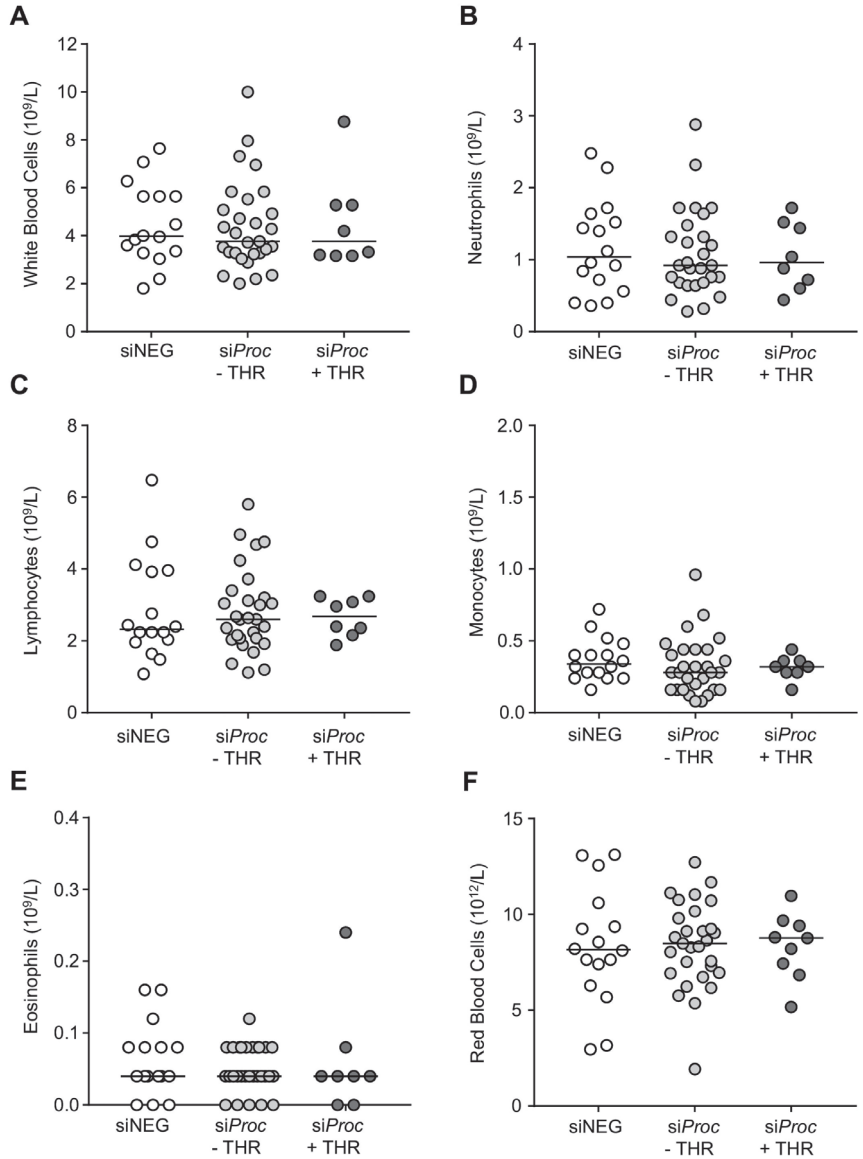
Supplemental figure 3 | Atypical thrombi in the aortic root of si*Proc* treated *Apoe*$^{-/-}$ mice. (A) Atypical thrombus which associated with an atherosclerotic plaque. In contrast to other thrombi, it did not have a layered structure and did not contain leukocytes. (B) Atypical thrombus associated with a valve within a sinus of the aortic root, and not with the atherosclerotic plaque. The composition of the thrombus was similar to other thrombi. Both mice were included in the analysis in the si*Proc* group without a thrombus (-THR). Black bars represent 200 µm. NCS: Non-coronary sinus, RCS: Right coronary sinus, LCS: Left coronary sinus. *: Thrombus.



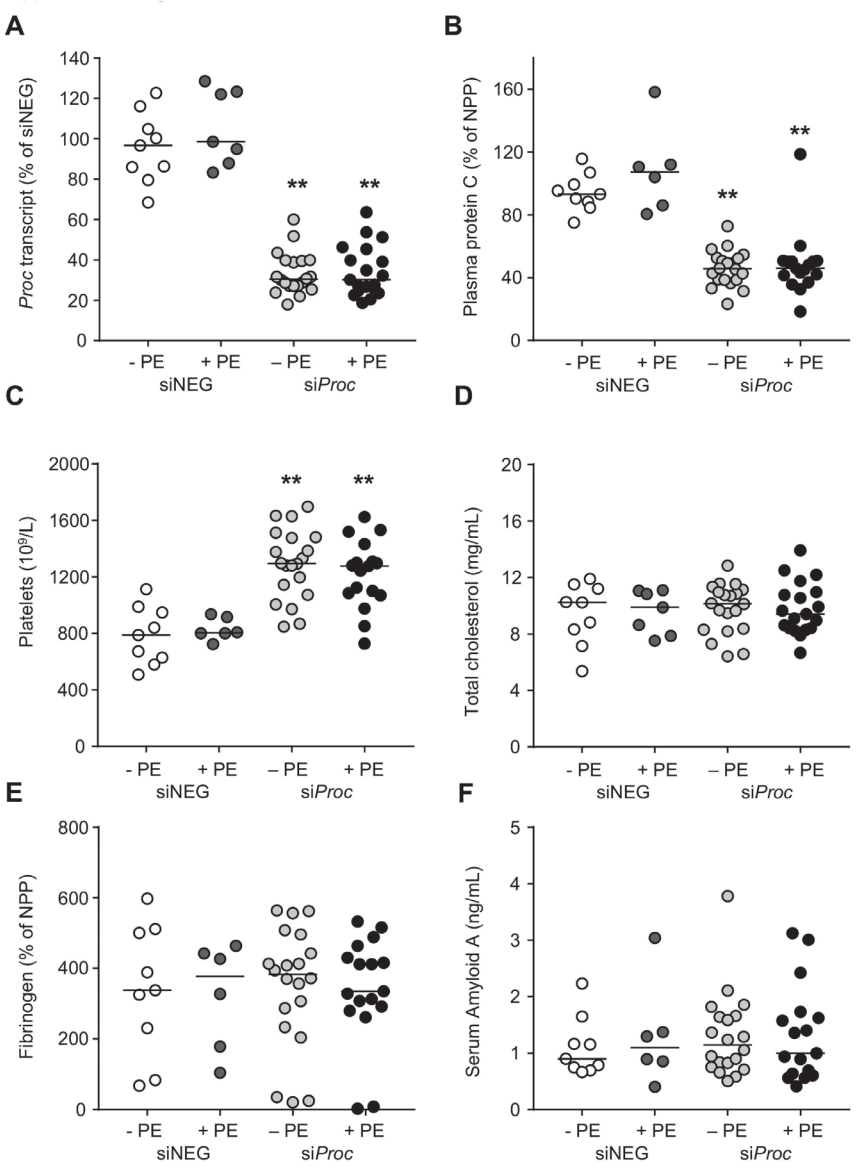
Supplemental figure 4 | Thrombosis-associated with atherosclerotic plaques upon collar placement in the common carotid arteries. Overview of sectioned common carotid arteries, at the site of maximal stenosis. (A) Two representative sections of common carotid arteries of mice treated with siNEG (- PE), (B) Two representative sections of common carotid arteries of mice treated with siNEG (+ PE), (C) Sections of common carotid arteries of mice treated with si*Proc* (- PE), (D) Sections of common carotid arteries of mice treated with si*Proc* (+ PE). All sections were HE stained. Black bar represents 200 µm. *: Thrombus (for enlargement, see figure 1F).



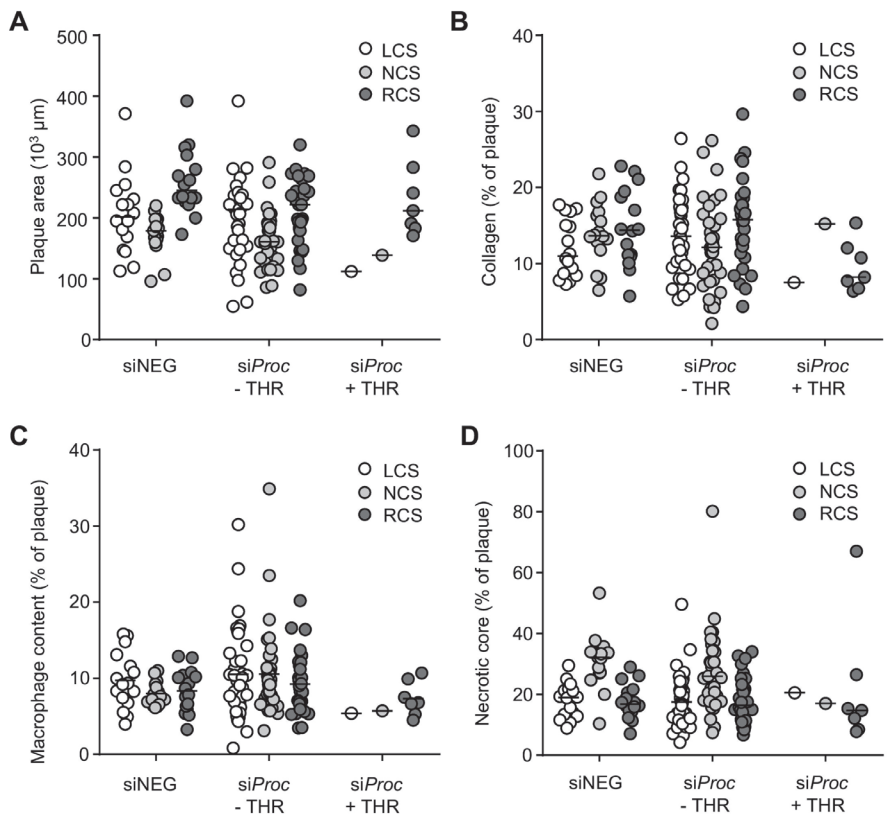
Supplemental figure 5 | Atherosclerotic plaque composition of siNEG and si*Proc* treated *Apoe*$^{-/-}$ mice, with and without phenylephrine (PE). (A) Total plaque area, (B) Collagen, (C) Macrophage content, (D) Necrotic core. For all panels, the indicated values represent an average measurement of three sections. Black bars indicate the median.



Supplemental figure 6 | Blood cell populations of siNEG and si*Proc* treated *Apoe*$^{-/-}$ mice. Blood cell numbers from the si*Proc* treated group is divided in plaques without a thrombus (si*Proc* – THR) and plaques containing a thrombus (si*Proc* +THR). (A) Total white blood cells, (B) Neutrophils, (C) Lymphocytes, (D) Monocytes, (E) Eosinophils, and (F) Red blood cells are displayed. Black bars indicate the median.



Supplemental figure 7 | Liver, blood and plasma analysis of siNEG and si*Proc* treated *Apoe*$^{-/-}$ mice, with and without phenylephrine (PE). (A) *Proc* transcript in the liver upon sacrifice (7 days after si*Proc* treatment), compared to the mean value of siNEG treated (100%), (B) Plasma protein C levels., compared to the mean value of siNEG treated (100%), (C) Whole blood platelet levels, (D) Plasma total cholesterol levels, (E) Plasma fibrinogen levels, measured by ELISA, and expressed as % of normal pool plasma. We did not have any reason to exclude outliers with a low value (e.g. due to coagulation upon blood withdrawal), (F) Serum Amyloid A levels. Black bars indicate the median. – PE: PBS control treated mice, + PE: Phenylephrine treated mice **: *P*<0.01 for siNEG vs. si*Proc*.



Supplemental figure 8 | Composition of atherosclerotic plaques formed in the LCS, NCS, and RCS. (A) Total plaque area, (B) Collagen, (C) Macrophage content, (D) Necrotic core. LCS: Left coronary sinus, NCS: Non-coronary sinus, RCS: Right-coronary sinus. For all panels, the indicated values represent an average measurement of three sections. Black bars indicate the median.

8

General Discussion and Perspectives

Marco Heestermans

The aim of the research which is summarized in this thesis was to gain further insight into venous and arterial thrombosis pathophysiology. To reach this goal, we induced spontaneous thrombotic phenotypes in mice, by introducing a transient imbalance in the coagulation profile. Introducing a transient imbalance circumvented the use of invasive methods to introduce thrombosis, such as by damaging or inducing stasis of blood vessels. This allowed us to study the pathophysiology of thrombosis in a setting which mimics human scenarios for thrombosis, where an imbalance (e.g. genetic profile or obesity) is a major contributor. In the current chapter, the most relevant findings are discussed in more detail and the perspectives for further research are outlined.

PLATELETS, NEUTROPHILS, AND FXII IN SPONTANEOUS VENOUS THROMBOSIS

Recently, platelets, immune cells, and coagulation factor XII (FXII) have been introduced as vital players in experimental venous thrombosis (VT) pathophysiology. These three components are involved in the initiation of coagulation via the contact activation pathway in a mouse model for VT, which is provoked by partial ligation of the inferior vena cava to induce stasis of the blood vessel (1). In chapters 3 and 4 of this thesis, we induced VT in mice by the acute lowering of natural anticoagulants antithrombin and protein C. Here, platelets are rate-limiting for the development of spontaneous VT. Conversely, neutrophils and FXII were not rate-limiting, and the transient lowering of these components did not result in the reduction of VT onset.

Platelets have received much interest in arterial thrombosis research because of their crucial role in the formation of thrombi on atherosclerotic plaques. In contrast, platelets in relation to VT have received lesser attention because for this form of thrombosis secondary hemostasis (e.g. fibrin formation) is considered more important. This concept is translated towards the clinic, where patients at risk for arterial thrombosis are treated with platelet inhibitors, while patients at risk for VT are treated with anticoagulants (2-4).

The importance of coagulation in VT pathophysiology is not debatable, but recent data obtained in preclinical models of VT point towards a more crucial role for platelets than thought previously (5, 6). In 2017, a study was published in which mice that carry lethal prothrombotic mutations were subjected to a mutagenesis screen to improve their survival. One novel modifier gene to rescue the spontaneous lethal phenotype was identified; A loss-of-function mutation in the *Actr2* gene led to the rescue of the lethal thrombotic phenotype (7). *Actr2* is part of a complex which is required for actin polymerization during platelet shape change (8). Hence, using this unbiased approach to discover novel VT genes a protein associated with normal platelet function was identified to be essential for development of the disease in mice. In line with these results, we

and others found that platelets are vital for experimental VT development (1, 9-11). Remarkably, compared to e.g. the inferior vena cava stenosis model of von Brühl et al., the role of platelets in VT pathogenesis in our spontaneous VT model was slightly different. We showed that platelets were involved in the progression of the disease, while others reported that platelets are pivotal for initiation of VT (1, 9). The reason for this discrepancy is currently unknown, although it is clear that the nature of experimental VT is different in both models. Spontaneous VT induced by the acute depletion of natural anticoagulants antithrombin and protein C in mice may represent a situation of VT in humans where manifestation of the disease is associated with an imbalance of coagulation. Other mouse models where experimental VT induced by vascular damage or stasis may represent a situation where human VT is triggered by something else than an imbalance of coagulation, such as by surgery or immobility.

Preclinical data show that pharmacological global inhibition of platelets can prevent VT. Since specific inhibitors of platelets, such as acetylsalicylic acid, $P2Y_1$ inhibitors, and indobufen, are FDA-approved and widely-available drugs, it would be interesting to test these specific platelet inhibitors in different mouse models for VT. Because of the suggested role of platelets in initiation or progression of the disease, different platelet inhibitors may or may not be effective in inhibiting experimental VT induced by stasis, vascular damage, or an imbalance of coagulation. This may be translated towards the clinic, where VT patients can receive more personalized antiplatelet therapy based on their history or risk of VT.

Based on preclinical studies, the leukocyte population of neutrophils is an interesting candidate to serve as a therapeutic target for VT treatment (12-14). It has been shown that deficiency or inhibition of neutrophils can prevent experimental VT, and this does not coincide with bleeding (15, 16). Bleeding as a side effect of anticoagulant treatment remains to be the major problem with the current generation of therapeutic anticoagulants. In contrast to previous studies using different mouse models, in our preclinical study depletion of neutrophils from the circulation of mice using a Ly6G-specific antibody did not result in a different onset and progression of spontaneous VT. Hence, the proposed crucial role for neutrophils in experimental VT pathophysiology does not hold true for conditions where endothelial activation and/or vessel wall inflammation are considered absent (i.e. not triggered by surgical handlings).

Neutrophils are involved in immunity and injury repair. In the context of VT, a specialized cell death program where so-called neutrophil extracellular traps (NETs) are excreted is particularly interesting. Besides the role of NETs in targeting certain pathogens, it has been proposed that NETs can initiate coagulation. For clinical purposes, neutrophil or NETs markers are currently tested for their usefulness as a biomarker for diseases such as VT (17, 18). However, the pleiotropic effect of targeting neutrophils or NETs to prevent VT can be disadvantageous for a patient.

Inhibition or deregulation of neutrophil function can cause a disturbed immunological profile in patients, which may lead to e.g. sepsis. For now, a suitable drug target which can exclusively prevent VT without interfering with the neutrophil primary physiological function in inflammation has yet to be identified.

The final player in VT pathophysiology which we studied in chapter 3 and 4 of this thesis is coagulation factor XII (FXII). Similar to neutrophils, we concluded that FXII was not rate-limiting in spontaneous VT in mice. This result contradicts reports in other preclinical VT models, where absence or inhibition of FXII coincides with thromboprotection without bleeding (1, 19, 20). Indeed, in the human population individuals deficient in FXII do not suffer from bleeding, unlike patients deficient in other coagulation factors (21, 22). However, FXII deficiency has never been convincingly associated with protection from VT in humans (12, 23). Remarkably, pulmonary embolism contributed to the death of railroad worker John Hageman, the first individual identified to be deficient in FXII (24). This may be partly explained by FXII's involvement in several VT-related processes besides initiation of coagulation. FXII is known to be involved in fibrinolysis, complement activation, and the kallikrein-kinin pathway (25).

The discrepancy between the lack of association of VT and FXII within the human population and the vital role for FXII in multiple preclinical models for VT might be explained by the mechanism via which FXII initiates coagulation. FXII is converted to the active form (FXIIa) when it comes into contact with a negative surface (26). Subsequently, FXIIa can activate FXI, which marks the initiation of coagulation via the contact activation pathway. In a rabbit model for an extracorporeal membrane oxygenation (ECMO) cardiopulmonary bypass system, inhibition of FXII using a specific antibody resulted in thromboprotection (27). ECMO is used during severe surgical interventions to take over the patient's heart or lung function for a period of time (28). During this time period, circulation is redirected through the ECMO machine and blood comes directly into contact with the ECMO's bio-incompatible surface, which is highly prothrombotic due to its negative surface. Because the surface of this system (very likely) induces coagulation via FXII and contact activation, FXII blockade is highly effective. The same principle might hold true for mouse models of VT where thrombus formation is initiated after another surgical intervention.

In contrast to mouse models where VT is provoked by a surgical intervention, spontaneous VT in mice is induced by a transient imbalance of the coagulation system. Onset of spontaneous VT was not altered when the contact system of coagulation (FXII) was inhibited. These preclinical data are in line with the lack of association between FXII and VT in human epidemiological data, and suggest that FXII does not play a crucial role in all forms of VT. Our mouse model may represent a situation of VT in which manifestation is associated with an imbalance of coagulation (e.g. thrombophilia via antithrombin deficiency), rather than a situation where VT is primarily induced

by vascular damage or stasis. In line with these results, FXII might not be the most feasible target to prevent VT in humans. Coagulation factor XI (FXI), one of the main targets of FXIIa, may be more interesting. Besides being a target for FXIIa, FXI can be activated by thrombin via a feedback loop mechanism, which implies a more global role in coagulation for FXI as compared to FXII. Interestingly, mice with a complete FXI deficiency were partly protected from spontaneous VT (own observation). Human epidemiological data show that FXI deficient individuals are protected from cardiovascular and venous thromboembolism events, although complete deficiency for FXI coincides with minor bleedings (29, 30). Recently, it has been shown that inhibition of FXI prevents thrombosis in humans (31).

MOUSE MODELS FOR VENOUS THROMBOSIS

In this thesis, the mouse (genera: *Mus musculus*) is used as an animal model to study experimental thrombosis. Mice are mammals and have a fundamentally similar coagulation system as compared to humans (32). Advantages of working with mice are that they are small, cheap, and easy to handle, house, and genetically manipulate. Additionally, because of an extensive inbred program experimental mice have a similar genetic background, which limits confounding effects when interpreting experimental data. However, mouse and human differ in multiple ways: Species differences, such as size, behavior, and roughly every component of the triad of Virchow, can contribute to false interpretation of experimental data in the context of human VT. These differences are important to consider, since the goal of using animal models and studying their pathophysiology is to translate the findings to humans.

In chapter 1 of this thesis, several mouse models for VT are introduced. VT in mice can be initiated by disturbing one of the three elements of the triad of Virchow. Thrombus formation can be triggered by injuring the venous vessel wall or by inducing stasis in a large vein (33, 34). Our group has shown in (35) and chapter 3 and 4 of this thesis that altering the composition of the blood by transiently lowering natural anticoagulants antithrombin and protein C results in spontaneous VT. The nature of inducing VT is fundamentally different in various mouse models for VT, because thrombus formation is induced by another trigger. This can cause changes in the importance of various players in coagulation (figure 1). In our mouse model for spontaneous VT, thrombus formation is induced upon the transient depletion of natural anticoagulants antithrombin and protein C. Here, VT is dependent on thrombin and platelets (9, 35). Neutrophils, FXII, von Willebrand factor, and coagulation factor VII (unpublished data) are not rate-limiting, while FXI-deficient mice were partly protected from spontaneous VT (unpublished data).

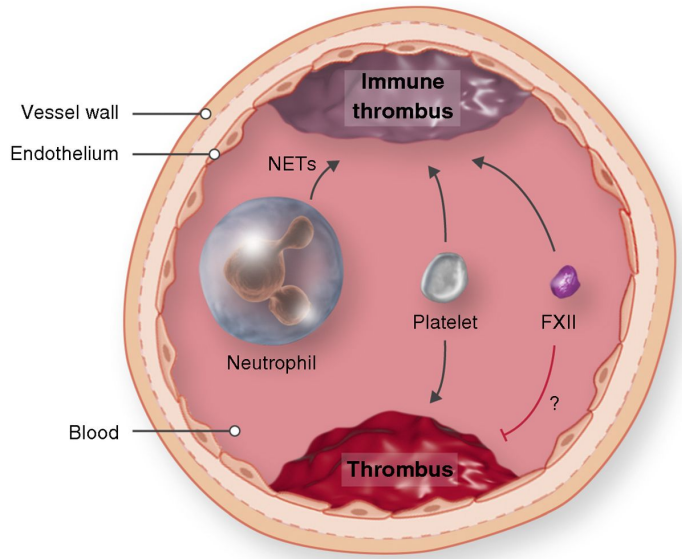


Figure 1 | Comparison of two mouse models of venous thrombosis. In the inferior vena cava stenosis model of venous thrombosis, the inferior vena cava is ligated to reduce blood flow by ~90%. Platelets, FXII, and neutrophils contribute to immune thrombosis (1). In the spontaneous venous thrombosis model, mice are treated with siRNAs to reduce levels of the anticoagulants antithrombin and protein C. Platelets contribute to thrombosis in this model, whereas there is no role for neutrophils, and decreasing FXII increases thrombosis (9). Image from (73).

The thrombotic coagulopathy associated with experimental spontaneous VT is highly reproducible. However, the timing and severity of the onset of the phenotype can differ between experiments. When small interfering (si)RNA-mediated lowering of protein C and antithrombin is insufficient, mice will not develop VT. When inhibition of both natural anticoagulants is exacerbated, mice will develop VT more rapidly (in some occasions within 48 hours) and form large thrombi in locations other than the large veins of the head ((35) and unpublished data). These observations imply that by lowering natural anticoagulants at a certain threshold a delicate balance of coagulation factors is disturbed. Based on these observations, we suspect that the magnitude of the disturbing factor i.e. the effective dose of siRNA can influence the timing and manifestation of the onset of spontaneous VT.

In most VT mouse models specific veins are injured or blocked to induce thrombus formation at a predefined location. When in mice an imbalance of coagulation is introduced by the transient lowering of natural anticoagulants antithrombin and protein C, spontaneous VT occurs in the large veins of the mandibular area of the head. Here, the location of thrombus formation is not

predefined. The consistent occurrence of thrombosis at this peculiar location implies that there must be an additional factor involved in thrombus formation, besides the alteration of blood composition. Currently, it is unknown which additional factor(s) are involved in preference for the specific venous vascular bed of the head. One interesting candidate which may determine the location in spontaneous VT is the blood flow. In humans, venous thrombi are mainly formed in the legs, and it is well-established that local low blood flow and hypoxia contribute to the manifestation of the disease at this location (36). Due to the anatomical differences between mice and humans, and the lack of evidence for local hypoxia and stasis in mice, the role of blood flow is unclear in mouse spontaneous VT. The veins which run from the snout of the mouse to the heart, where thrombosis takes place in spontaneous VT, are relatively long. Here, blood flow may be relatively low and thus more prone for thrombus development. Another factor which may play a role for local thrombus formation in the large veins of the mandibular area of the head is that this location might be prone for minor vessel damage and subsequent generation of thrombin. Mice are rodents and because of their chow diet, their jaw muscles are well developed and compose a large part of their head. Because of the intensive muscle activity in this area, mice may suffer from minor vascular damage and subclinical tissue factor activation. When natural anticoagulants are present at a physiological concentration in the blood, minor tissue factor activation and initiation of coagulation can be inhibited sufficiently. However, when natural anticoagulants are acutely lowered by siRNAs, minor initiation of coagulation can lead to uncontrolled thrombus formation.

RNA INTERFERENCE FOR GENE TARGETING

In this thesis, small interfering RNAs (siRNA) have been used as a tool to inhibit protein production in the liver of mice (37). Repeatedly, it has been shown that mRNA and plasma protein are significantly lowered within two days of siRNA injection. Lowering of hepatic plasma proteins enabled us to study the phenotype of mice with a transient (although incomplete) deficiency of this protein. Besides introducing a complete genetic deficiency in mice, the siRNA-approach is an alternative method to study gene function. Besides the lower costs of siRNA experiments as compared to generate a knockout mouse, mice deficient in certain genes are not always viable. For instance, mice with a full deficiency in the liver-transcribed genes antithrombin, protein C, and coagulation factor VII (the latter is not discussed in this thesis) die perinatally (38-41). Using siRNAs to study genes *in vivo* may be beneficial because of the cost- and time-efficiency, and the ability to gain novel insights in hepatic gene function.

The major advantage of siRNA over direct protein inhibitors, such as antibodies or small molecules, is the intrinsic trait of siRNAs to target different mRNAs and genes by introducing a

simple nucleotide change. Moreover, siRNAs are relatively easy and cheap to produce. Antibodies and small molecules that interfere directly with proteins require a more extensive process of specificity testing. When targeting plasma proteins, one of the major disadvantages of using siRNAs as compared to direct protein inhibitors is the delivery of the product to the correct cell type (where the plasma proteins are produced). So far, only the liver is well-established as a target for siRNA delivery. Since the liver produces most coagulation proteins, using siRNAs to study these proteins is a realistic approach.

An additional disadvantage of using siRNAs as compared to direct protein inhibitors is their potential for off-target effects. Targeting short mRNA sequences (in siRNAs, approximately 20 base pairs) is less specific than targeting complex protein structures (37). Multiple studies on off-target effects of siRNAs (and oligonucleotides) have been reported, and it is acknowledged that including an internal siRNA control is crucial for correct interpretation of the results (42). When using a control siRNA, potential off-target effects introduced by the chemical structure of the siRNA or the carrier are taken into account. These off-target effects are independent of the siRNA sequence. In this thesis, we used an siRNA without an mRNA target on the mouse transcriptome (designated siNEG), to correct for the effect of exposure to the chemical components to the mice.

As described in chapter 4, treating mice with siNEG appeared insufficient as a control in our FXII study. Within this study, initially we found that siRNA-mediated lowering of FXII exacerbated the onset of spontaneous VT (part of chapter 3). Because of this unexpected result with implication for a new role of FXII in thrombus formation, we treated mice solely with siRNA against *F12* without inducing VT. Interestingly, compared to siNEG treated mice si*F12* treatment caused a transient subclinical prothrombotic state in plasma (measured by thrombin generation on plasma). To test whether this response was FXII-dependent, we designed a new siRNA: si*F12*-$1^{C9/11}$. This siRNA had the same sequence as the si*F12*, except for a minor mutation in the seed region of the siRNA which leads to the loss of its ability to target *F12* mRNA (43). Remarkably, treatment of mice with si*F12*-$1^{C9/11}$ also lead to the prothrombotic state in plasma, as determined by thrombin generation assays.

These results imply that the prothrombotic effect of si*F12* treatment was independent of FXII, but specific for the siRNA sequence; Using the C9/11 approach, false positive si*F12* off-target effects will mostly maintain their activity, whereas true positive si*F12* on-target effects will lose their activity. Currently, the mechanism behind the off-target prothrombotic response remains to be determined. It has been reported that oligonucleotides (of which siRNA is a subclass) can cause prothrombotic responses, such as the activation of platelets via the platelet-specific receptor glycoprotein VI (44, 45). However, these studies suggest that prothrombotic responses depend on the chemical structure of the oligonucleotide backbone and not on the siRNA sequence,

which was the case in our study. Because of our unexpected findings described in chapter 4, we recommend to control for sequence-specific elements that are not covered by BLAST analysis in siRNA experiments and trials.

SLC44A2 AND VENOUS THROMBOSIS

A recent meta-analysis of twelve genome wide association studies (GWAS) discovered several novel genomic loci associated with VT, that have not been associated (yet) to the hemostatic system (46). One of these loci is in the *SLC44A2* gene, a gene which has been linked to auto-immune hearing loss and transfusion-related acute lung injury (47, 48). The association between *SLC44A2* and thrombosis was recently confirmed in a separate study (49). The SLC44A2 protein does not play a role in the traditional coagulation cascade and the mechanistic link with VT is currently unknown.

Identifying risk genes for VT or other diseases with GWASs is a strategy that has emerged as a result of the recent advances in genome sequencing (50). Genome sequencing gets cheaper and faster each year, which exponentially increases the pile of available genomic data to identify more SNPs that are associated with VT. However, with an increasing amount of genomic data the odds ratio (OR) of SNPs will also become lower, since the SNPs OR negatively correlates with group size. Moreover, SNP frequency will approach 0.5 when groups of VT patients and healthy individuals get larger. Because of the low OR in newly identified SNPs, it has been questioned whether for VT the limit of identifying novel SNPs has been reached (51). For *SLC44A2*, the top risk coding SNP rs2288904 had an OR of 1.21 and the risk allele has a frequency of 0.785 in the normal population (46). Hence, although the SNP represents a functional difference of the SLC44A2 protein, the small OR and high frequency in the human population make it an unfeasible therapeutic target. However, the notion that this specific SNP was related to an auto-immune and transfusion-related disease where an immune response is involved, tempted us to investigate whether the protein plays a role in immunothrombosis. The biomarkers we measured for neutrophil activation and NET formation are thought to be essentially involved in this novel concept of VT (13). Also the report of a direct interaction between the von Willebrand factor protein and SLC44A2 fueled our interest to understand its exact role in thrombosis (52).

In chapter 5 of this thesis, we attempted to link the top exonic single nucleotide polymorphism (SNP) rs2288904 to markers for neutrophil activation and neutrophil extracellular traps (NET) formation. Previously, these markers have been measured in a study where plasma samples were taken from individuals suspected of VT and actual VT patients, to compare both groups for biomarkers of VT (53). In this study, levels of circulating nucleosomes and elastase α1-antitrypsin

complexes (markers for neutrophil activation and NET formation, respectively) were increased in VT patients. In our study, we did not find an association between the levels of circulating nucleosomes and elastase α1-antitrypsin complexes and the top exonic SNP rs2288904. This finding implies that rs2288904 is not involved in immunothrombosis.

To pursue the novel finding of an association of *SLC44A2* with thrombosis, within our group multiple studies were initiated to investigate *SLC44A2* normal gene function and its role in the pathophysiology of thrombosis. Moreover, we started international collaborations with experts in the field of genetics and immunology on *SLC44A2*. Of note, we used the mouse VT model for spontaneous VT (siRNA-mediated depletion of antithrombin and protein C) to test the thrombotic profile in *Slc44a2*$^{-/-}$ mice. Also, we plan to use other mouse VT models to further elucidate the association between *Slc44a2* and VT.

SPONTANEOUS ATHEROTHROMBOSIS IN MICE

In chapters 6 and 7 of this thesis, we reported that transient inhibition of natural anticoagulant protein C leads to spontaneous atherothrombotic events in apolipoprotein E deficient (*Apoe*$^{-/-}$) mice. The thrombi were directly associated with atherosclerotic plaques in the sinuses of the aortic root, were rich in fibrin, and had a layered structure. Although the incidence of atherothrombosis was low (in three independent studies; 25%, 12%, and 17%), this unique event was robust for three studies.

Mouse plasma possesses a stronger anticoagulant potential, as compared to human plasma (54). This may contribute to the absence of atherothrombosis in mice. The rationale for lowering natural anticoagulant protein C was to introduce a more prothrombotic milieu, which may allow events of thrombus formation in mice. In line with this rationale, a pilot experiment was performed in which the natural anticoagulant antithrombin was also lowered in atherosclerotic *Apoe*$^{-/-}$ mice, using a specific siRNA (si*Serpinc1*). Interestingly, *Apoe*$^{-/-}$ mice treated with si*Serpinc1* developed spontaneous VT within 48 hours, which is more rapid than wild type (*Apoe*$^{+/+}$) C57BL/6 mice (35). This precluded follow-up studies on atherothrombosis in an antithrombin-low environment, since the spontaneous VT phenotype is lethal. The early and severe onset of spontaneous VT in atherosclerotic mice suggests that these mice are more prone to develop VT, an observation which has not been described before and may be of interest for future studies. Within the human population, potential associations between venous thrombosis and atherosclerosis are described and common risk factors are known (55, 56). However, a clear mechanistic link has not been established.

It is currently unknown whether protection from atherothrombosis in atherosclerotic mice is dependent on natural anticoagulant activity or on protein C specifically. Besides protein C's role as an anticoagulant, it is involved in multiple cytoprotective actions, which can prevent cellular injury (57, 58). This means that lowering levels of protein C may interfere with cellular integrity of e.g. endothelial cells, which may predispose to atherothrombosis in mice. In order to increase the incidence of atherothrombosis in low-protein C atherosclerotic mice, it would be interesting to decrease cellular integrity in addition to the si*Proc*-treatment. Also, additional disturbance of the balance of coagulation in atherosclerotic mice to assess atherothrombosis incidence would be of interest.

Spontaneous atherothrombosis in the aortic root in atherosclerotic *Apoe*$^{-/-}$ mice can be induced by the transient lowering of protein C plasma levels (using specific siRNAs; si*Proc*). In addition, we found that differences in the composition of the plaque and the location within the aortic root of the plaque were associated with atherothrombosis. Moreover, platelet numbers were significantly increased upon si*Proc* treatment. Within the si*Proc* groups, the mice with atherothrombosis even showed significantly elevated levels of circulating platelets compared to the group without atherothrombosis. The crucial role for platelets in atherothrombosis has been well-established and most therapies for individuals at risk for atherothrombosis are focused on platelet inhibition (2, 59-61). Also in an independent experiment in normal female C57BL/6 mice, platelets were elevated upon si*Proc* treatment (unpublished data). For now, the mechanism behind the increase in platelets upon si*Proc* treatment and whether the transient platelet increase contributes to atherothrombosis, is unknown. Pharmacological platelet inhibition to prevent atherothrombosis in si*Proc*-treated atherosclerotic mice would be a logical follow-up of the current studies as described in chapters 6 and 7.

Upon transient protein C lowering thrombi are formed spontaneously and exclusively on atherosclerotic plaques in the aortic root of the mice. As described in chapter 7 of this thesis, the blood flow and hemodynamics might be important contributors to the formation of thrombosis at this location. Thrombotic events were found preferentially in the right carotid sinus of the aortic root, which suggests that atherothrombosis in mice depends on the sheer stress of a specific sinus. The observed sinus preference for the development of atherothrombosis also suggests that in mice local hemodynamics and wall shear stress are not only involved in atherogenesis (62, 63), but also in the development of atherothrombosis, as has been proposed for the human disease (64, 65). It is known that development of atherosclerotic plaques in mice are formed in the cusps of the aortic root due to the local oscillating shear stress, which occurs because of the opening and closing of the valves (66). Future studies will have to further elucidate the exact mechanism between atherothrombosis in mice, but our studies indicate that natural anticoagulant protein C, plaque composition, and hemodynamics are key players in the process.

The robustness of low protein C-mediated atherothrombosis in mice with a different genetic background than *Apoe*-deficiency is currently not clear. For this reason, studies in both *APOE**3-Leiden.CETP and scavenger receptor class B, type 1-deficient (*Srb1*$^{-/-}$) mice have been initiated. In contrast to *Apoe*$^{-/-}$ mice, *APOE**3-Leiden.CETP mice have a more human-like lipid metabolism and atherosclerosis formation. Moreover, this mouse strain is responsive for lipid-lowering interventions (67). For instance, in *APOE**3-Leiden.CETP statin treatment lowers non-HDL cholesterol in plasma and reduces atherosclerosis (68, 69). The *Srb1* gene encodes for a receptor which mediates the uptake of HDL to cells (70). For this reason, mice deficient in *Srb1* (*Srb1*$^{-/-}$) have abnormal HDL levels and an increased susceptibility to atherosclerosis (71). Compared to atherosclerotic plaques formed in *Apoe*$^{-/-}$ mice, plaques formed in *Srb1*$^{-/-}$ mice have lower collagen content and a larger necrotic core, both characteristics of instable plaques. Moreover, it has been reported that Srb1 in platelets is protective for thrombosis (72), suggesting that plaques of *Srb1*$^{-/-}$ mice are more prone for atherothrombosis.

CONCLUSIONS

The goal of the research performed in this thesis was to gain new insights in the pathophysiology of venous and arterial thrombosis in mice. The tool we used to achieve this goal were siRNAs that inhibit the production of natural anticoagulants antithrombin and/or protein C. This causes a transient imbalance in the coagulation profile, which can lead to spontaneous thrombotic phenotypes. The main findings from chapters 3 and 4 were that platelets were crucial in a mouse model for spontaneous VT, while neutrophils and FXII were not rate-limiting. Chapters 6 and 7 showed that in a mouse model for atherosclerosis transient inhibition of protein C can lead to spontaneous atherothrombosis. This approach may be the first step towards a novel mouse model of spontaneous arterial thrombosis, which is currently not available.

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9

Nederlandse Samenvatting

Marco Heestermans

Het stollingssysteem van de mens zorgt voor de vorming van bloedstolsels, om bloedverlies te beperken in het geval van een lichamelijke verwonding. Het stollingssysteem is relatief goed geconserveerd door het gehele dierenrijk, en zelfs vissen bezitten een vergelijkbaar mechanisme om bloed te laten stollen. Het behoud van dezelfde fundamentele principes om bloedverlies tegen te gaan benadrukt de noodzaak van het systeem. Het impliceert bovendien dat een verstoring van het stollingssysteem ernstige gevolgen kan hebben, wat ook het geval is. In het geval dat er een bloedstolsel wordt gevormd op het moment dat dit niet nodig is (er is dus geen bloeding), kunnen er bloedpropjes in de circulatie komen. Trombose is de ziekte die de vorming van bloedpropjes en de complicaties die dit met zich mee brengt beschrijft. Als een individu in het geval van vaatschade geen bloedstolsel kan vormen door een niet-functionerend stollingssysteem, kunnen er bloedingen optreden. De instandhouding van een evenwicht in het stollingssysteem is dus cruciaal om te voorkomen dat een individu trombose of bloedingen ontwikkelt.

Trombose kan worden onderverdeeld in twee categorieën: veneuze en arteriële trombose. Bij veneuze trombose ontstaat er een bloedprop in de aderen van een patient (meestal de benen). Dit kan lokaal problemen veroorzaken, maar ernstige complicaties treden vooral op wanneer het lokaal gevormde bloedstolsel los schiet en via het hart in de longen belandt. Dit ziektebeeld wordt een longembolie genoemd en kan uiteindelijk zelfs de dood tot gevolg hebben. Arteriële trombose vindt plaats in de slagaderen. Als hier een bloedpropje wordt gevormd, kan het bloedvat worden afgesloten en zullen de distaal gelegen organen niet worden voorzien van zuurstof. Een beroerte of een hartinfarct zijn ziektebeelden die het gevolg kunnen zijn van arteriële trombose.

Tijdens mijn promotieonderzoek, waarvan in deze PhD thesis de belangrijkste bevindingen zijn uiteengezet, was het doel om nieuwe inzichten te verkrijgen in de initiatie en progressie van veneuze en arteriële trombose. We hebben dit getracht door in een levend systeem factoren die de stolling tegengaan (anti-coagulanten) te remmen. Deze remming verstoort de balans van het stollingssysteem, waardoor een intern milieu wordt gecreëerd waarin factoren die de stolling bevorderen (pro-coagulanten) de overhand krijgen. Dit kan trombose veroorzaken. Het proefdier wat tijdens ons onderzoek is gebruikt om veneuze en arteriële trombose te bestuderen is de muis (*Mus musculus*). Hoewel er geen misverstand kan bestaan over de diverse verschillen tussen mensen en muizen, zijn beide organismes evolutionair gezien nauw verwant en wordt de muis beschouwd als een geschikt *in vivo* model om stolling en trombose (en verschillende andere ziektes) in te bestuderen. Gecontroleerde en specifieke remming van anti-coagulanten wordt gerealiseerd door gebruik te maken van zogenoemde gen-specifieke *small interfering RNAs* (siRNAs). siRNAs kunnen worden geïnjecteerd in de bloedbaan van de muis en belanden, nadat ze zijn ingepakt in lever-specifieke liposomen, in de lever, waar ze de transcriptie (en dus

productie) van specifieke genen kunnen remmen. De anti-coagulanten waarvan we de productie remmen worden exclusief geproduceerd in de lever.

Hoewel siRNAs tijdens mijn promotieonderzoek uitsluitend zijn gebruikt om de invloed van bepaalde genen in een modelsysteem te bestuderen, kunnen ze ook worden toegepast om klinische vraagstukken te beantwoorden. Door middel van remming van de productie van specifieke (lever)eiwitten kunnen bepaalde ziektebeelden worden verbeterd of zelfs worden verholpen. In **hoofdstuk 2** wordt dieper ingegaan op de chemische eigenschappen van siRNAs, en in welke situaties siRNAs gebruikt kunnen worden. In het vervolg van dit hoofdstuk is het gebruik van siRNAs (en gerelateerde remmers van eiwitten gebaseerd op RNA of DNA oligonucleotides, tot welke groep van therapeutica siRNAs behoren) voor zowel fundamentele als therapeutische doeleinden in het kader van trombose en bloedingen uiteengezet.

De anti-coagulanten antitrombine en proteïne C worden exclusief in de lever geproduceerd. Zoals eerder vermeld, wordt in de muis door remming van dergelijke anti-coagulanten een pro-coagulant intern milieu gecreëerd. Als we antitrombine en proteïne C tegelijk remmen in een gezonde muis, ontwikkelt deze veneuze trombose. In de aderen in het gebied rondom de kaakspieren worden spontaan (zonder chirurgische interventies) grote trombi gevormd. Bovendien vindt in de lever afzetting plaats van fibrine, een product van pro-coagulante eiwitten en een van de belangrijkste bestandsdelen van een trombus. Deze manier om veneuze trombose te veroorzaken met behulp van siRNAs is uniek en is fundamenteel anders dan bij andere diermodellen om de ziekte te bestuderen. In andere diermodellen wordt namelijk veneuze trombose geïnduceerd door middel van vaatschade of stase (stilstand van het bloed). Voor deze modellen zijn complexe chirurgische ingrepen noodzakelijk. Hoewel deze invasieve diermodellen ons veel informatie hebben opgeleverd over het ontstaan en de progressie van (experimentele) veneuze trombose, kan onze manier om veneuze trombose te veroorzaken vernieuwende inzichten geven over fundamentele processen van de ziekte, met als uiteindelijke doel de ontwikkeling van nieuwe therapieën.

In **hoofdstuk 3** hebben we in het muismodel voor spontane veneuze trombose de invloed van drie nieuwe kandidaat-hoofdrolspelers getest: bloedplaatjes, neutrofielen en coagulatie factor XII. Remming van bloedplaatjes voorkwam spontane veneuze trombose. In tegenstelling tot studies gedaan met andere diermodellen, bleek dat remming van neutrofielen en coagulatie factor XII niet cruciaal was bij de ontwikkeling van veneuze trombose. Dit impliceert dat experimentele veneuze trombose zich op verschillende manieren kan manifesteren, en dat hierbij verschillende factoren betrokken kunnen zijn. Of dit bij patienten met veneuze trombose ook het geval is, zal verder onderzoek moeten uitwijzen. In **hoofdstuk 4** gaan we dieper in op de rol van coagulatie factor XII in spontane veneuze trombose. In dit hoofdstuk bevestigen we

allereerst dat deze factor niet betrokken is bij spontane veneuze trombose in muizen. Bovendien tonen we in dit hoofdstuk aan dat het gebruik van siRNA om de rol van bepaalde eiwitten te bestuderen resultaten kunnen opleveren die misleidend kunnen zijn, een belangrijke observatie in het kader van verder siRNA onderzoek.

Veneuze trombose wordt niet alleen in muizen onderzocht, maar ook in de menselijke populatie. Door grote groepen van mensen met en zonder de ziekte te vergelijken, kunnen bepaalde genetische varianten worden geïdentificeerd die geassocieerd zijn met de aanwezigheid van het ziektebeeld: komt een bepaalde genetische variant relatief vaak voor in een patiëntenpopulatie, kan dit duiden op de aanwezigheid van een biologische variant waardoor een individu een verhoogde kans op trombose heeft. De snelle ontwikkelingen op het gebied van *genome sequencing* heeft ervoor gezorgd dat het tot in detail in kaart brengen van de genetische code van het DNA steeds sneller en goedkoper kan, waardoor steeds grotere populaties met elkaar kunnen worden vergeleken. Het sneller genereren van meer data in grotere populaties heeft als gevolg dat steeds meer genetische varianten worden geassocieerd aan het optreden van trombose. Een recent geïdentificeerde variant is gelokaliseerd op het gen *SLC44A2*, een gen wat tot voor kort nog nooit geassocieerd was met trombose. In **hoofdstuk 5** is getracht de aanwezigheid van de variant met de verhoogde kans op veneuze trombose te koppelen aan biomarkers voor neutrofiel activatie (een belangrijke marker voor trombose), met als doel om een beter inzicht te krijgen in de rol van *SLC44A2* met betrekking tot trombose.

Arteriële trombose is net als veneuze trombose een ziekte waarover nog veel onbekend is, en meer kennis is noodzakelijk om behandeling van de ziekte te verbeteren. Om de ziekte te onderzoeken in een levend systeem wordt er wederom veelvuldig gebruik gemaakt van muizen. Het grootste probleem is echter dat muizen normaal gesproken geen arteriële trombose ontwikkelen; er zijn weliswaar muismodellen beschikbaar voor het verdikken en verharden van de slagaders (de beginfase van de ziekte, ook wel atherosclerose of (slag)aderverkalking genoemd), maar deze muizen ontwikkelen nooit (zichtbare) arteriële trombi in de verkalkte slagaderen.

De exacte oorzaak van het uitblijven van arteriële trombose in muizen in tegenstelling tot mensen is onbekend, hoewel er waarschijnlijk meerdere factoren een rol spelen. Een van de vele verschillen tussen mensen en muizen is de samenstelling van het bloed. Het is bekend dat de anticoagulatie in muizen sterker is. Observaties hieromtrent hebben ons tot de hypothese geleid dat de sterke anti-coagulatie een belangrijke reden is waarom er geen arteriële trombose optreedt in muizen met ernstig verkalkte slagaderen. In **hoofdstuk 6** is het onderzoek beschreven naar het optreden van spontane arteriële trombose in muizen, door het remmen van anticoagulant proteïne C met behulp van siRNAs. Spontane arteriële trombose in muizen is nog nooit eerder beschreven, en onze studie toont bovenal aan dat onder normale omstandigheden proteïne C

een belangrijke rol speelt in het voorkomen van arteriële trombose in muizen. Bovendien kan dit muismodel wellicht worden gebruikt om arteriële trombose te bestuderen, met als ultieme doel de behandeling van de ziekte te verbeteren. In **hoofdstuk 7** wordt er dieper ingegaan op het fenomeen van spontane arteriële trombose in muizen, waarbij het duidelijk wordt dat vooral het stromingsprofiel van het bloed een cruciale rol speelt met betrekking tot de locatie van de trombosevorming.

Hoofdstuk 1 is een inleiding van het promotie-onderzoek voor een beter begrip van de specifieke hoofdstukken. In dit hoofdstuk wordt in meer detail het kader geschetst waarin het promotieonderzoek heeft plaatsgevonden. In de algemene discussie in **hoofdstuk 8** worden de belangrijkste ontdekkingen, inzichten, pijnpunten en perspectieven van het promotieonderzoek bediscussieerd.

CURRICULUM VITAE

Marco Heestermans was born in Waalre, the Netherlands, on the 20th of August 1987. After completing his secondary education in 2005 (van Maerlant Lyceum, Eindhoven, the Netherlands) he started the Bachelor study Biology at the Radboud University in Nijmegen (the Netherlands). After successful completion of the Bachelor study in 2009, he continued with a Master study in Medical Biology at the Radboud University. During his Master, from which he graduated in 2012, he completed two internships: In 2010, he was involved in a research project on the convergent evolution of Argonaute-2 slicer antagonism in two distinct insect RNA viruses, at the department of Medical Microbiology (virology) at the Nijmegen Medical Centre for Life Sciences in Nijmegen (now, Radboud Institute of Molecular Life Sciences). From 2011 to 2012, he completed an internship at the department of Immunology at the Centre for Neuroscience and Cell Biology in Coimbra (Portugal), where he worked on a project on the influence of peripheral B lymphocytes and TGF-β expression on regulatory T cell differentiation in Parkinson patients.

In December 2013, he started as a PhD candidate at the department of Internal Medicine (Division of Thrombosis and Hemostasis) in the Einthoven Laboratory for Vascular and Regenerative Medicine (Leiden University Medical Center, Leiden, the Netherlands), on a project entitled "The link between venous thrombosis and arterial thrombosis". This project was funded by the Leiden University Medical Center and the Leiden Academic Centre for Drug Research. During his PhD period, he worked under the supervision of Prof. Dr. Pieter Reitsma, Prof. Dr. Henri Versteeg, and dr. Bart van Vlijmen. His most important research findings are outlined in this thesis. Since December 2017, he is employed at the Universitätsklinikum Hamburg-Eppendorf (UKE, Hamburg, Germany) as a postdoctoral researcher in the lab of prof. dr. Thomas Renné.

PUBLICATIES

Heestermans M*, Ouweneel AB* et al. “Low Protein C-mediated Spontaneous Atherothrombosis in *Apoe*$^{-/-}$ Mice Localizes to the Right Carotid Sinus Independent of Plaque and Blood Composition”, *Manuscript submitted for publication,* (*: denotes equal contribution)

Heestermans M et al. “Venous Thrombosis in Mice after Silencing of Natural Anticoagulants Depends on Platelets, not Neutrophils or Factor XII”, *Manuscript submitted for publication*

Padro T et al. “Atherothrombosis and Thromboembolism: Position Paper from the Second Maastricht Consensus Conference on Thrombosis.”, *Thromb Haemost*. 2018 Feb;118(2):229-250.

Ouweneel AB*, Heestermans M* et al. “Silencing of Anticoagulant Protein C Evokes Low-Incident but Spontaneous Atherothrombosis in Apolipoprotein E-Deficient Mice”, *Arterioscler Thromb Vasc Biol*. 2017 May;37(5):782-785 (*: denotes equal contribution)

Heestermans M, van Vlijmen BJM, “Oligonucleotides targeting coagulation factor mRNAs: use in thrombosis and hemophilia research and therapy”, *Thromb J*. 2017 Mar 7;15:7

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Van Mierlo JT et al. “Convergent evolution of argonaute-2 slicer antagonism in two distinct insect RNA viruses”, *PLoS Pathog*. 2012;8(8):e1002872

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