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Novel insights in thrombosis pathophysiology using Mice with Impaired anticoagulation

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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).

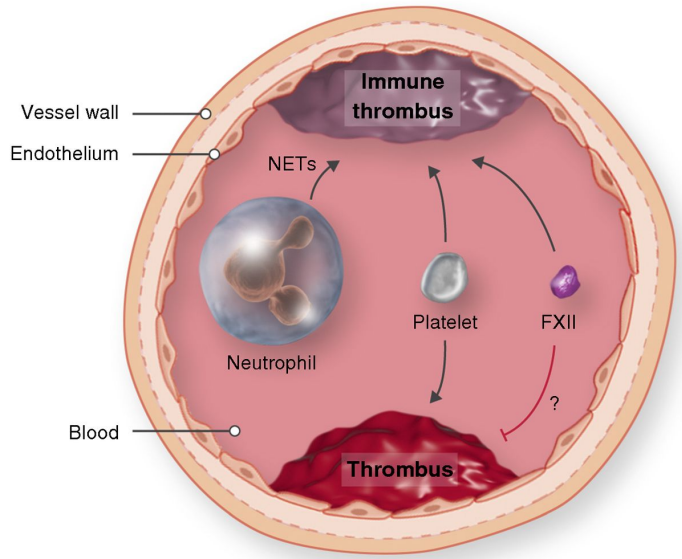

Figure 1 | Comparison of two mouse models of venous thrombosis. In the inferior vena cava stenosis model of venous thrombosis, the inferior vena cava is ligated to reduce blood flow by ~90%. Platelets, FXII, and neutrophils contribute to immune thrombosis (1). In the spontaneous venous thrombosis model, 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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