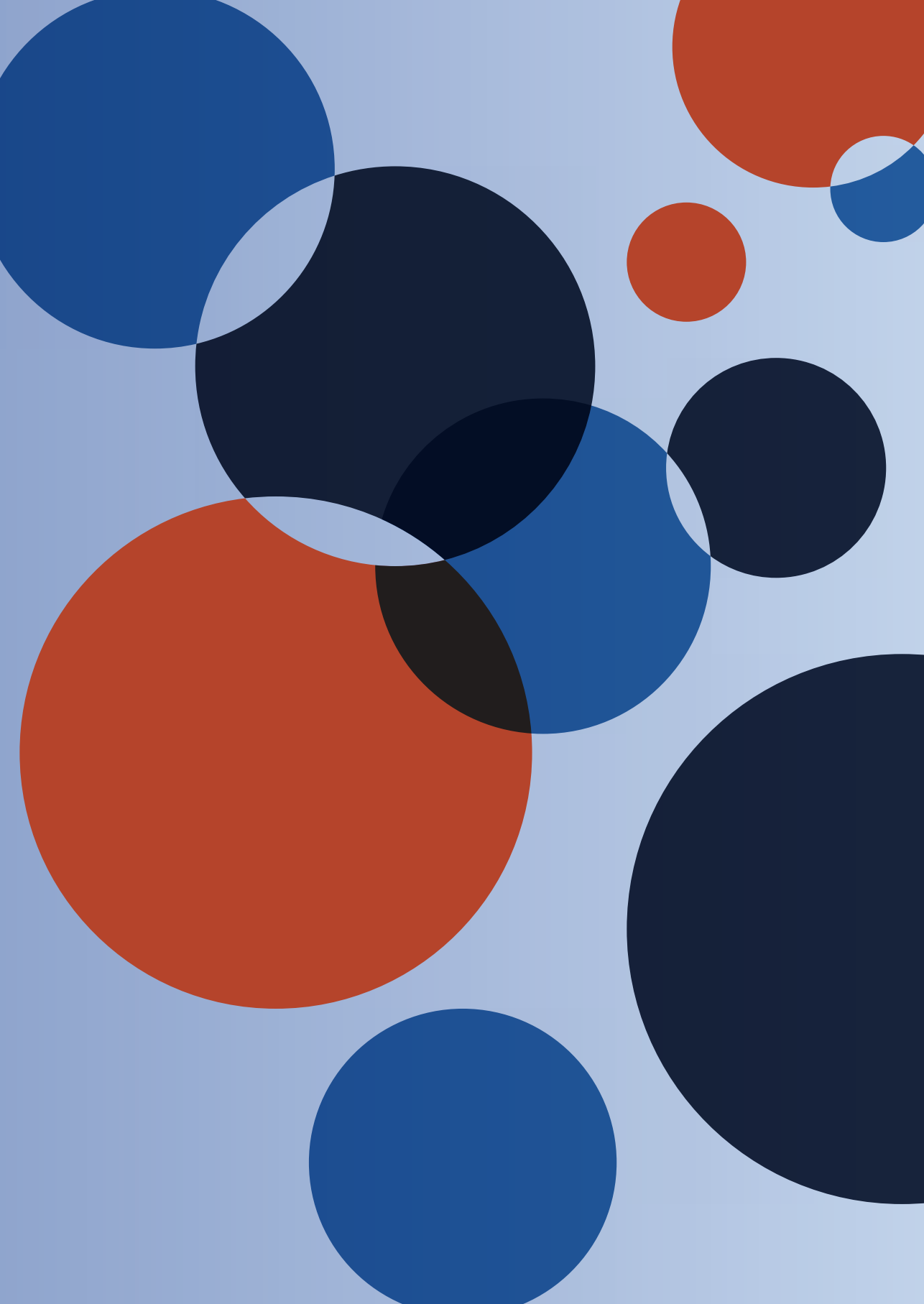

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Julia Tilburg

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Venous Thrombosis

Hemostasis of the blood is essential to life as this keeps blood within the circulation upon the damage of a blood vessel. This process ensures that the transport of oxygen, nutrients and other necessities throughout the body is retained (1). A delicate interplay between vasoconstriction, blood platelets and several pro- and anticoagulant factors is responsible for the maintenance of hemostasis (1). A shift in this balance may lead to a reduced propensity to coagulate, possibly resulting in a bleeding disorder (2). On the other hand, a shift towards a more procoagulant condition can result in unwanted blood clotting known as thrombosis (3). Thrombosis can occur either in the arteries (arterial thrombosis; AT), or within a vein (venous thrombosis; VT). Together AT and VT are the main causes of death worldwide and a major burden on global health (4, 5). VT has an incidence of 1-2 in 1.000 people per year and usually occurs within the deep veins of the leg (deep vein thrombosis), where a blood clot causes obstruction of the vein (4). When a part of the clot embolises towards the lungs, it may cause a life-threatening condition called pulmonary embolism (PE) (6). VT has a mortality rate of 6% one month after diagnosis. When a patient survives, the recurrence is 25% after 5 years, thereby leaving patients with a lifelong risk (7, 8). Anticoagulant treatment is commonly used, both in treating thrombosis and as a prophylactic treatment in high risk patients (9). All currently available anticoagulant treatments (heparins, vitamin K antagonists and direct oral anticoagulants (DOACs)) introduce an imbalance of hemostasis (10-12). This imbalance can lead to adverse side effects, being serious and sometimes fatal bleedings (13).

The general principles of hemostasis

The pathophysiology of VT is well studied and relatively well understood (3). One of the first landmarks in thrombosis research was established in 1856 by the German physician Rudolph Virchow, when he described the elements contributing to thrombosis, also known as Virchow's triad (14, 15). He observed that thrombosis may develop if one of the following occurs: 1) Stasis of the blood flow, 2) Endothelial injury or 3) Hypercoagulability (14). To understand how these elements are linked to thrombosis, it is of importance to comprehend the general principles of hemostasis and the several different proteins and cell types that contribute to the development of a blood clot (16). Under normal conditions, the endothelium has an anticoagulant character as the surface is covered with the anticoagulant protein thrombomodulin (17). Damage of the vessel, however, will lead to the activation of the endothelial cells and the release of procoagulant von Willebrand factor (VWF) from the Weibel–Palade bodies. In addition, the subendothelial matrix is exposed, consisting of several procoagulant proteins (18). Platelets from the blood circulation are captured by VWF and subendothelial matrix proteins, thereby leading to activation and aggregation of these platelets and the formation of a hemostatic plug (19). This process of platelet recruitment is referred to as primary hemostasis and is followed by the activation of coagulation known as secondary hemostasis. Activation of the coagulation cascade is established by exposure of the blood to the procoagulant protein tissue factor (TF) upon vessel damage (20). This cascade, consisting of the subsequent activation of several serine proteases eventually results in the conversion of prothrombin into thrombin (16). Thrombin is central

to coagulation as thrombin leads to the polymerisation of fibrinogen to fibrin. Here, primary and secondary hemostasis come together as fibrin fibers interact with platelets and form a blood clot (21). An anticoagulant and fibrinolytic system is in place to avoid excessive blood clotting and to remove clots after wound healing is achieved (22, 23). This current concept of blood clot formation has been around for decades but new insights into the pathophysiology of VT are still being discovered (24).

The concept of immunothrombosis

The role of immune cells in VT initiation and propagation has recently been investigated extensively, although mainly in the setting of experimental mouse models (25). Neutrophils play an important role in the initiation of hypoxia- mediated VT (resulting from blood stasis), as they are recruited to the endothelial surface at an early phase of thrombus development (26). This accumulation of neutrophils is partially dependent on the mast cells residing in the vessel wall. Upon activation by hypoxia, these cells secrete histamine leading to endothelial activation and an enhanced exposure of adhesion molecules (27). Recruitment of neutrophils to the endothelial layer will result in release of neutrophil extracellular traps (NETs) forming a scaffold for platelets, VWF and fibrinogen, thereby propagating VT (28-30). Furthermore, NETs can activate the coagulation system as they trigger the activation of coagulation factor XII and consequently the intrinsic coagulation pathway (26). Monocytes are recruited to the vessel wall upon endothelial activation in a similar way as neutrophils, although to a lesser extent (26). These monocytes are the carrier of procoagulant TF, which initiates activation of blood coagulation via the extrinsic pathway (26). Besides their roles in hemostasis platelets also have a role in immunothrombosis, as specific adhesion molecules on their surface bind and activate neutrophils leading to an increased thrombus formation (31, 32). Together these concepts indicate a role for the immune system in VT at least in a murine VT setting. The relevance of this concept for human pathophysiology is of interest, as these mechanisms imply that systems other than classical hemostasis are influencing VT and therefore new insights into VT disease and potential therapeutic targets are to be discovered.

GWAS as a tool to identify novel genetic contributors to venous thrombosis

Risk factors for venous thrombosis

VT is a multifactorial disease and risk factors impacting one or more elements of Virchow's triad are commonly identified using human population studies (33-35). Amongst others, cancer, age, trauma, obesity, oral contraceptives, pregnancy, surgery, and air travel are identified as common environmental risk factors (36). In addition to acquired risk factors for VT, inheritable genetic predispositions also contribute to disease development (37). Although congenital deficiencies of natural coagulation inhibitors, protein C, protein S and antithrombin are rare, these deficiencies are major genetic risk factors for VT leading up to a 10-fold increased risk of developing a VT (38). Another well-known, but less strong, genetic contributor is Non-blood type O (ABO), which is associated with higher levels of VWF and subsequently a 2-fold higher VT risk (39). The first genetic mutation associated

with VT was discovered by linkage analysis of inheritable activated protein C-resistance (40). This increased resistance and thereby 3-fold increased risk for VT was denoted to a polymorphism in the coagulation factor V gene (Factor V Leiden mutation, rs6025) (40). Mutations located in the genes coding for prothrombin (*F2*, G20210A/rs1799963, 1.5-fold increased risk), fibrinogen (*FGG*, rs2066865, 1.5-fold increased risk*)* and coagulation Factor XI (*F11*, rs4253417, 2.8-fold increased risk*)* were later also linked to VT using conventional linkage analysis (41-43).

GWAS for venous thrombosis

Collectively, the known genetic factors described above do not fully explain how the genetic landscape contributes to VT risk. Therefore, to increase our understanding, advanced genetic studies are performed. Moreover, genome wide association studies (GWAS) provide a potentially powerful tool to determine single nucleotide polymorphisms (SNP) across the genome associated with diseases (44). By comparing allele frequencies of SNPs from a cohort displaying a phenotype or disease to a control group, GWAS can link genetic loci to certain traits (45). The hypothesis-free character of these studies opens up an opportunity to unravel unsuspected associations. This is relevant as these associations can provide biological insights and, in addition, may lead to the identification of novel therapeutic targets. The first GWAS was performed in 2002 for myocardial infarction and ever since GWAS have a proven track record in cardiovascular disease (CVD) (46-49). In order to identify novel genetic risk factors for VT and novel therapeutic targets potentially circumventing bleeding side effects, GWAS was first applied to this field in 2009 (50). This study, including 453 VT cases and 1327 controls confirmed previously reported genetic risk factors for VT, including Factor V Leiden and ABO loci, but did not reveal additional factors that contribute to the extent of these previously described factors (50). As estimations showed that the by then known genetic risk loci together explained 35% of the genetic predisposition for VT, novel factors were to be revealed (51). With the population studies and GWAS further emerging and the number of included subjects and measured SNPs radically increasing, studies became larger and more powerful. In 2015 a meta-analysis was published which combined the results of multiple VT GWAS studies that included a total of 7507 VT case subjects and 52632 controls (52). This was the first study to show the capacity of GWAS as a discovery method for VT as two novel risk factors were identified; *TSPAN15* and *SLC44A2* (with top associated SNPs: rs78707713 and rs2288904, respectively) (52). Importantly, these two loci were successfully replicated in three independent case-control studies. In addition, a second independent GWAS conducted in the 23andMe cohort, showed associations between these genes and self-reported blood clotting events (52, 53). Interestingly, *TSPAN15* and *SLC44A2* were not previously linked to coagulation, in contrast to all the previously identified genetic risk factors. This is further supported by the lack of an association of these genetic loci with 25 related quantitative biomarkers included in the GWAS such as the activated partial thromboplastin time, endogenous thrombin generation and activity levels of numerous coagulation (related) factors (52). Therefore, the association of these loci with VT implies

that there are undiscovered mechanisms to be revealed regarding the pathophysiology of VT involving TSPAN15 and SLC44A2.

GWAS have been widely used in CVD and other diseases or genomic traits. As an illustration, at start of the writing of this thesis, over 4000 GWAS were published and this number keeps on rapidly growing (54). Together these GWAS have provided a plethora of data and are an invaluable tool in uncovering genetic dispositions and biomarkers (55). However, the translation of these associations into novel therapeutic strategies remains limited (56). For a successful GWAS bench to bedside approach it is important to comprehend that gene discovery should not be a goal on itself. GWAS should be combined with further functional studies and treatment development to fully capture the biological relevance and potentially improve the disease outcome (57, 58). To this end, the work described in this thesis provides a functional follow up on one of the genes identified by GWAS as a VT susceptibility locus namely SLC44A2 and its role in the pathophysiology of VT.

SLC44A2 and its relation to disease

SLC44A2 is a relatively unknown gene and questions regarding the function of SLC44A2 still arise. In the following section, SLC44A2 and its relation to disease is further described.

The solute carrier family 44 member 2

SLC44A2 or solute carrier family 44 member 2 is encoded by a 42,115 nucleotide, 22 exon spanning gene located on chromosome 19p13.1 (59, 60). *SLC44A2* has two transcript variants; P2 and P1 which lead to proteins differing in the region at the N terminus and these transcripts have alternative promotors (60, 61). The SLC44A2 gene encodes a 68-72kDa transmembrane glycoprotein which is characterized by ten hydrophobic membrane-spanning domains, five extracellular loops and six intracellular regions (62). It is expressed in several tissues including cochlea, tongue, heart, colon, lung, kidney, liver and spleen (60). SLC44A2 is shown to be widely expressed in different blood cells such as neutrophils, platelets and erythrocytes (61, 63, 64). As the classification as a solute carrier transporter implies SLC44A2 is a presumed transporter enabling the passage of choline thought the cell membrane (65). Hence the alternative name of SLC44A2 is choline transporter-like protein 2 (CTL-2). Interestingly, however, this transport capacity is only associated with the longer transport variant P2 which is expressed on lung endothelial tissue (60). This was experimentally confirmed *in vitro* using brain microvascular endothelial cells (66). Neutrophils and platelets only express the P1 variant and therefore SLC44A2 on these components is not able to transport choline and the function here remains unknown (61, 64).

Human neutrophil antigen-3

In addition to the properties described above, SLC44A2 harbours one of the human neutrophil antigens (HNA), which were recently extensively reviewed (62). There are five HNAs (HNA1-5) and antibodies targeting these sites are known to be involved in immune diseases (62). SLC44A2 harbours the HNA3 epitope to which alloantibodies can arise. These

antibodies may lead to transfusion related acute lung injury (TRALI) or autoimmune hearing loss, both further described below (59, 67). The HNA3 epitope has two allelic variants, HNA3A and HNA3B, determined by a single point mutation SLC44A2*455G>A (SLC44A2*457G>A for transcript variant P2) (68). Interestingly, the same polymorphism, rs2288094, is linked to VT (52). More commonly, antibodies targeting HNA3A cause adverse effects as antibodies targeting HNA3B are less pathogenic (68). The prevalence of the HNA3A genetic variant, which is associated with an increased VT risk, is high with 0.79, 0.93 and 0.74 in Caucasians, African Americans and Chinese respectively (68-70). In the German population 64.1% is homozygous for HNA3A, 5.5% for HNA3B and 30.4% is heterozygous (68).

Transfusion Related Acute Lung Injury

HNA3 antibodies can arise in pregnant women, specifically when carrying a child with the opposite allelic variant (71). Upon blood or plasma transfusion with antibody contaminated blood from these women to recipients expressing the epitope targeted by these antibodies, an immune response can occur within hours after transfusion (67). Upon binding of the antibodies to the HNA3 epitope present on the SLC44A2 protein on neutrophils, these cells will become activated resulting in agglutination and the release of NETs (72, 73). Subsequently, the release of reactive oxygen species (ROS) from the neutrophils causes severe damage to the lung endothelium and when left untreated, this condition referred to as TRALI, can be fatal (74). A pre-existing pulmonary pathology is required for neutrophils to be present in the lung tissue and for TRALI to occur (67). TRALI is the most common transfusion related cause of death, therefore to lower the prevalence, women who delivered children should be excluded as a blood donor (75). The role of SLC44A2 is of interest as the transcript variant involved in TRALI is the same as the one associated with VT. Moreover, the cell type which is driving TRALI, neutrophils, has a known role in VT.

Autoimmune hearing loss and Slc44a2 deficient mice

In addition to its role in TRALI, SLC44A2 is also the target of antibodies inducing autoimmune hearing loss (59, 76, 77). Human autoantibodies can bind to the HNA3 epitope on SLC44A2 in the organ of Corti resulting in dysfunction of this organ (78). The organ of Corti, located in the cochlea of the inner ear, is pivotal to transduction of auditory signals and malfunctioning leads to severe hearing impairment (79). To study the role of SLC44A2 in hearing loss *Slc44a2* deficient mice were generated (80). By the insertion of *flox* sites surrounding the exon 3 to 10 region of the *Slc44a2* gene, and crossing of these mice to mice carrying a *Cre* recombinase behind the EIIa promotor (expressing *Cre* to the early mouse embryo) a full body knockout was developed. The mice appeared healthy and fertile and did not exhibit aberrant behaviour, size differences or other atypical features. However these mice exhibited extensive hair cell and spiral ganglion cell loss in the cochlea and subsequently progressive hearing loss (80). This finding further underlines a role for SLC44A2 in long term hair cell survival and in the maintenance of normal hearing.

SLC44A2 and von Willebrand factor

Further studies into the localization of SLC44A2 in the inner ear reported that this protein colocalizes with cochlin, one of the predominant proteins of the inner ear (81). Since cochlin harbours two VWF binding domains it was hypothesised that this colocalization is mediated by VWF (82). Bayat and colleagues created a human embryonic kidney (HEK293) cell line overexpressing SLC44A2. Interestingly, SLC44A2 positive cells displayed enhanced binding to VWF (83). Subsequently, by blocking specific VWF domains, SLC44A2 was shown to bind the A1 domain, the domain required for platelet-VWF interaction. Furthermore, it was established that neutrophil agglutination, induced by antibodies targeting SLC44A2, is reduced in plasma containing lower levels of VWF. Because of the predominant role of VWF in hemostasis the binding capacity of SLC44A2 to this protein is of interest when investigating the role of SLC44A2 in VT.

Modelling thrombosis using murine models

Selecting a study approach to investigate the role of SLC44A2 in the pathophysiology of VT is challenging as in VT several factors i.e. the endothelial, blood flow, coagulation factors and cellular components of the blood synergize to form a blood clot. Therefore, to study SLC44A2 in this interplay of different elements and the circulation, advanced experimental setups are necessary. The mouse is a frequently used model organism in VT research because of several advantages. First, the coagulation system is highly conserved in the mouse and all elements of Virchow's triad are present, making it suitable in mimicking VT (84). In addition, mice express SLC44A2 and the gene encoding SLC44A2 can be easily genetically manipulated to produce a mouse deficient in this protein (80, 85). Lastly, mice are also fast breeders, have low costs of maintenance and are relatively easy to handle (86). Although hemostasis is highly conserved, mice appear to be resistant to the development of a venous thrombus in the absence of any genetic, surgical or chemical manipulations. Therefore, to study thrombosis in a mouse model, a pro-thrombotic trigger has to be applied. As there are several factors that play a role in thrombosis, i.e. the elements of Virchow's triad (endothelial damage, blood flow and coagulation) there are also various ways to introduce thrombosis in mice (87). Endothelial activation is one method to modulate vascular injury or thrombosis in mice. Models such as the ferric chloride model, the laser injury model, or more systemic endothelial activation are widely accepted (88-90). Also models manipulating the blood flow are common practise, for instance by completely (stasis) or partially (stenosis) ligating the large veins in a mouse (26, 91, 92). Mouse models which interfere with expression of genes related to coagulation are used to study Virchow's third element of hemostasis; coagulation. For instance mice harbouring the Factor V Leiden mutation or tissue factor pathway inhibitor (TFPI) heterozygosity display mild to moderate pro-coagulant phenotypes (93, 94). Mice with more severe genetic deficiencies such as deletion of antithrombin, Protein C or TFPI will die perinatally (95-97). To overcome this early mortality, a mouse model using a transient imbalance of anticoagulant factors was established (98, 99). Overall, there are several different VT models available which all have advantages and disadvantages (100). A convenient algorithm including the 7 most used murine VT models was recently published

and although not complete, this guideline forms a solid foundation for VT model selection (101, 102). The murine VT models that are used throughout this thesis are summarized below and their advantages and disadvantages are discussed.

A challenge of hemostasis by endothelial damage of the cremaster arterioles
The recruitment of blood cells and other blood components towards the site of injury at the vessel wall is an important element in the maintenance of hemostasis (1). Intravital video microscopy upon laser injury of the cremaster muscle arterioles provides an opportunity to monitor this process live (103). In short; a laser is used to introduce injury on the luminal vessel wall of the cremaster muscle arterioles and within seconds, accumulation of fluorescently labelled elements at the site of injury may be recorded (89). This model was previously shown to be dependent on intravascular tissue factor, platelets and neutrophils (104, 105). Important to note, this model is not dependent on VWF. Thus to study VWF dependent mechanisms, other models might be more suitable (106). As the dissection of the cremaster muscle is a delicate procedure, a skilled animal surgeon is required to prepare the animal. One of the main advantages however, is that multiple injuries can be applied to one mouse leading to large experimental numbers per mouse (107). It should be pointed out that in this model the injury is applied to arterioles meaning that hemostasis is measured under arterial flow, therefore this model is not fully representative of VT. Besides its limitations, this model has been widely used to elucidate the contribution of several molecular components involved in thrombosis (107).

Neutrophil adherence to histamine stimulated endothelium
The model described above induces injury to monitor blood cell recruitment to a specific site. To introduce a more systemic endothelial activation resembling a state of inflammation, histamine can be used. In this model the venules of the mesentery bed are exposed and using intravital microscopy the recruitment of blood cells can be visualized and quantified (108, 109). Prior to laparotomy of the mesentery the mice is injected with histamine, resulting in endothelial degranulation and the release of VWF from the Weibel-Palade bodies (110). To monitor neutrophil adherence to the vessel wall these cells are fluorescently labelled. The advantages are again that multiple sites can be monitored in one mouse and the venous flow makes it suitable for studies on VT. As this model monitors the interaction of immune cell with the endothelial it is mainly used to study immunothrombosis.

Thrombosis induced by stenosis of the inferior vena cava
The inferior vena cava (IVC) is the largest vein in mice and is therefore a commonly used venous location to induce thrombosis. In the stenosis model, a partial ligation of the IVC is achieved by placing a ligature around the vein. A spacer is used to retain a small opening, resulting in a ~10% residual blood flow (92). In contrast to the previously commonly used St. Thomas model where a clip is used, the stenosis model is supposed to be endothelial damage free and endothelial activation is solely the result of hypoxia resulting from the reduced blood flow (101). Current understanding indicates the following sequence of events to result

in thrombus formation in this model. Upon endothelial activation, VWF-mediated platelet adhesion to the vessel wall occurs. Activation of these platelets leads to platelet-platelet aggregates which form a scaffold for coagulation activation via FXII activation into FXIIa (111). In addition, monocytes and neutrophils accumulate during the initiation phase. Remarkably within two hours after ligation neutrophils localize at the vessel wall (26, 112). Upon neutrophil activation NETs are formed, that will trap platelets and red blood cells forming the scaffold for a thrombus (29). Within 6 hours after ligation in the stenosis model a visible thrombus is formed and after 48 hours a multi layered thrombus consisting of white and red regions is developed (26). Because of the pivotal role of neutrophils and NETosis in the stenosis model, it is currently primarily used to study immunothrombosis.

Thrombosis induced by a hypercoagulable state

All models described above require advanced surgical procedures. On the contrary, the model driven by downregulation of anticoagulants is induced by a simple tail vein injection (98, 113). Following injection of small interfering RNAs (siRNA) against two anticoagulants, antithrombin and protein C, hepatic expression of these proteins is downregulated resulting in a hypercoagulable state. Within two days after treatment, the mice start to develop thrombi within the veins of the mandibular area of the head. Histologically, these obstructions present as a fibrin rich thrombus. As a secondary phenotype, mice display haemorrhaging in the area surrounding the eye which is likely due to rupture of the veins because of thrombus formation. This model was previously shown to be highly dependent on platelets and tissue factor (113). As neutrophils do not influence thrombosis in this model this model is not suitable for studies in immunothrombosis (113).

Mass spectrometry based targeted plasma proteomics

Mouse models for VT may be used to gain insight into the role of SLC44A2 in the pathophysiology of VT. To investigate a possible physiological effect of SLC44A2 on VT, the composition of the blood may be analyzed by various methods. For the work described in this thesis, we utilized a novel methodology to measure the plasma proteome in mice. Below, this method is introduced and the advantages above current assays are discussed.

The plasma proteome

Analysis of blood components yield important information regarding the state of health and disease in an individual. Blood counts and characteristics of the cellular components of blood i.e. erythrocytes, lymphocytes and thrombocytes may yield important information, for instance about ongoing inflammation, kidney malfunction or bleeding tendencies (114-116). Blood plasma contains, amongst others, proteins that are involved in blood coagulation, the acute phase response, cholesterol metabolism and the complement system (117, 118). In addition to these functional plasma proteins, the plasma proteome also contains proteins as a consequence of tissue leakage and signaling proteins (119). As the concentrations and activity of plasma proteins reflect the disease state, measuring these proteins in clinical diagnostics is common practice (120, 121). To measure protein abundance, antibody and

activity-based protein assays are nowadays frequently used. These assays have the ability to precisely determine protein concentrations, but often do not have the possibility to measure multiple proteins at once e.g. measure in multiplex (120). Another disadvantage of antibody based protein detection is the reliability on the availability and quality of an antibody for the protein of interest (122, 123).

Mass spectrometry based targeted plasma proteomics

As an alternative to the conventional protein assays, mass spectrometry can be applied to measure protein concentrations in multiplex (124). In short, a digested protein sample is loaded on a reversed phase column attached to a liquid chromatographer (125). After sample separation the peptides are converted to gas phase ions by electrospray ionization and the samples are then fragmented on a mass spectrometer (126). The originating mass spectra of these ions are used to identify and quantify the peptides and corresponding proteins (127). This method serves as a good tool for discovery-based proteomics, but is not suitable when a high precision is required, due to the spectral overlap in protein mass spectra (126). Mass spectrometry-based targeted proteomics, also called multiple reaction monitoring (MRM), incorporates stable isotope-labeled peptide standards to select and quantify specific peptides thereby overcoming the spectral overlap (127-130). By this means, MRM offers a technique to quantify preselected proteins with high precision and opens an opportunity to measure low abundance proteins in small volumes of for instance plasma (131, 132).

Murine plasma proteomics

Previously our group showed that protein quantification of several coagulation factors in human plasma by MRM strongly correlated with quantification by conventional antibody and activity assays (133). The protein signatures allowed for successful separation of patients with the diagnosis of VT and cancer-related VT emphasizing the potential of this technique for both diagnostics and for biomarker detection (133). MRM also holds potential in murine research as plasma volumes in mice are small and available antibodies required for conventional protein assays are limited. Therefore, recently, a large peptide panel was developed to quantify 500 plasma proteins in mouse using MRM (134). This panel was successful in separating different mouse strains based on the plasma proteome and in addition sex-dependent differences were reported (135). In the work described in this thesis, a modified version of this peptide panel is used to study the effects of VT and SLC44A2 on the plasma proteome in mice.

Aims and outline of this thesis

Within the context of this thesis, we aim to unravel the mechanism underlying the association between SLC44A2 and VT. As SLC44A2 does not belong to the coagulation or fibrinolytic system, dissecting these mechanisms is a powerful strategy to generate new biological insights into the pathophysiology of VT. In addition, as all current therapeutic strategies come with a risk for bleeding, novel pathways might present possibilities for new therapies.

From literature the following hypothesis arose regarding SLC44A2 and VT 1) SLC44A2 does not influences hemostasis, as GWAS do not link SLC44A2 2288904 SNP variances to haemostatic biomarkers included in the study. 2) SLC44A2 on endothelial and/or blood cells mediate the effect of SLC44A2 on VT, as these cell types express SLC44A2 and have a central role in VT. 3) SLC44A2 exhibits its effect on VT by neutrophil activation and NET release, similar as in TRALI, which also has a role for SLC44A2. 4) SLC44A2 exhibits its role in VT by binding of SLC44A2 on cells to VWF, as previous reports revealed these as binding partners. To test these hypotheses, a mouse deficient in SLC44A2 is used and throughout the chapters of the thesis, we will explore different aspects of hemostasis and thrombosis. To study the role of SLC44A2 rs2288904, human neutrophils expressing variations in this SNP are used.

Despite the deletion of the *Slc44a2* gene, mice appear relatively normal and no aberrancies were previously detected with the exception of hearing loss upon age. In **chapter 2** these mice are subjected to a general characterization of hemostasis to investigate whether SLC44A2 affects this process. Several parameters of hemostasis were studied i.e.: thrombin generation potential, expression of coagulation related genes, VWF characteristics and platelet aggregation. In addition a challenge of hemostasis was applied by introducing endothelial damage to the cremaster muscle arterioles.

In **chapter 3** we took our research a step further and moved from hemostasis to thrombosis. *Slc44a2* deficient mice were subjected to two independent models of VT. First, a model which challenges coagulation by siRNA-mediated inhibition of protein C and antithrombin resulting in a procoagulant state was used. Second, a model of VT that is driven by an inflammatory response upon flow restriction of the IVC was employed. To further elucidate the underlying mechanism, several elements of VT, i.e. blood, neutrophils and platelets were further investigated *ex vivo*.

Plasma protein levels hold important information on health and disease, and MRM can be used to measure the plasma proteome. In **chapter 4** we have employed a recently developed multiplex panel of peptide standards allowing measurement of 375 proteins (unbiased to pathway) in murine plasma. To first show the validity of this methodology, the plasma proteome was determined in wild type mice with VT induced by siRNA mediated inhibition of Protein C and antithrombin. Next, to determine the effect of SLC44A2 on the plasma proteome, we applied this panel to plasma of *Slc44a2* deficient mice.

In **chapter 5** we investigate the association between SLC44A2 and VWF in the setting of VT and move our research from mice to humans. To test the experimental set up and to replicate previous findings, HEK293 cell overexpressing the minor or major SLC44A2 rs2288904 allelic variant were perfused over VWF-coated flow chambers. Next, human neutrophil adhesion, activation and NET release was investigated in this perfusion set up. To study neutrophil adhesion in a more complex setting in *Slc44a2* deficient mice, neutrophil

mobilization towards the venules of the mesentery veins was determined upon endothelial activation.

In **chapter 6** the results described in this thesis are further discussed and **chapter 7** provides a Dutch summary of the findings.

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