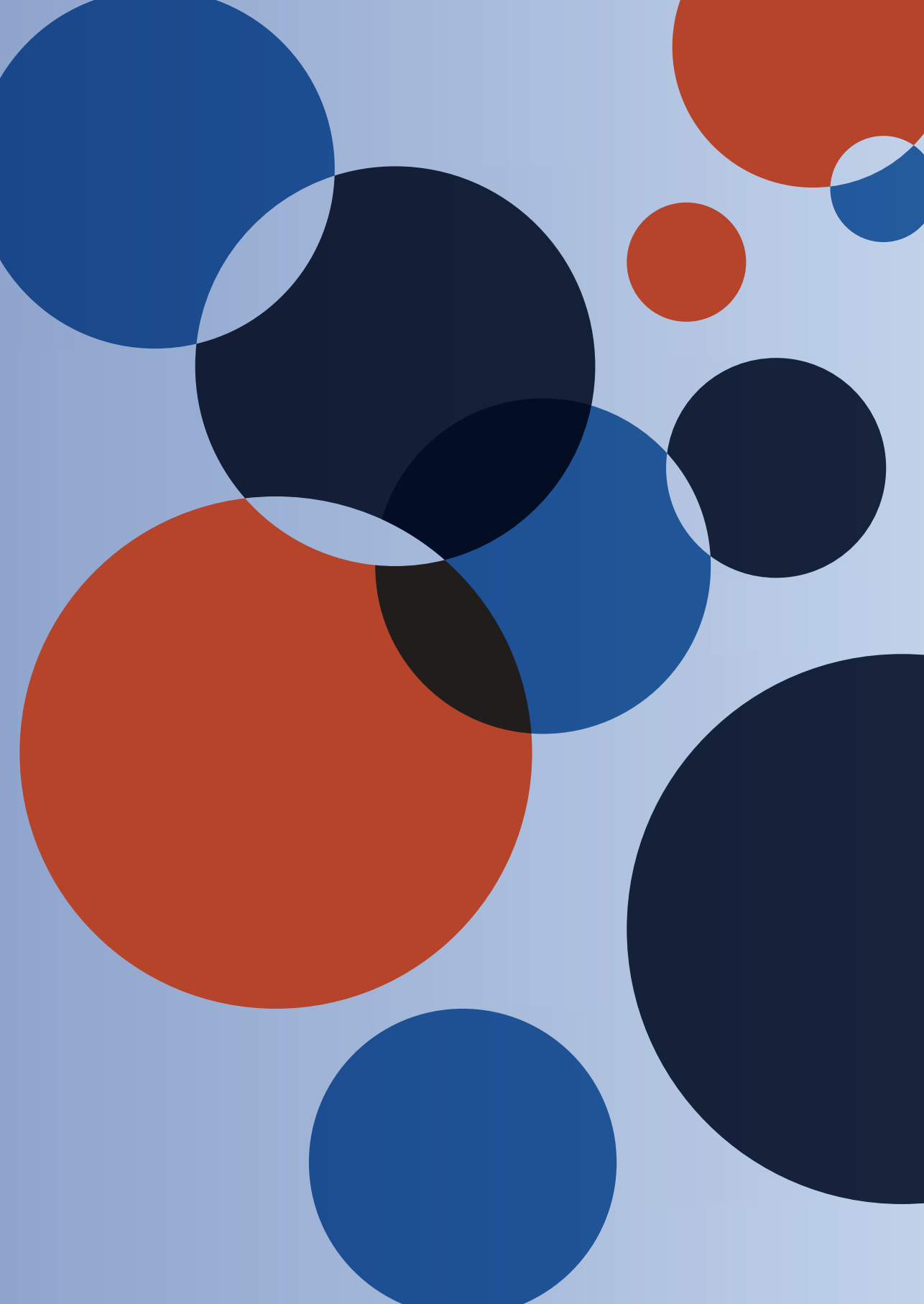

GENERAL DISCUSSION AND PERSPECTIVES

Julia Tilburg

6

Venous thrombosis (VT) is a multifactorial disease characterized by the development of an unwanted blood clot. In addition to several environmental risk factors, genetic predisposition contributes to VT development. Genome wide association studies (GWAS) were performed to identify novel VT susceptibility genes and to thereby increase our knowledge on the genetic landscape of VT (1, 2). Besides confirming the associations with known genetic risk factors, two novel loci were revealed, one of them harboring the *SLC44A2* gene. *SLC44A2* encodes the solute carrier family 44 member 2 (SLC44A2), a protein that does not belong to the conventional pathways of thrombosis (3). This is of interest as this association implies that there are undiscovered mechanisms to be revealed regarding the pathophysiology of VT. In addition to functional insights into thrombotic disease, these mechanisms might also present therapeutic targets, potentially without a risk for bleeding. The work described in this thesis aimed to provide functional insights into the mechanism underlying the association between SLC44A2 and VT.

SLC44A2 in hemostasis and thrombosis

We started our investigation into this mechanism by examining the effects of *Slc44a2* gene deletion on hemostasis in mice. Previously, it was shown that the deletion of genes with a crucial function in the hemostatic process may lead to a severe hemophilic or thrombotic phenotype in mice. For instance, it was shown that mice with deletions of the anticoagulant genes tissue factor pathway inhibitor (*Tfpi)* or antithrombin (*Serpinc1*) die during embryonic or gestational days as a consequence of thrombotic features (4, 5). However, mice with a *Slc44a2* gene deletion were fully healthy without clinical phenotype or aberrant development (6). This gave us a first indication that SLC44A2 is not vital to normal hemostasis. Subsequently, we observed that RNA expression (Chapter 2) and plasma protein levels (Chapter 4) of coagulation (related) factors were not altered by SLC44A2 deficiency. This coincided with normal thrombin generation potential in plasma of these mice (Chapter 2), which is indicative of a normally functioning hemostatic system (7). Both these findings are in line with observations from the GWAS, which show that the VT linked small nucleotide polymorphism (SNP) rs2288904 in *SLC44A2* did not affect hemostatic biomarkers included in the GWAS study such as thrombin generation potential and coagulation factor levels (1). The modest drop in von Willebrand factor (VWF) levels in SLC44A2 deficient mice as reported by our group, does not correlate with findings in a human setting that show that VWF levels are unaffected (chapter 2). Together, these observations indicate that SLC44A2 is not involved in normal hemostasis and imply that other mechanisms are underlying the association of this gene with VT.

We took our research a step further and moved from normal hemostasis to pathology to further investigate the mechanism by which SLC44A2 influences thrombosis. To this end, four different murine models representing different elements of thrombosis were used (Chapter 2, 3 and 5). Interestingly, SLC44A2 deficiency resulted in smaller thrombi or reduced neutrophil adhesion in models driven by damage of the cremaster arterioles, inflammation of the mesenteric venules, or stenosis of the inferior vena cava (IVC). In

contrast, the onset of VT was unaffected when *Slc44a2* knockout mice were exposed to VT driven by a hypercoagulable state. The main difference between the various models used is the site of action. The cremaster and stenosis model both have a predefined location of blood clot formation, the site of vessel injury and ligature respectively. The inflammation of the mesentery vein model has a more systemic mode of action but also here, endothelial activation is driving the adhesion of blood cells. In contrast to these three models, endothelial activation or interaction is not the main driver of VT in the hypercoagulability model (although this might occur). Here, it is the imbalance of the pro- and anticoagulant factors in the blood causing the spontaneous development of thrombi. Together, the results from these models provide a compelling argument towards a mediating role for the endothelium, but not coagulation, to drive the effect of SLC44A2 on VT. Interestingly, SLC44A2 is expressed on both the endothelium and on cells that are known to interact with the endothelium in VT, i.e. platelets and neutrophils (8-10). This led us to hypothesize that 1) the endothelium or 2) the interaction between the endothelium and blood cells is involved in the association of SLC44A2 and VT. A systemic (Lipopolysaccharides) and local (ligation of the IVC) stimulus prompting endothelial activation was applied to determine the effect of SLC44A2 in this activation. In both settings activation of the endothelium was found unaffected by SLC44A2 status (Chapter 3). The effect of SLC44A2 on the endothelium was also measured *in vitro* using human umbilical vein cord endothelial cells. Neither overexpression nor knockdown of the *SLC44A2* gene showed an effect on endothelial activation markers measured, including secretion of VWF (unpublished data). Together, these observations in mice and human settings are indicative of a normal endothelial response upon alterations in SLC44A2. Therefore, we focused on our second hypothesis i.e. alterations in the endothelium-blood cell interaction are the contributing factor of SLC44A2 leading to VT.

SLC44A2 mediates Von Willebrand factor neutrophil interaction

SLC44A2 is highly expressed on neutrophils and it is well accepted that neutrophils have a pronounced role in the VT models in which we observe an effect of SLC44A2 (11). In addition, previous studies demonstrated that the A1 domain of endothelial VWF is a SLC44A2 (rs2288904) binding partner (12). These observations, together with our hypothesis that SLC44A2 influences VT through the endothelial-blood cell interaction, piqued our interest. Using flow channel systems, we explored the interaction of neutrophils with VWF. We identified SLC44A2 on neutrophils to be responsible for binding of these cells to VWF resulting in the activation and release of neutrophil extracellular traps (NETs), a process referred to as NETosis (Chapter 5). In addition, we showed that neutrophils homozygous for the minor allele of *SLC44A2* (rs2288904-AA) display a reduction in this adhesion. Our observations thereby form a mechanistical explanation for the reduced VT risk in rs2288904-AA individuals (Odds Ratio (OR): 1.21) (1, 2). Moreover, this possible mechanism also reveals novel insights into the pathophysiology of thrombotic disease itself (Figure 1). It is important to note that for the interaction between VWF and neutrophilic SLC44A2, presence of VWF on the endothelium is required. We therefore envision that this mechanism is augmenting thrombus propagation rather than being a trigger for thrombosis.

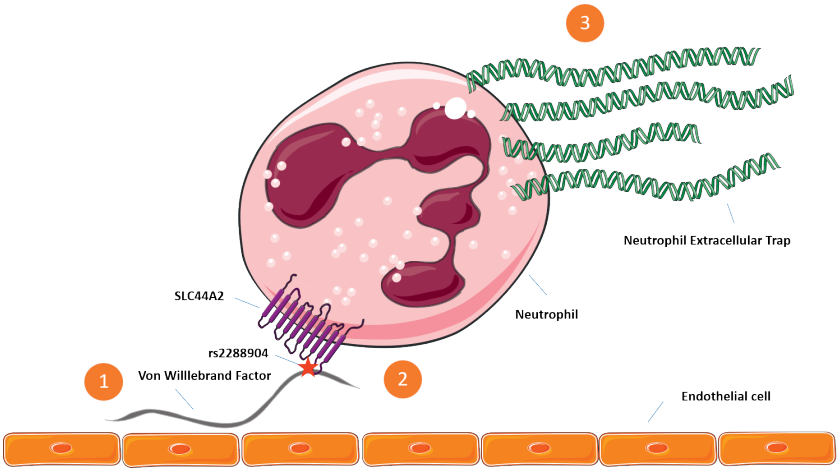

Figure 1 Proposed mechanism underlying the association between SLC44A2 and venous thrombosis.
1) Upon endothelial activation, Von Willebrand Factor is secreted from endothelial cells or captured from circulation and uncoiled, exposing the A1 domain.
2) Neutrophils bind to VWF via the SLC44A2 receptor to the A1 domain on VWF.
rs2288904-G, located within the receptor, has less affinity for this binding compared to rs2288904-A.
3) Neutrophil activation and release of neutrophil extracellular traps propagate thrombosis.

Interestingly, Constantinescu-Bercu and colleagues recently postulated another mechanism by which SLC44A2 contributes to VT involving platelet-neutrophil crosstalk (13). It is well established that platelets and neutrophils bind to each other through P-selectin/P-selectin glycoligand-1 and CD40L/CD40 interactions (14-16). Both these interactions require the activation of platelets to present P-selectin and CD40L on their outer membrane. Remarkably, in low flow microfluidic channel systems, VWF-bound-platelets can bind neutrophils in the absence of full platelet activation. This indicates that alternative mechanisms of platelet-neutrophil interactions are occurring under venous flow. The work of Constantinescu-Bercu and colleagues reveals that upon binding of platelets to VWF, adhesion of neutrophils to these platelets is mediated by platelet integrin αIIbβ3. Interestingly, they identified SLC44A2 as the counter-receptor on neutrophils for the interaction with platelet integrin αIIbβ3. Similar to our observations (Chapter 5), neutrophils with SLC44A2 rs2288904-AA (minor allele) show a reduction in binding and a subsequent reduction in neutrophil activation and NETosis. In contrast to our findings and previous studies by Bayat and colleagues, their experiments do not show a direct interplay between SLC44A2 and VWF (12). One difference in experimental set up is the venous flow applied by Constantinescu-Bercu and colleagues (25 and $50s^{-1}$), which is lower compared to the flow we used ($100s^{-1}$). Possibly, the lower flow does not permit the neutrophils to directly interact with VWF. Overall, these differences observed in SLC44A2-mediated neutrophil vessel wall interplay are of interest and warrant further investigation.

Despite this difference of neutrophil binding, either to VWF or to VWF-bound-platelets, the proposed mechanisms by which *SLC44A2* rs2288904 SNP variants impact VT is similar i.e. altered neutrophil activation and NETosis. Of interest, NETosis is also the driver of another disease with a pronounced role for *SLC44A2* rs2288904; transfusion related active lung injury (TRALI). Evidence for this was previously presented in mice, and disruption of NETs by DNase-1 treatment improved outcome in a TRALI disease model (17). Neutrophil activation and NETosis are also known drivers of immunothrombosis in an experimental mouse model. In the stenosis VT model, both the deletion of peptidyl arginine deiminase 4 (PAD4), a protein crucial in NET formation, and the treatment with DNase-1 resulted in a reduction of thrombus formation (18, 19). The effect we observed of SLC44A2 and the pronounced role of NETs in this model together is therefore supportive of our proposed mechanism by which SLC44A2 influences VT. Interestingly, however, while looking into the composition of the thrombi developed following stenosis of the IVC, we made some observations contradicting NETosis as a driver of the effect of SLC44A2. When neutrophil binding occurs, activation and NETosis is impaired in SLC44A2 knockouts, one would expect that neutrophils and/or NETosis in these thrombi is reduced. Remarkably, we find similar levels of neutrophil and NET markers both at 6 hours (initiation) and 48 hours (propagation) post stenosis (Chapter 3). This raises the question whether the methodology we used to investigate these components is adequate. At 6 hours (thrombosis initiation), we observe variation in neutrophil and NET positivity due to small thrombus size. At 48 hours (thrombosis propagation), the thrombi are fully formed, potentially disallowing us to detect a possible effect occurring in early phases. To overcome these limitations, neutrophil adhesion to the vessel wall upon stenosis can be captured using intravital microscopy of the IVC (11). This is a key experiment to visualize a delay in neutrophil adhesion to the vessel wall, which is expected in SLC44A2 knockout mice. Blocking the integrin αIIbβ3 receptor on platelets will provide answers as to whether SLC44A2 on neutrophils binds VWF directly, via the αIIbβ3 on platelets or perhaps a combination of both. To exclude a role for SLC44A2 on other cell types (for example the endothelium or platelets), follow up murine experiments should preferably be performed using mice deficient for *Slc44a2* specifically in neutrophils. This can be established by crossing mice with a conditional (floxed) *Slc44a2* allele with mice expressing *Cre* recombinase under control of the neutrophil specific Ly6G promotor, which are both available mouse lines (20). These proposed experiments will complement the knowledge we have on the mechanism involving VWF/neutrophil SLC44A2-associated NETosis and determine whether this mechanism is underlying the observed reduction in thrombosis observed in SLC44A2 deficient mice.

Thus far our murine studies have investigated full deficiency of SLC44A2. The GWAS findings however, identified *SLC44A2* rs2288904 as a susceptibility allele for VT and our performed microfluidic flow channel experiments indicate this SNP to be responsible for altered adhesion of neutrophils (Chapter 5). It would be of interest to further study these *SLC44A2* rs2288904 findings in relation to our observations regarding experimental VT in our knockout mice. Laboratory mice are inbred which makes them ideal as experimental

animals as they show little variability in their genetic composition. However, this also means that they do not have allelic variance in *SLC44A2* rs2288904, which would be of interest for our study. Knock in mice expressing human SNP variances of genes involved in coagulation have been previously generated. For instance, mice with the Factor V Leiden variation have been widely used and have led to novel insights into this SNP and its role in thrombosis (21, 22). Development of a 'humanized' *SLC44A2* knock in mouse with the minor *SLC44A2* rs2288904 allele would serve as an interesting tool to further investigate the role of the rs2288904 SNP in VT and, potentially, other diseases such as TRALI.

These knock in mice together with additional flow chamber experiments will enable further dissection of the role of SLC44A2 rs2288904 in NETosis and VT. In addition to these *ex vivo* and *in vivo* experiments, it would be of interest to translate our findings back to the human population. Neutrophil activation and NETosis can be monitored in human plasma by measurement of elastase α1-antitrypsin complexes and circulating nucleosomes, respectively. Interestingly, a previous study detected elevated levels of these markers in VT patients (23). This study did not provide genetic information, hence these levels could not be correlated to *SLC44A2* rs2288904 status. Conversely, the GWAS associating SLC44A2 with VT did not include plasma biomarkers for neutrophils activation or NETosis. A study by Heestermans and colleagues aimed to combine both plasma markers for NETosis and *SLC44A2* rs2288904 data, to see whether there is a relation between SLC44A2 and these levels. In this study, elevated plasma levels of both circulating nucleosomes and elastase α1-antitrypsin complexes were found in VT patients, which is in accordance with previous findings (24). Against expectations however, no association was found between neutrophil/NETosis markers and *SLC44A2* rs2288904 status. One limitation of this study is the grouping of rs2288904-GA and rs2288904-AA (minor allele), which was essential because of the low number of included rs2288904-AA carriers. Our data would argue against this grouping as rs2288904-GA neutrophils still present rs2288904-A, although to a lesser extent. One might expect that the interaction of rs2288904-GA neutrophils with VWF is possibly still occurring to a similar rate as rs2288904-GG because of the presence of this one allele. Overall, the approach presented in the study of Heestermans is of great interest, but an increased sample size and further genotype stratification will be necessary to capture a possible effect of SLC44A2 rs2288904 on NETosis in the human population.

Alternative hypotheses explaining the mechanism underlying the association of SLC44A2 and venous thrombosis

Overall, our research suggests that neutrophilic SLC44A2 is mediating the interaction of neutrophils with VWF. We thereby look at the function of SLC44A2 as a binding partner but overlook two other important features of SLC44A2; the suggested transporter of choline and a protein presenting the human neutrophil antigen-3 (HNA-3). Neutrophils only express the P1 variant of SLC44A2, which is unable to transport choline (10). Therefore, it is not expected that SLC44A2 deficiency or rs2288904 allelic variances will alter choline transport and related functionality of neutrophils. It would be of interest however, to investigate

whether SLC44A2 rs2288904 allelic variances can alter the transport of choline in cells which do express the P2 variant such as endothelial cells. This is of interest, because if alteration in transport capability is observed for the SLC44A2 rs2288904 allelic variants, this might reveal potential insights into VT susceptibility of this SNP. Building on this suggested choline transport function of SLC44A2 is the group of Dr. Lowenstein from Rochester University. Their unpublished research indicated that altered transport of choline by SLC44A2 is affecting acetylcholine levels in platelets (The hemostasis GRC, 2018, poster presentation). Acetylcholine is an endogenous inhibitor of platelets and thereby levels can influence hemostasis (25). Remarkably, their research implies that SLC44A2 on platelet has choline transport capacities. This is in contrast with previous reports showing that platelets do not present the SLC44A2 P2 variant on their membrane (10). In addition, the group of Lowenstein, investigated the effect of SLC44A2 on tail bleeding time in mice, which is an overall measurement of the hemostatic system. Their preliminary data shows elongated tail bleeding time in *Slc44a2* knockout mice, which is in consensus with our observations of SLC44A2 deficient mice showing a reduction in clot formations (Chapter 2 and 3). It would be exciting to see if they can specifically relate the elongated tail bleeding time to platelet SLC44A2 mediated choline uptake. Although multiple aspects of the hemostatic system influence tail bleeding time it should be noted that mice that do not produce NETs, as a result from PAD4 deficiency do not display aberrancies in this hemostatic challenge (18). Therefore, the by Lowenstein and colleagues observed elongated tail bleeding time does not fit the mechanism proposed by us (Figure 1).

Beside the suggested role of SLC44A2 as a choline transporter, SLC44A2 forms the HNA-3 epitope. As the same SNP, rs2288904, and codes this epitope and is associated with VT, an interest is sparked for studying the role of anti-HNA-3 antibodies in thrombotic disease. One might envision similar mechanisms as in TRALI where HNA-3 antibodies bind to SLC44A2 on neutrophils resulting in neutrophil activation and NET release (17, 26). This phenomenon can then result in a higher susceptibility for VT. HNA-3A targeting antibodies are more common than HNA-3B targeting antibodies (27). Also, it is the HNA3A epitope which is associated with elevated VT risk, supporting a potential mechanism involving HNA-3 antibodies in VT (1). Our collaborators at the university of Marseille have started analysis to detect the presence of HNA3 antibodies in VT patients. Results from these studies are awaited with high interest, as elevated levels of these antibodies would reveal a novel element in the pathophysiology of VT.

Clinical implications for SLC44A2

VT has an incidence of 1-2 in 1000 people per year and together with the high rate of recurrence, this results in a large amount of people at high risk for developing VT (28). The anticoagulant treatment often prescribed to these people does not come without a cost, as all currently implemented treatments have a risk of bleeding (29). Because of these side effects, careful consideration of prescribing treatment is necessary, which is achieved by the use of prediction models that combine multiple risk factors (30). These risk prediction

models include, among others, sex, age, D-dimer levels, hormone therapy, body mass index and a history of previous VT (30, 31). Unfortunately, these predictions still suffer from limitations and the discriminating performance is often not optimal. Therefore, further optimization of these models is needed. The findings described in this thesis corroborate the GWAS study and thereby further substantiate the evidence for *SLC44A2* rs2288904 as a genetic risk factor for VT (1). *SLC44A2* rs2288904 status, however, would not make an attractive genetic candidate to be included in prediction algorithms for VT risk. The relatively small OR paired with *SLC44A2* risk (OR 1.21) and the large prevalence of the risk allele in the population (0.785), make SLC44A2 an inefficient discriminating biomarker (1). Moreover, the predictive value of testing for other genetic risk factors for VT is also debatable. Factor V Leiden (rs6025) and variation in prothrombin (rs1799963), both have a larger OR than *SLC44A2* rs2288904, 3.25 and 2.29 respectively, and are also currently not included in VT risk prediction (32). *SLC44A2* rs228904 is therefore not efficient for VT risk prediction. The mechanism by which *SLC44A2* influences VT, however, has potential clinical implications as a target for novel anticoagulant therapies. Our findings show that SLC44A2 does not influence hemostasis, which paves the way for a therapy without bleeding risk. We thereby envision that by blocking SLC44A2 rs2288904, a reduction of VWF/neutrophil SLC44A2-associated NETosis occurs, leading to a protection against VT.

Targeting NETs as a treatment for both arterial and venous thrombosis and other diseases such as TRALI, autoimmune diseases and cancer, has been previously suggested (33). Currently, this is mainly established in mice and *ex vivo* models, but so far, the studies yield promising results. The mode of action of these treatments is comprised of either 1) the degradation of NETs, by DNAse or 2) the blockage of NETosis. Blockage of NETosis can be accomplished in multiple ways, such as through the targeting of PAD4 or Janus kinase 2 (JAK2), another protein involved in NET formation, or by the lowering of reactive oxygen species resulting in a reduction of neutrophil activation (18, 34, 35). As described above, DNAse treatment and PAD4 inhibition have both resulted in a reduction of thrombus formation in the stenosis VT mouse model (11, 18, 19). This is also evident for the JAK2 inhibitor Ruxolitinib, which is an FDA approved drug for myelofibrosis in humans (35). Remarkably, it was observed that patients receiving Ruxolitinib had lower VT incidence in the polycythaemia vera (blood cancer) RESPONSE trial (36). This provides evidence of an anti-NET treatment that results in protection for VT in humans. Of note, this study was not designed for investigations into VT and therefore additional trials are needed (37). In addition to implementation of anti-NET treatment in thromboprophylaxis, the targeting of NETs has also been investigated in thrombolysis as a treatment to disrupt an existing thrombus. The benefits of NET targeting strategies in thrombolysis were recently shown in mouse models for myocardial ischemia and renal ischemia-reperfusion injury (38, 39). Here, the combination of the fibrin degrading protein tissue plasminogen activator (tPA) together with DNAse resulted in improved survival compared to tPA alone, which is the currently used treatment .

Together, these therapies hold a promising future in VT management, but as NETs hold an important biological function in the fight against bacterial infection, implementing these strategies in human can be challenging (40, 41). As follows, it is undesirable to develop novel VT therapies that cause an increased risk for bleeding (as with the current thrombotic therapies), or an increased risk for bacterial infection (as a result of NET inhibition). Therefore, it is important to take into careful consideration this beneficial function of NETs when further exploring NET targeting therapies in VT. A promising recent study in mice showed that PAD4 deficiency did not result in an increased risk of bacterial infection (42). These findings were contradicted, however, by a study that investigated PAD4 in a mouse model for myocardial infarction (43). Here, PAD4 deficiency results in aggravated acute inflammation as a consequence of impaired NET formation. Overall, we may conclude that substantial effort will need to be undertaken to determine safety and efficiency before NET therapies can be implemented for VT (44). Nevertheless, NET targeting therapies form an exciting field with a possible role for SLC44A2. One might speculate that by not blocking NETosis in general, but solely blocking VWF/neutrophil-SLC44A2 mediated NETosis, neutrophils can still exhibit their antibacterial NETosis function, while prothrombotic phenotypes are reduced. This is obviously a speculative assumption, but forms an interesting platform for further investigation into possible therapeutic applications of SLC44A2.

Plasma proteomics in thrombosis research

Plasma protein concentrations hold important information regarding the state of health and disease of an individual. A compelling tool to measure multiple plasma proteins with high precision is mass spectrometry based targeted proteomics. For the work described in this thesis, we have employed this technique to measure 375 proteins in mouse plasma. The experiment provided a wealth of information regarding the effect of the hypercoagulability driven model of VT and *Slc44a2* deficiency on the plasma proteome in mice (Chapter 4). It was observed that the plasma proteome of SLC44A2 deficient mice was weakly affected and the only changes were observed in proteins which are involved in cellular mechanisms. This is in line with the conclusion drawn above, i.e. SLC44A2 influences VT by affecting cellular components and not the coagulation system. Interestingly, we observed that the *Slc44a2* plasma proteome signature was overruled by the effect of sex. Females and males showed distinct plasma profiles which advocates for the careful selection of animals in research or preferably the inclusion of both sexes. It should also be noted that scientific journals currently support the use of both sexes unless arguments for the inclusion of one sex are presented (45). In addition to this observed effect of sex on the plasma proteome, previous reports also showed a variety in plasma proteome signatures in five different mouse strains, commonly used in murine research (46). Interestingly, mouse strain specific effects are also noticeable when looking at their coagulation potential, as differences were observed in coagulation assays such as tail bleeding time (47, 48). Together, the findings by both our group and others further substantiate the importance of taking the plasma proteome into consideration when selecting mouse strains and sex in murine research.

Currently, several initiatives are presented by our department to incorporate mass spectrometry based targeted plasma proteomics in human VT research. Showing the capabilities of this technique, plasma proteome dynamics were determined for patients with VT and the combination of VT and cancer (49). The two groups showed a clear separation based on the plasma proteome and moreover the measured plasma protein levels nicely correlated with levels measured by the conventional assays. Work presented at the International Society of Thrombosis and Hemostasis Congress 2019 described plasma proteomics signatures for patients that developed VT upon a plaster cast or knee arthroscopy (50-52). Findings from these signatures will potentially be incorporated in an expansion of the current prediction models for VT occurrence in these patients. In addition, research is performed to determine the plasma proteome of patients which have major bleeding events during treatment with Vitamin K antagonists. The researchers aim to discover novel biomarkers for bleeding events in these patient group to improve prediction (53). Together, these efforts will expand our knowledge of the plasma proteome in clinical and fundamental VT/bleeding research and further establish a solid mass spectrometry based targeted proteomics workflow (54).

The contribution of the genetic landscape to VT

Within this thesis, we aimed to provide a functional follow-up to the association between *SLC44A2* and VT which was identified by GWAS (1, 2). Moreover, our data successfully corroborated the GWAS and provided insights into the mechanism underlying this finding. In order to give a clinical implication to the GWAS findings, besides solely biomarker discovery, functional follow-ups are necessary. To avoid a discrepancy between the novel gene associations and the gain in functional insights, it is important to keep this overarching goal in mind. For instance, with respect to the other novel VT locus *TSPAN15*, no functional follow up has been published so far and the mechanism by which that gene might influence VT therefore remains elusive (1).

During our functional follow up to the previous GWAS finding, new actions have been undertaken to expand the knowledge on the genomic landscape and VT by Lindström and colleagues. They performed a large meta-analysis of several VT GWAS, which included 30.234 VT cases and 172.122 controls (55), a large increase compared to the previous study, which consisted of 7.507 VT cases and 52.632 controls (1). In addition to the increased population, the included genetic traits (SNPs measured) were increased from 6.751.884 to 12.923.718 SNP variants. Furthermore, this study goes beyond the genome alone by also including a transcriptome-wide association study (TWAS) on liver and whole blood expression data of these subjects. The GWAS and TWAS combined identified a total of 16 novel VT susceptibility loci, in addition to replication of the previously identified loci, including *SLC44A2* (55). This complements our current knowledge and leads to a list of 37 VT susceptibility genes which are summarized in Figure 3 (56). Altogether, the VT GWAS performed over the past decade made enormous contributions to our understanding of the genetic landscape of VT, but this list is still not complete. Together, these 37 genes

are estimated to be responsible for 15% of the VT heritability, while previous twin studies showed that the overall heritability of VT is 40-60%. Two methodologies can be employed to further reveal these genetic contributors; increase the SNPs and number of subjects of GWAS or use genome wide sequencing. The latter is currently used in other research fields and shows promising results (57).

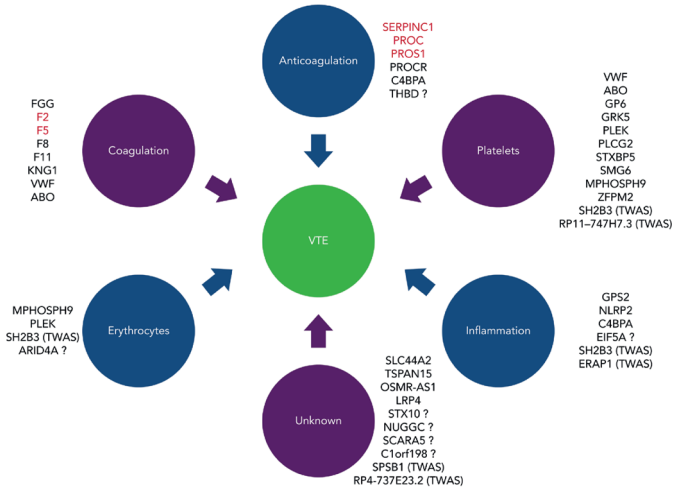

Figure 2 Genes associated with VTE. Genes are grouped according to biological links to VTE: coagulation, anticoagulation, platelets, erythrocytes, inflammation, and unknown. Genes associated with major thrombophilia are marked in red. Question marks denote non-replicated genes. TWAS shows that the gene was identified with transcriptome-wide association study. Adapted from: Zöller, Blood, 2019

The novel VT susceptibility genes identified by the latest GWAS include candidate genes, not related to coagulation. Interestingly, Lindström and colleagues included correlations of these associations with blood cell trait. Several of the novel genetic risk factors were correlated with either platelet or red blood cell traits, indicating that these variances exhibit their contributing role to VT through one of these cell types. From the genes correlating with platelet traits *GP6*, *ZFPM2*, and *VWF* are known, whereas *ABO*, *PLEK*, *GRK5*, *MPHOSPH9*, *PLCG2*, *SMG6*, and *SH2B3* are newly associated loci. Indeed, in line with these GWAS findings are mechanistic insights into the role of platelets in VT. It is suggested that alterations in platelet formation by megakaryocytes, elevated platelet aggregation, platelet derived microparticles, and higher platelet counts and volumes are linked to VT (58). Together, these observations and the presented GWAS findings are indicative of a larger role for platelets than originally thought, which encourages further research into the consideration of antiplatelet therapy in VT. Recent clinical trials are also advocating for the implementation of these therapies in VT, as these trials showed a reduction in VT upon antiplatelet treatment such as aspirin (59-61). In addition to the genes influencing platelet traits, associations were also found with

red blood cell traits. This sparked interest for a role of this cell type in VT risk, which is not unanticipated as they are a major constituent of the venous thrombus. From a mechanistic point of view, red blood cells are suspected to contribute to VT by several mechanisms. This includes altering blood viscosity, formation of platelet aggregates, adhesion to the vessel wall, involvement in the increase of thrombus size and their potential to promote thrombin generation, among others (62-64). The findings of Lindström and colleagues encourages further investigation into the role of red blood cells in the pathophysiology of VT. To summarize, the findings from the most recent VT GWAS hold potential for further functional follow-up studies. Thereby, the overall wealth of genomic data available opens up an opportunity to further dissect the pathophysiology of VT and form a foundation for potential novel therapeutic targets.

Concluding remarks

GWAS identified *SLC44A2* as a novel susceptibility gene for VT and the work in this thesis has corroborated these findings. Moreover, in mice, SLC44A2 deficiency was determined to not affect hemostasis. This is in line with our plasma proteome findings as we did not find an effect of SLC44A2 on plasma protein levels of proteins involved in coagulation. Interestingly, we observed a protective effect of SLC44A2 deficiency in multiple VT mouse models. Furthermore, our research provided evidence that SLC44A2 influences VT by altering the interaction of endothelial-VWF and neutrophils, mediated by SLC44A2. Neutrophils with the *SLC44A2* minor allele, previously identified as a protective SNP for VT, had reduced neutrophil-VWF interaction. Our study thereby revealed a novel mechanism in VT pathophysiology, involving cellular components of the blood. This paves the way for further investigation into novel therapeutics for VT targeting SLC44A2. Furthermore, our research underlines the potential of GWAS to reveal novel genetic contributors to this disease and encourages functional follow-ups to further dissect the pathophysiology of VT.

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