The endothelial compartment as a disease modifier in bleeding disorders

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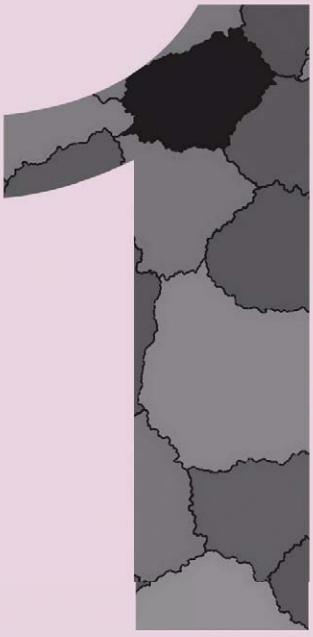
General introduction and outline of the thesis



Hemostasis is the vital physiological process that prevents excessive bleeding by maintaining the integrity of vessels and controlling blood coagulation. This involves an intricate and highly regulated interplay between many factors. The balance within this interplay, between pro- and anticoagulant and pro- and antifibrinolytic mechanisms, is sensitive to fluctuations and can lead to disease if changes are not corrected accordingly. Tipping too far to one side might lead to defective or overly active coagulation which increases the chance of bleeding or thrombosis respectively.

The role of endothelial cells and von Willebrand factor in hemostasis

One of the major players in the anti- and procoagulant as well as fibrinolytic mechanisms are the endothelial cells, which both passively and actively contribute to hemostasis. Primarily, the endothelium forms a physical barrier between blood and tissues. The barrier blocks binding to the underlying subendothelial matrix thus preventing activation of platelets and circulating coagulation factors (1). One of the main procoagulant roles of endothelial cells is the production and secretion of von Willebrand factor (VWF). 85% of this large multimeric protein is produced by endothelial cells and in lower levels by megakaryocytes (2). VWF produced by endothelial cells is stored in cell specific secretory organelles called Weibel-Palade bodies (WPB) (3). VWF multimers are coiled and stored in WPBs as long tubules which is determinant of the characteristic rod like shape of WPBs (4, 5). Once stimulated, the endothelial cells secrete the WPB and release large quantities of VWF into the vessel lumen (6, 7). VWF is also continuously secreted by endothelial cells to maintain steady state levels in the plasma. At sites of vascular injury, VWF can bind to the exposed subendothelial matrix and become activated. Subsequent binding to circulating platelets causes aggregation and the start of platelet plug formation (8). Steady state levels of VWF in the blood also protect coagulation factor VIII (FVIII) from degradation and ensure sufficient levels of circulating FVIII (9). In this general introduction we only scratch the surface of the intricate processes involved. See **Chapter 2** for a more elaborate and detailed review of endothelial cell functions, VWF and their role in the hemostasis (10).

Von Willebrand disease

When defects in the hemostatic process occur, bleeding disorders can arise. These can lead to excessive bleeding or faulty clotting. In von Willebrand disease (VWD), VWF is either defect or absent in varying degrees causing mild to severe bleeding abnormalities (1, 11). When defects occur in VWF, as a carrier protein of FVIII, the levels of FVIII can also be reduced. Defects or low levels of FVIII lead to a bleeding disorder called hemophilia A. VWD is found in roughly 1 in 100 people with symptomatic disease that requires specific treatment occurring in 1 in 10.000 people, making it the most common bleeding disorder worldwide (12). VWD can be divided into three subtypes. Type 1 and type 3

VWD are caused by defects leading to a quantitative deficiency of VWF. Type 1 VWD patients have reduced but not absent levels of VWF, usually presenting with a mild or moderate bleeding phenotype. A subtype of type 1 VWD, Type 1C, has also recently been recognized and is associated with an increased clearance of VWF from the plasma (13). Type 3 VWD is characterized by a complete absence of VWF, making it the most severe type. Type 2 VWD is associated with qualitative defects in VWF (1, 11) which can be further divided into subtypes. Type 2A is characterized by lack of the highest molecular weight multimers, type 2B by enhanced or spontaneous binding to platelets, type 2M by decreased binding to platelets or collagen and finally type 2N, where VWF has decreased binding to FVIII.

Large variation in bleeding phenotype between but also within the subtypes of VWD exist. Over the past three decades more than 750 unique mutations in *VWF* have been found (14). Interestingly, approximately 30-50% of patients with type 1 VWD do not have pathogenic *VWF* gene variants (15-17). The reduced levels may be caused by increased clearance of VWF or due to impaired synthesis or secretion of VWF and it is hypothesized that other genetic modifiers may be the cause of the reduced VWF levels and bleeding phenotype in those patients (18). Previous genome-wide association studies have studied genetic determinants of VWF levels outside of the *VWF* gene. They have found genetic variability in genes which correlated with VWF levels either by affecting the WPB secretory processes, like *STXBP5* and *STX2* (19) or by affecting clearance (*CLEC4M*) (20). This suggests that exocytosis of WPB is important in the regulation of VWF levels. However, these findings only explain a limited part of the regulation of VWF levels and thus encourages further research into additional genetic modifiers. In this thesis, one of the research aims was to identify determinants of lower VWF levels in patients without pathogenic *VWF* variants (**Chapter 7**) and we hypothesized that defects in the components of the secretory machinery of VWF are causative for those lowered levels.

Treatment of von Willebrand disease

Most treatment options for VWD aim to prevent or treat the bleeding when it occurs. Logically, one of the treatment options aims to increase the levels of functional VWF and/or FVIII in the plasma by administering factor concentrates (21). Alternatively, VWD and other bleeding disorders can be treated by 1-deamino-8-D-arginine vasopressin (DDAVP) also known as desmopressin (22). This synthetic vasopressin analogue can activate the vasopressin V2 receptor (V2R) on endothelial cells (Figure 1). This is a G-protein coupled receptor (GPCR) on the membrane of endothelial cells in blood vessels. Upon binding of DDAVP to V2R, the receptor undergoes a conformational change that activates the cyclic adenosine monophosphate (cAMP) pathway (23). cAMP,

in turn, activates protein kinase A (PKA) which plays a central role in various cellular responses. One of which is the induction of exocytosis of WPBs in endothelial cells which increases the levels of VWF and FVIII in the plasma. Alternatively, cAMP can activate Epac1 which also induces WPB secretion (24). In endothelial cells, it has been shown that VWF and FVIII are co-trafficked to the WPBs (25-27) although the majority of FVIII is synthesized and stored in the liver sinusoid endothelial cells (LSECs) (28-30). It has also been shown that FVIII is produced in other endothelial beds, such as in kidney (30), and in lung (31). Interestingly, vascular endothelial cells, especially lung endothelial cells express V2R (23), but it is unclear whether LSECs express this receptor. Furthermore, it has been shown that DDAVP increases VWF levels, but not FVIII levels in hemophilia A patients after receiving a liver transplant (32). Collectively, this suggests that FVIII release after DDAVP administration is mainly caused by secretion of FVIII together with VWF by vascular endothelium and not liver endothelial cells.

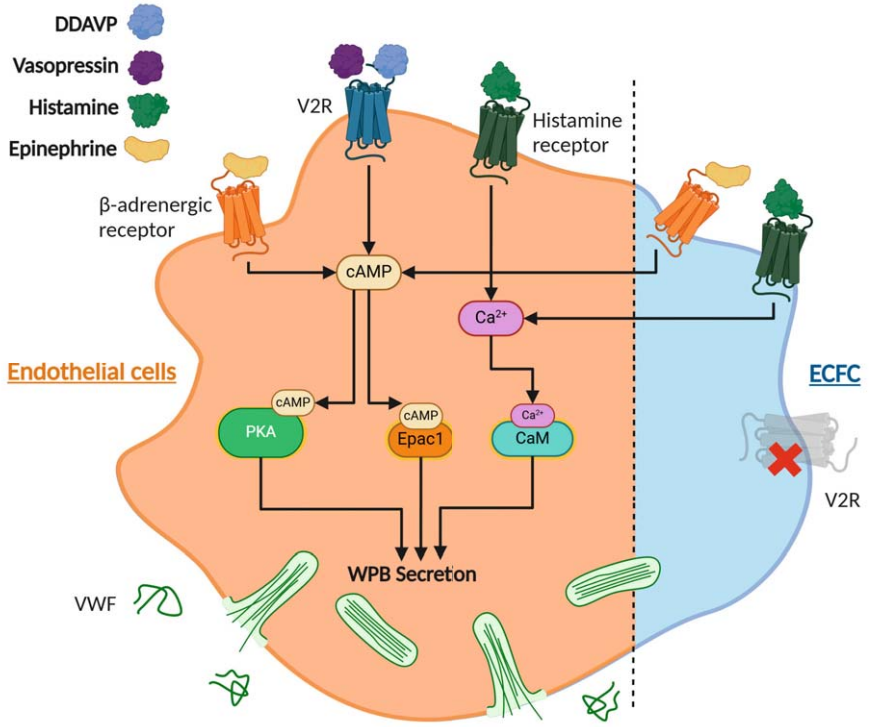


Figure 1. Simplified overview of the secretory pathways in endothelial cells. The schematic model depicts general endothelial cells (orange) and endothelial colony forming cells (ECFC) (blue). Abbreviations: VWF, von Willebrand factor; WPB, Weibel-Palade body; DDAVP, 1-deamino-8-D-arginine vasopressin; V2R, vasopressin 2 receptor; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; CaM, calmodulin; Epac1, exchange factor directly activated by cAMP 1. Created with BioRender.com

The stimulation of endothelial cells by DDAVP increases the levels of both FVIII and VWF in the plasma by 2-5 fold (33). This fold change is largely dependent on the amount of protein stored within the cells, the efficacy of secretion and the rate of clearance. Therefore, DDAVP is often administered in patients with low, but not deficient levels of VWF, like type 1 VWD or mild to moderate hemophilia A. DDAVP is one of the most readily available treatments for patients with bleeding disorders. Furthermore, because it can be administered intranasally by patients at home, it greatly improves quality of life. However, large inter-individual differences in response to DDAVP have been observed. Studies have been performed to elucidate the determinants of response like age, blood group, disease severity or mutation type (**Chapter 6**). Unfortunately, the cause of the variation is not yet fully understood. Comparatively to the unexplained low levels of VWF in VWD patients without a *VWF* variant, the variation and/or lack of response to DDAVP could be caused by undetermined genetic modifiers that affect the endothelial secretory pathway. A second research aim in this thesis was to identify determinants of DDAVP non-response in VWD patients (**Chapter 7**). The question then is, how can we study and understand this variation?

Endothelial cell models

Over the years, a number of approaches have been developed to investigate the pathophysiology and the underlying cause of bleeding in patients with bleeding disorders. For example, plasma coagulation factor levels can be measured in the plasma taken from the patients. These factors can also be functionally tested using aggregation or activity assays. However, these tests do not take into account how the factors are synthesized, stored and secreted by the endothelial cells. Model systems have been developed that can reflect the cellular context from which the pathogenic mechanisms could be investigated. VWF can be brought to expression through transfection into non-endothelial cells like Chinese hamster ovary and human embryonic kidney 293 cells (HEK293). The ectopic expression of VWF in HEK293 cells also induces the formation of pseudo-WPBs. These models can be used to study VWF *in vitro* and the pathogenic effect of mutations can be analyzed by transfecting a mutant version of VWF (34). However, these models are limited as they do not have a native environment wherein VWF is produced and the complete secretory machinery. Furthermore, only known mutations can be tested in these models.

Endothelial cells from the patient can be directly collected from various vascular beds. However, these invasive procedures are not ideal as they burden the patient. Fortunately, two methods exist where endothelial cells can be derived from whole blood via venipuncture. In the first model, peripheral blood mononuclear cells can be reprogrammed into induced pluripotent stem cells (iPSCs) (35). These can then be

differentiated into iPSC endothelial cells (iPSC-ECs) which have the great advantage of carrying the genetic background of the patient it was derived from. Unfortunately, maturation of WPB is impaired in iPSC-ECs, making it difficult to study this aspect of endothelial function (36) (**Chapter 5**). The second model cell is named endothelial colony forming cells (ECFCs), previously known as blood outgrowth endothelial cells. ECFCs also carry the genetic background of the donor from which the whole blood was collected. Additionally, in ECFCs, WPB mature fully allowing studies to be performed on their formation, maturation and secretion. ECFCs have already been used extensively as a model to study VWD patient specific defects (37-42) (reviewed in **Chapter 2**). Although ECFCs do not express the V2R receptor (42) and can thus not be stimulated by DDAVP, they can be stimulated by other agonists like epinephrine or histamine to activate the cAMP or Ca^{2+} pathways respectively (Figure 1). This means that ECFCs can also be used to study stimulated secretion defects like in VWD patients that do not respond to DDAVP. Despite these advantages, ECFCs can also be challenging to work with. Large heterogeneity exists not only between ECFC clones derived from healthy controls, but also between clones from the same donor. Variation has been observed in cell size, VWF production, WPB quantity and size, cell growth, migration and RNA expression (43-45). It is currently unclear what causes these differences warranting further research. Another research aim of this thesis was to elucidate the extent and the potential cause of this variation (**Chapter 4**).

The importance of hemostasis and the challenges of managing bleeding disorders underscore the need for ongoing research. Understanding both the clinical aspect and the fundamental pathophysiology of bleeding disorders on a cellular level is therefore essential in improving outcomes and developing innovative treatments for affected individuals. Therefore, in this thesis we studied various aspects of the endothelial compartment as a disease modifier in bleeding disorders. This is further elaborated on in the outline of the thesis.

Outline of the thesis

In this thesis, the endothelial compartment and its role in the pathophysiology of bleeding disorders is analyzed in multiple ways. First, in **Chapter 2,** the use of ECFCs in analyzing the pathophysiology in bleeding disorders is reviewed. There, we go into detail what contribution endothelial cells play in the primary and secondary hemostasis. Specifically, how the endothelial cell model, ECFCs, was used in previous research to explore pathogenic effects, but also mechanical aspects in patients with various disorders. Following this, **Chapter 3** introduces the concept of automated

quantification techniques which can be used to analyze and quantify the morphology of many parameters of the cell. In this chapter, we developed an extensive automated pipeline using CellProfiler for the quantitative analysis of cell and organelle morphology, localization, and content. We show that the pipeline can be used on various cell models and organelles, specifically endosomes and WPBs. Furthermore, we show the quantitative power of the pipeline by comparing different groups of ECFCs clones. It is known in literature that large heterogeneity between ECFC clones exists. We used this heterogeneity as an example in **Chapter 3** to display the quantification capabilities of the pipeline. In **Chapter 4**, however, we dive into this heterogeneity and the challenge it poses for all research groups using this model. There, we look beyond the basic morphologic characterization and explore the transcription signature of a large panel of control-derived ECFCs. Using this signature, we created a minimal qPCR panel which can be used to classify and cluster ECFC clones. Between the clusters we show that inflammation and endothelial to mesenchymal transition may act as potential drivers for the phenotypic heterogeneity.

ECFCs offer a great way to get an inside look at the potential endothelial defects of patients. However, they are not the only endothelial model that offers this advantage. iPSC-ECs are another model that can be used to investigate patient specific defects. Our group has shown that one major disadvantage of the model is that the iPSC-EC do not seem to mature fully, showing low levels of synthesized VWF and small, round WPBs. In **Chapter 5**, we strived to optimize the cell culture, isolation and differentiation protocol to create iPSC-ECs that fully mature and especially, synthesize sufficient amounts of VWF and WPB with typical cigar-shaped morphology.

Patients with bleeding disorders are usually treated with appropriate factor concentrates or, when effective, with DDAVP. This drug is widely used for patients with VWD and hemophilia A as it quickly increases the plasma levels of both VWF and FVIII. However, a substantial portion of patients do not or only partially respond to this medication. In **Chapter 6** we undertake a systematic review and meta-analysis to elucidate the rate of response and possible determinants of DDAVP response in patients with bleeding disorders, offering valuable insights into therapeutic approaches.

Chapter 7 can be seen as the culmination of all chapters in this thesis. Here, we analyze the ECFCs derived from two groups of patients with VWD. Patients with VWD without a pathogenic gene variant identified within *VWF* and patients with VWD that do not respond to DDAVP. ECFCs were characterized and clustered according to our minimal qPCR panel (**Chapter 4**). The ECFC panel was analyzed extensively with various assays including imaging and subsequent image quantification (**Chapter 3**). In this study we

found patient specific functional defects which explain the bleeding phenotype in some patients. Furthermore, we correlated the large panel of functional aspects of the endothelial cells with their proteome, revealing processes and proteins of interest.

In **Chapter 8** the findings from the preceding chapters are summarized and discussed, paving the way for future research directions and potential implications in clinical practice. Through this structured journey, the thesis contributes to the growing body of knowledge in endothelial biology and its relevance in the context of bleeding disorders, offering valuable insights and avenues for future investigation.

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