The endothelial compartment as a disease modifier in bleeding disorders

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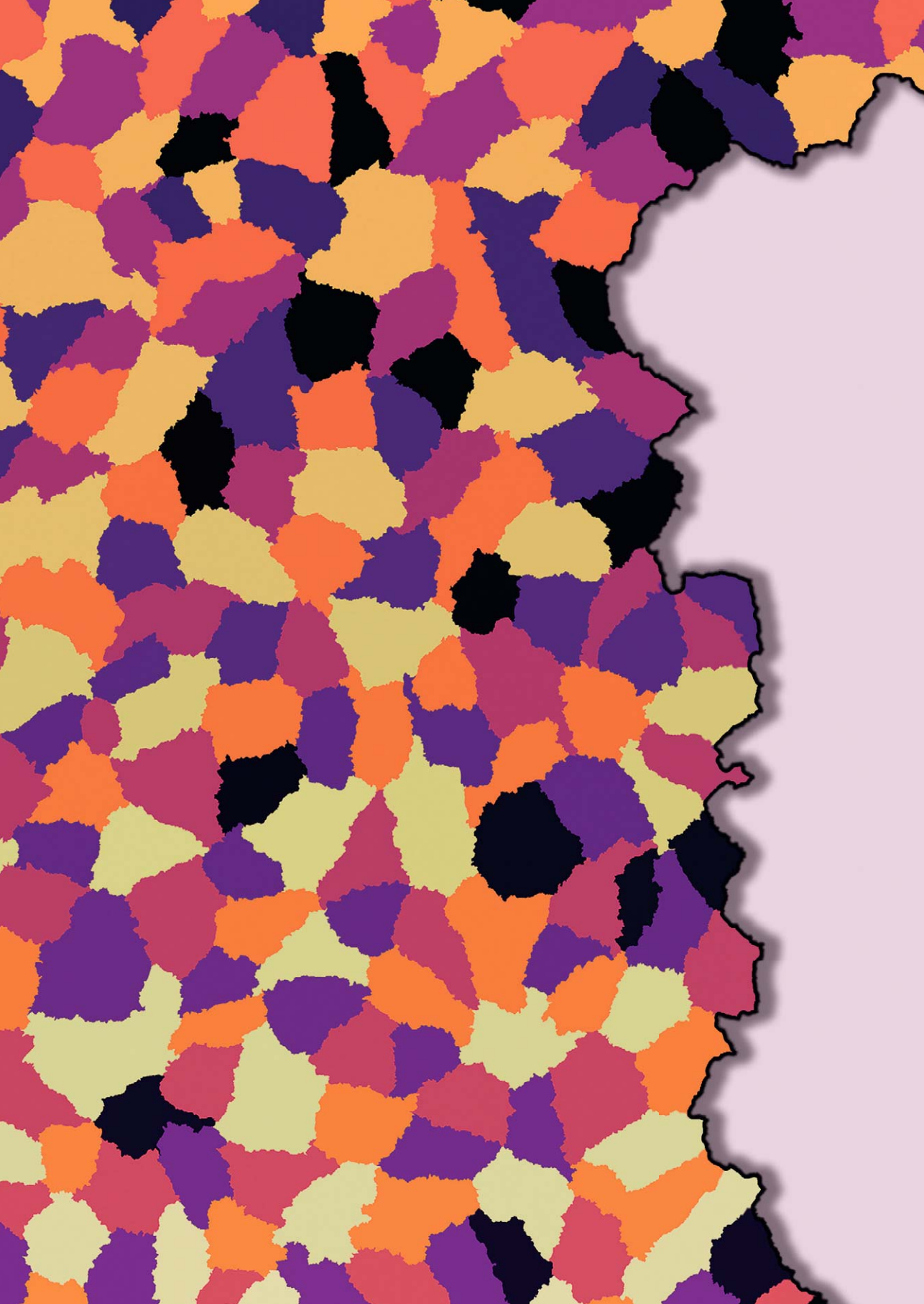
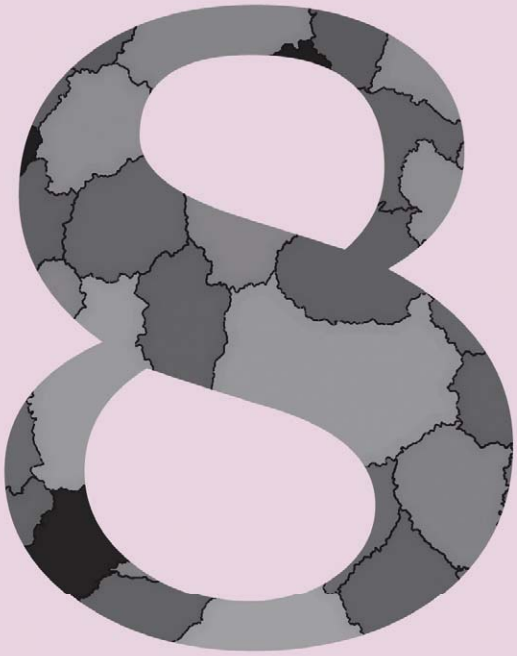


General discussion and perspectives



Study objectives

In this thesis, several aspects related to treatment in bleeding disorders, endothelial cell function and endothelial model systems in the context of studying bleeding disorders have been studied. The main aim of this thesis was to find defects in the endothelial secretory pathway that could potentially lead to bleeding which could not be explained through standard clinical testing. Specifically, we investigated patients with von Willebrand disease (VWD) with low von Willebrand factor (VWF) levels without a known pathogenic *VWF* variant or patients with VWD that did not respond to 1-deamino-8-D-arginine vasopressin (DDAVP). We also studied and optimized the method of using the endothelial colony forming cell (ECFC) model for research and how to automatically quantify morphological parameters of those cells. Each of the factors studied in this thesis contribute to our understanding of endothelial cell function in health and disease, but they also raise important questions about the methodologies used, the models chosen, and the future direction of this research which we will discuss here.

Where to look, methods for studying bleeding disorders

Bleeding disorders are complex due to the variety of underlying causes, ranging from genetic mutations to acquired conditions affecting clotting factors, platelets, or the vascular endothelium. For example, VWD is the most common bleeding disorder in humans (1), but it is difficult to diagnose due to multiple assays being needed and variation within those assays. The large heterogeneity and number of mutations or deletions in the *VWF* gene observed within patients further complicate the diagnosis (2). First level diagnostic tests (FVIII:C, VWF:Antigen and VWF:Activity plasma measurements) are performed on patients that present with bleeding symptoms or elevated ISTH-BAT to confirm or reject a VWD diagnosis (3). Second level tests are then performed to determine the subtype of disease. This includes; VWF multimer analysis, VWF:Collagen binding, VWF propeptide and VWF:FVIII binding assays, ristocetin-induced platelet aggregation and genetic testing. These second level tests can give insight into the quantitative or qualitative defects in VWF which allows for more efficient and effective treatment of most patients.

Interestingly, approximately 30-50% of patients with type 1 VWD do not have pathogenic *VWF* gene variants that could explain the lowered levels of VWF (4-6). Although these patients can be treated with factor concentrates or DDAVP (7), current assays and genetic testing often do not fully explain the cause of bleeding in these patients. Furthermore, a substantial portion of patients do not or only partially respond to DDAVP. Several potential determinants for DDAVP response, like disease subtype, mutation, age or blood group (8-17) have been reported, but the cause of the variation is not fully understood. We undertook a systematic review and meta-analysis to elucidate the

rate of response and possible determinants of DDAVP response (**Chapter 6**). We found large differences in response to DDAVP between disease subtypes, which was largely determined by the baseline levels of FVIII:C for Hemophilia A and VWF:Antigen for VWD. The importance of baseline levels of VWF has also been described in other studies. For example, Heijdra *et al.* studied DDAVP response in type 1 VWD patients and concluded that a baseline of 34 IU/dL VWF:Activity or higher will lead to complete response in all patients (18). Clear determinants of response and associated cut-offs can allow clinicians to predict likelihood of response and choose the appropriate medication. A cut-off value based on baseline levels was not determined in our systematic review due to limited individually reported patient data and varying definitions of response. This further emphasizes the need for a standardized definition of response to DDAVP to aid not only clinicians but also fundamental research on DDAVP response. Furthermore, our systematic review highlights that it is still not fully known what determines DDAVP response. It is hypothesized that other modifiers of VWF can cause the low levels and associated bleeding in patients without a *VWF* defect (19). Potentially, modifiers of VWF could also play a role in the response to DDAVP and cause non-response in patients. Further testing on these cohorts that could identify these modifiers is thus needed.

Broad, unbiased methods and studies are effective in finding potential candidates, but not for functional validation of those candidates. Luckily, various *in vivo* and *in vitro* models exist that are used to study VWF and VWD (20). Naturally occurring VWD has been described in mammals such as dogs and pigs (21-23), which offered valuable insight by studying the disease in a complete system. Although, cost of housing limited the use of these models. Mouse VWD models offer a cheaper, easier to handle model but have the challenge of small blood volumes and vessel which make experimentation more difficult (24). Alternatively, a rat VWD model can be used which offers relatively simple housing and larger blood volume than mice (25). VWF can also be expressed in non-endothelial cell types to study pathogenic *VWF* variants and their effect on VWF biosynthesis, storage and secretion (26). However, those models lack endothelial cell specific gene expression and thus lack a true endothelial environment. Unfortunately, none of the models mentioned above could be used in this thesis as they all require knowledge of the pathogenic variant of VWF or other factor that causes the unexplained low levels of VWF or non-response to DDAVP in our patient cohort. It is therefore almost inevitable to analyze the endothelial cells from the patients themselves.

Looking inside, endothelial cell models with intrinsic patient characteristics

Patient endothelial cells can be isolated via invasive surgery or they can be derived directly from whole blood. This can be done by differentiating late appearing endothelial progenitor cells into ECFCs (27) or by reprogramming peripheral blood mononuclear

cells (PBMCs) into induced pluripotent stem cells (iPSCs) and then differentiating those into iPSC endothelial cells (iPSC-ECs) (28). These are excellent model systems to investigate vascular defects as they carry the mutation(s) of the patient (27) and have been used to study pathogenic disease mechanisms (**Chapter 2**). In this thesis we extensively tested ECFCs derived from patients with VWD with either no *VWF* mutation or no response to DDAVP (**Chapter 7**). In three of those patients, clear retention of VWF in the endoplasmic reticulum was found. These three patients all have known *VWF* mutations. In patients carrying the p.Arg924Gln or p.Arg1374Cys mutation, ER retention of VWF had not been described before (29, 30) and was thus a novel finding. For the other patient, carrying both p.Asp141Asn and p.Arg2313Cys mutations, ER retention had been previously shown in HEK293 cells transfected with the p.Asp141Asn mutation (31). Although the retention of VWF can explain the non-response to DDAVP in these patients, it is still unclear what causes the retention. Proteomic analysis on these cells did not reveal other candidates that hint towards other defects in the early secretory pathway or defects in the synthesis of VWF. To continue this research, the known *VWF* mutation in the ECFCs could be corrected through genetic modification or blocked through the use of allele-specific small-interfering RNA (32). Alternatively, through a recently published novel approach, the mutation can be introduced into healthy ECFCs by base editing to confirm the patient phenotype (33). These methods could confirm whether the ER retention is caused by the *VWF* mutation or additional factor in these patients.

There were also ECFC clones from which no clear defect was observed that could explain the bleeding phenotype, especially in ECFC derived from patients without *VWF* mutation. In those patients the defect could be present in an aspect of the endothelial compartment that we did not test like the angiogenic capacity, proliferation, apoptosis or endothelial barrier function. However, as the patients reported with lowered VWF levels we did test the aspects most likely to directly or indirectly affect VWF such as; cell and organelle morphology, VWF synthesis, storage and secretion, cell migration and the proteomic signature. Alternatively, lower VWF levels could be caused by something outside of the endothelial cells. For example, in the clearance of VWF which has been shown in VWD patients with gene variations in CLEC4M (34), or in the later functioning in the primary hemostasis. Defects in these cases cannot be detected by solely analyzing the ECFCs of these patients. In future research we could use models that allow for a more complete analysis of the endothelial function like the vessel-on-a-chip model (35) or the even more intricate hemostasis-on-a-chip model (36). These models allow the simulation of endothelial injury and hemostatic plug formation. A specific example of the hemostasis-on-a-chip, developed by Lam *et al.* (37) is currently being tested with healthy control- and patient-derived ECFCs (data not published). These models can

give crucial insight into the interplay between plasma, endothelial cells, platelets and hemodynamic forces which conventional 2D cell cultures lack.

Alternatively, modifiers of VWF can be found through genome wide association studies (GWAS). Previous GWAS have studied genetic determinants of VWF levels and found a correlation between VWF levels and variants in *VWF*, ABO and genes encoding for WPB secretory machinery components like *STXBP5* and *STX2* (38, 39). The latter have been shown to play an important role in the secretion of WPBs (34, 40). In line with these findings, we found a positive correlation between the quantity of WPBs and secreted VWF in ECFCs and several secretory pathway components such as VWF, Rab27A, Rab3D (41), and SYTL4 (42) (**Chapter 6**). This suggests that the exocytosis machinery of WPB is important in the regulation of VWF levels and should be further studied as it could contain other modifiers of VWF levels. Alternatively, the plasma proteome can be analyzed (43). The plasma proteome contains coagulation factors and secreted proteins by cells. Plasma proteomics has previously been used to identify proteoforms of VWF to aid in VWD diagnostics (44). In patients with unexplained bleeding, the cause could lie in the clearance of coagulation factor levels, or the lack of certain secreted factors. Plasma proteomics could give insight into those aspects. Furthermore, the role of platelets can be analyzed by performing platelet proteomics (45). A next step forward could be to perform plasma proteomics in the patients cohorts also included in this thesis.

Weighing the odds, the challenges of the ECFC model

In previous research, and in this thesis, ECFCs have been shown to be a powerful tool to investigate patient specific defects (**Chapter 2**). However, some challenges regarding their intra- and interindividual phenotypic heterogeneity must be addressed. Firstly, the availability and expansion capacity of ECFCs can be problematic. About 45-70% of all attempts to obtain ECFCs from whole blood are successful (46), which is in line with the success rate of our lab. Secondly, this success rate is further exasperated by situations where repeated isolations are not possible due to patients age or health, or by limited blood volume being available for collection in children. Thirdly is the observed heterogeneity in morphology, proliferation and endothelial markers (47, 48). Using the specifically developed automated quantification pipeline (**Chapter 3**) we showed significant differences in cell size, WPB quantity and shape, and migratory cell speed between ECFC clones. Furthermore, between healthy control-derived ECFCs we found distinct differences between clones based on their transcriptome (**Chapter 4**). The difference between these clusters yielded new insights into the potential cause of heterogeneity as we showed that inflammation and endothelial to mesenchymal transition (EndoMT) may act as potential drivers. Specifically, we showed that *CXCL8*

(IL-8*), TGFBi, TGFB2, BMP2* and *SMAD1* were differentially regulated between ECFC clones, which are pro-inflammatory (49) or EndoMT associated genes (50). Collectively, our transcriptional, morphological and functional data was used to categorize ECFCs as either cluster 1 with a standard endothelial morphology, and cluster 2, which presented with large, more mesenchymal like cells. We also measured this difference in the proteome of a different cohort of control and patient ECFCs further supporting this observation. To deal with this heterogeneity, we developed a qPCR panel based on the transcriptomic signature of the ECFCs that can aid other researchers.

This panel does however, not fix or prevent the heterogeneity from occurring. One of the potential drivers of heterogeneity is EndoMT which is the transformation of endothelial to mesenchymal cells. This results in loss of endothelial markers, reduced VWF synthesis and increase in extracellular matrix proteins (51-54). It has also been shown that inflammation can cause EndoMT in endothelial cells (55-57). We speculated that the expression of inflammatory and EndoMT associated genes is part of an autocrine/paracrine loop that could initiate or maintain the change from cluster 1 to cluster 2 ECFCs. We performed a pilot experiment on the secretome of a small subset of healthy ECFC clones in cluster 1 and cluster 2 (data not shown). We observed that cluster 1 ECFCs secreted more VWF while cluster 2 ECFCs secreted higher levels of EndoMT and inflammation markers ABI3BP, SERPINE1 and IL-8. However, this has to be confirmed with further testing before conclusions can be drawn. The insight that inflammation and EndoMT could drive the phenotypic differences between ECFC clones offers an interesting new direction of research. Members of the transforming growth factor (TGF)-β cytokine superfamily and bone morphogenetic proteins (BMPs) play a crucial role in EndoMT (58). TGF-β has been shown to be a potent inducer of EndoMT (59). Furthermore, it has been shown that inhibition of TGF-β by inhibitors significantly increased endothelial cell function, delayed cellular senescence and increased proliferation in human iPSC-ECs (60). Therefore, we attempted to induce and inhibit EndoMT in ECFC derived from healthy controls (data not published, performed by Britte Lenderink). We exposed a small panel of cluster 1 ECFCs clones (C10 and C22) and cluster 2 ECFC clones (C05 and C07) to EndoMT inducers (TGF-β2) or inhibitors (TGF-βi). We observed that TGF-βi caused slightly higher production of VWF in cluster 1 ECFCs as measured by ELISA in the lysates of the cells compared to the control (Figure 1). Cluster 2 ECFCs did not show any clear changes. Furthermore, ECFCs expressed a more cluster 1-like RNA expression pattern after TGF-βi exposure (data not shown). This suggests that ECFCs might be affected and even improved through this method. However, this pilot has to be repeated with a larger panel of ECFCs, optimizing the dose and duration of exposure and testing various functional assays. Control over ECFC morphology or potentially only generating cluster 1 ECFCs would greatly reduce the

heterogeneity between clones, which is especially useful in cases where there is a low yield of clones or only slowly proliferating clones. Another option to improve the use of slowly proliferating clones would be the immortalization of the cells. Telomerase activity can be prolonged through nucleofection using SV40 large T antigen which has been shown to effectively immortalize human umbilical vein endothelial cells (HUVEC) (61, 62) and chicken intestinal epithelial cells (63) while retaining their normal phenotype. This has been shown in a pilot experiment to effectively prolong the normal phenotype in ECFCs until ~passage 11 after transfection. (Isabel Bär, personal communication). Alternatively, cord-blood (CB) ECFCs have been shown to have significantly higher proliferation rate and remain stable for more passages when compared to whole blood derived ECFCs (46). However, these can only be derived from possible new-born patients if the mother has been diagnosed before or during the pregnancy.

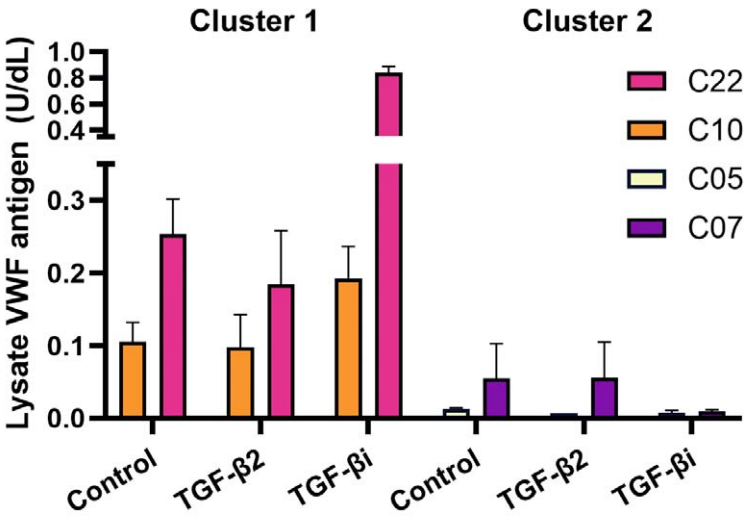


Figure 1. VWF production increased in ECFCs after exposure to EndoMT ligands and inhibitors. VWF antigen levels were determined by ELISA in ECFC lysate after exposure to ligands and inhibitors for 6 days. Abbreviations: von Willebrand factor, VWF; Transforming Growth Factor, TGF. Figure adjusted from data generated by Britte Lenderink.

iPSC-ECs versus ECFCs, which is better?

Other models, such as using iPSCs to generate iPSC-ECs, provide an alternative way to analyze endothelial pathology in cells that carry the patient mutation (64). ECFCs can be difficult to acquire due to a low success rate or limited patient material as previously mentioned. However, iPSCs can be generated from almost any somatic cell type in about 8-10 days and are capable of self-renewal (64). This allows iPSCs

to be acquired despite limited material and can be quickly expanded to very large amounts. IPSCs have the potential to differentiate into many cell types like erythroid, megakaryocytic, myeloid cells (65, 66) or endothelial cells, called iPSC-ECs (67-69). Generated iPSC-ECs have typical endothelial like characteristics and have been shown to produce and secrete VWF (70). However, levels of VWF production in iPSC-ECs are significantly lower than in ECFCs. iPSC-ECs thus lack some of the phenotypic and functional maturity of ECFCs, particularly in producing fully formed WPBs. We showed that small adjustments to existing protocols partially improve the VWF production and elongation of WPBs, although still significantly less than ECFCs (**Chapter 5**). Maturation of WPBs in iPSC-EC is thus a hurdle that must be overcome to use the model in VWF and VWD focused research. Interestingly, it has been shown that the donor cell type influences the epigenome of iPSCs (71) suggesting that the donor material could influence the differentiation of iPSCs. Indeed, one study did show elongated WPBs in iPSC-ECs which were generated from HUVECs (72) although iPSC-EC derived from CB-ECFCs did not seem to produce typical elongated WPBs (73). Thus, starting material could have beneficial results in the maturation of WPB in iPSC-EC, but more research on this is needed. Alternatively, EndoMT has been shown to occur at later passages in iPSC-ECs (74) and inhibition of TGF-β has shown to increase proliferation in human embryonic stem cell endothelial cells (75). Perhaps control over EndoMT as described earlier could yield interesting results in iPSC-EC as well.

The question of which cell model is best suited for endothelial research is complex and largely depends on the research goals. ECFCs, on the one hand, have the advantage of being representative of a mature endothelial state and fully formed WPBs, but present with various challenges in heterogeneity that need be taken into account. On the other hand, iPSC-ECs offer scalability and versatility but do not fully represent normal VWF production and secretion. Ultimately, the research question determines which model is best used and in this thesis, where the production, storage and secretion of VWF is key, the ECFCs take the crown.

How to look, Quantitative imaging and the role of artificial intelligence

One widely used method of analyzing cells and tissues is microscopy. Ranging from relatively simple light microscopy to intricate Cryo-confocal or electron microscopy. These techniques yield insight into the inner workings of cells, protein location and shape and number of organelles. Qualitative observations can be easily made by viewing the output. However, considerable heterogeneity exists between cells and their contents, which can make subsequent analysis difficult and prone to subjective bias (76). Especially the crowded intracellular environment in combination with optical and immunostaining limitations presents an additional, technical challenge. Therefore,

quantification of parameters is usually performed using automated methods to reduce bias and improve the quality of the results.

There are several methods available for the quantification of cellular structures. For instance, Fiji (ImageJ) can make adjustments and measurements to images and allows the use of macros to automate image analysis (77). Other, more specialized software such as CellProfiler offer a more user-friendly module-based approach for high-throughput image analysis (78). Additionally, programs within Python, such as scikit-image are widely used for customized analysis pipelines and allow the user full control and even add functionalities (79). In this thesis we describe a method for automatically quantifying WPBs in ECFCs using CellProfiler (**Chapter 3**). We developed this pipeline with the quantification of WPBs and our own research in mind but it can also be used for other cell types and other organelles. We opted to make this pipeline in CellProfiler, instead of for example ImageJ, so that other groups could easily use and adjust the pipeline method without the need to understand and adjust the underlying code/macro. Despite optimization of software parameters, perfect identification of targets is not always possible. These imperfections may lead to incorrect identification of targets, which could lead to over- or underestimations of numbers and dimensions. Another challenge often encountered in image quantification is that the images are not perfectly suited for analysis due to, for example, inconsistent sample preparation, varying laser settings, or different operators. These variations can lead to small but significant differences between images. It is therefore important that a bioimaging experiment is well thought out before the microscope comes into play (80).

Recent advancements in the use of artificial intelligence (AI) in the field of imaging offer a solution to these problems. AI can "learn" to accurately characterize images through neural network training if supplied with a large, high-quality dataset. There has been considerable progress in the development and application of various AI-based tools that are now available for public use (81). AI is already being used in this field in the diagnosis, prognosis, and distribution of various types of thrombocytopenia (82), diagnostic testing for hematologic Disorders (83) and in analysis of endothelial cells (84, 85). While AI is undoubtedly a hot topic, it's important to recognize its limitations. AI models require large, high-quality datasets for training which are not always available in rare disease research. Another challenge in the use of AI is the "black box" nature of many AI algorithms (86). It is often unclear to the user how AI models analyze features within the images, which makes it difficult for users to trust and/or adjust the models if needed. It is thus needed that the functioning of AI is clearly explained to users. In contrast, traditional image analysis software, such as ImageJ and CellProfiler, offer a more transparent approach from which users can more easily understand the

processing steps being applied to the images. Finally, another aspect of the increasing use of AI that must not be overlooked is the environmental cost (87). Data centers which are needed to run and train AI require vast amounts of energy and water for cooling and are expected to account for over 3% of global carbon emissions in 2025. It is therefore vital that the cost of AI is considered when choosing to use it.

In the context of this thesis, AI could have been used to standardize measurements of endothelial cell function, reducing variability objects and enabling more reliable comparisons across studies. However, considering the complexity, the substantial computing power necessary and the need for a high-quality training data set, the question remains; is it necessary? For many purposes and research questions, an exact answer, or perfectly segmented cells are not necessary. Despite small percentages of false positives or false negatives, strong differences between subsets of data can still be discerned using non-AI tools. To conclude, it is vital for image quantification that the exact question or analysis methods is determined before the microscope is used (80). Furthermore, the use of AI can be a powerful tool to complement traditional methods if needed, but should not be a replacement.

Conclusion

The findings in this thesis contribute to our understanding of endothelial function in bleeding disorders, although there is more to learn. Future research should focus on refining and improving the available endothelial cell models, validating the patient specific defects observed in VWD patients and finally, identify new candidates that could determine VWF levels in VWD patients.

It is important to realize that the isolation, culturing and experimentation on iPSC-ECs and ECFCs is challenging and time consuming, although the lessons learned from these models can be invaluable. Despite the in depth, personalized approach to identify determinants of VWF used in this thesis, many patients remain without a clear cause of either low VWF levels without *VWF* mutation, or cause of non-response to DDAVP. For those patients and others, a wide scale approach like plasma and platelet proteomics might reveal interesting candidates which can then be further studied in ECFC or iPSC-ECs derived from those patients.

To conclude, the endothelial compartment can be studied on a personal scale, or on a larger, population wide scale to reveal novel insights into disease pathology. A good interplay between these methods is needed and by building on the foundations laid here, we can continue to push the boundaries of endothelial research and its clinical applications.

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