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**Author:** Groeneveld, Dafna Jordana  
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Introduction

Almost 90 years ago, the first case of what is now known as von Willebrand disease (VWD), was published in a Finnish scientific journal [1,2]. Prof. dr. Erik von Willebrand described in this journal his first case of an inheritable bleeding disorder. Almost 30 years later, Nilsson and co-workers concluded that the impaired clot formation was due to the lack of a plasma factor, the von Willebrand factor (VWF)[3,4]. VWF is best known for its three classical hemostatic functions: (i) as a carrier protein for coagulation factor VIII, (ii) binding to exposed collagen upon vascular damage and (iii) as a mediator of the recruitment of platelets to sites of vascular injury [5]. However, in recent years it has become clear that VWF has a versatile function beyond hemostasis, and has been implicated in several pathophysiological processes, such as tumor metastasis, angiogenesis, cell proliferation and inflammatory processes [6]. So, apart from its evident role in hemostasis, it has recently become clear that VWF is a much more complex multifaceted protein than initially thought. Several aspects – from synthesis, secretion and clearance, to functions in the circulation – of this protein have been studied and the results are described in this thesis.

Part 1: Aspects of synthesis and secretion of VWF

VWD is the most common inherited bleeding disorder and is characterized by reduced plasma VWF levels or functionally abnormal VWF [7,8]. VWD is divided into three groups based on the type of defect. Type 1 VWD is characterized by a partial quantitative loss of VWF, while type 3 VWD corresponds to a virtually complete loss of VWF. Type 2 VWD is encompasses all qualitative dysfunctions of VWF and is subdivided into 4 subtypes: 2A, 2B, 2M and 2N. Low plasma levels in VWD patients are the result of mutations in the VWF gene that lead to decreased synthesis, impaired secretion, increased clearance or a combination thereof. However, in approximately 30 to 40 % of the index cases no (causative) mutations were found [9-11], suggesting that other factors outside the VWF gene can determine VWF plasma levels. In Chapter 2 we gave an overview of factors that can influence VWF plasma levels. Factors such as ABO blood group [12], platelet α2β1 polymorphisms [13], age [12,14], and hormonal alterations [15,16] have major influences on VWF plasma levels and/or the bleeding phenotype of patients. This illustrates that VWD is a complex multifactorial disease, with inter-relating genetic and environmental components contributing to the variable phenotype of the disease. So despite the growing understanding of the pathophysiology of VWD, the diagnosis is often still difficult because of the many factors influencing VWF levels.

The VWF molecule contains a large number of cysteine residues which all form either intrachain or interchain disulfide bonds. Intrachain disulfide bonds are important for the VWF monomer structure, while interchain disulfide bonds are essential for the formation of the high molecular weight multimers (HMW)[17]. Cysteine mutations in VWF have been frequently identified in VWD patients (VWF database, accessible
Most cysteine mutations lead to a loss of intrachain disulfide bonds and are associated with low VWF plasma levels. Interestingly, the 5 identified cysteine mutations that are involved in interchain disulfide bonds (p.Cys1099Tyr, p.Cys2771Ser/Tyr and p.Cys2773Arg/Ser) are associated with normal VWF plasma levels. These distinct effects of the two types of cysteine mutations made us hypothesize that impaired intrachain or interchain disulfide bond formation in VWF may have different effects on the biogenesis of Weibel-Palade bodies (WPB), the endothelial cell-specific storage organelles for VWF. By analyzing formation of pseudo-WPB upon expression of VWF variants with disrupted intrachain (p.Cys1130Phe and p.Cys2671Tyr) or interchain (p.Cys2773Ser) disulfide bond formation in Chapter 3, we concluded that natural mutations of cysteines involved in the formation of interchain disulfide bonds do not affect the storage in WPB and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds will lead to reduced VWF storage and secretion due to endoplasmic reticulum retention.

Some type 1 VWD patients are known to have a reduced response to DDAVP treatment [18]. Interestingly, most of the VWF mutations identified in those patients with reduced response were located within the A1-A3 domains. As the DDAVP-induced increase in plasma VWF is due to exocytosis of WPB, the formation of WPB in those patients might be impaired by VWF mutations. However, expression studies of variants located in the A domains of VWF are limited. We therefore characterized in Chapter 4 the biosynthesis of six VWF mutations, located in the VWF A1-A3 domains, which were found in families diagnosed with VWD. We found that all mutants, except the p.Arg1583Trp, showed impaired formation of pseudo-WPB when transfected in HEK293 cell. In addition, two mutations (p.Leu1307Pro and p.Val1822Gly) also showed reduced numbers of pseudo-WPB and increased endoplasmic reticulum retention. Regulated secretion upon stimulation of transfected cells reproduced the in vivo situation, indicating that HEK293 cells expressing VWF variants found in patients with VWD can be used to properly assess defects in regulated secretion. The effects of the mutations p.Ser1285Pro, p.Leu1307Pro and p.Tyr1584Cys on WPB formation and regulated secretion as seen in transfection experiments with HEK293 cells were confirmed in the Blood Outgrowth Endothelial Cells (BOECs) derived from the corresponding patients [19].

**Part 2: Aspects of clearance of VWF**

As mentioned in Chapter 2, one of the major determinants of VWF plasma levels are the ABO blood groups. VWF is one of the few plasma proteins that is decorated with ABO blood group sugars. Those ABH antigens are attached to the N-linked oligosaccharide chains of VWF [20]. Individuals with blood group O have approximately 25% lower VWF plasma levels compared to non-O individuals [21]. Individuals with the Bombay blood group phenotype, who lack expression of the ABH antigens, even have lower plasma levels than individuals with blood group O [22]. The exact mechanism behind the relationship between ABO blood group and VWF levels remains unknown,
although effects on clearance have been postulated [23]. We therefore examined in Chapter 5 whether clearance of VWF depends on the ABH antigens present on the VWF molecule. By purifying human VWF from blood group A, B and O donors, we were able to study the clearance of VWF containing the different blood groups in VWF deficient mice. However, we observed no difference in clearance rate between A, B or O human plasma-derived VWF. In our human model, we infused three type 3 VWD patients with a VWF concentrate (Haemate-P®). Haemate-P® contains VWF from donors with all blood groups, which allowed us to study the clearance of A-VWF, B-VWF and O-VWF simultaneously in one individual. Faster clearance of O-VWF present in infused type 3 VWD patients would result in a relative increase in the loading of VWF with A and B antigens over time. Interestingly, we found that the loading of VWF with A,B and H antigens decreased 2-fold during clearance of VWF in 2 out of 3 patients, in the third patient the loading remained the same. Those changes in ABH antigen loading of VWF were confirmed by Western blot. Based on the results from our mouse- and human model we concluded that there is no direct effect of ABH blood group antigens on VWF in VWF clearance. We demonstrated that in a direct comparison within one individual O-VWF is not cleared faster compared to A- or B-VWF. Preliminary results on the pharmacokinetic data of type 3 VWD patients with blood group O and non-O that were infused with Haemate-P®, seems to substantiate our new hypothesis that it is the blood group O status of the individual himself that determines the faster clearance of the VWF molecule, and that this is independent of the ABO blood group antigens on VWF.

Faster clearance of the VWF protein is one of the mechanisms leading to decreased VWF plasma levels and subsequently bleeding phenotypes. Mature VWF and VWF propeptide (VWFpp) are released in equimolar amounts in the circulation, but the half-lives differ. The ratio between these two parameters can therefore be used to discriminate between normal and accelerated VWF clearance. While VWFpp and VWF are cleared differentially, FVIII is in complex with VWF and therefore their half-lives are related. The ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag (FVIII:C/VWF:Ag) can be used to identify defects in VWF synthesis. The FVIII:C/VWF:Ag is increased when VWF synthesis is reduced but the ratio remains 1 when VWF is cleared faster [24]. Opposing results are obtained for VWFpp/VWF:Ag, which will remain unchanged with reduced synthesis, but will increase with reduced half-life of VWF. Using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios, we have investigated in Chapter 6 the pathophysiological mechanisms involved in type 1, 2 and 3 VWD in patients included in the Willebrand in the Netherlands (WiN) cohort [25]. We found that in type 1 VWD both reduced synthesis (increased FVIII:C/VWF:Ag) and accelerated clearance (increased VWFpp/VWF:Ag) contribute to the pathophysiological phenotype in these patients. This is in line with previously published data in a large European cohort of type 1 VWD patients [24]. Interestingly, in type 2B patients increased clearance was frequently observed, probably because type 2B VWF spontaneously binds to platelets in the circulation. The VWF-platelet complex is removed from the circulation, leading
to lower VWF:Ag levels but normal VWFpp levels. Another, unexpected, finding was the measurable level of VWFpp in a large group of type 3 VWD patients, while their VWF:Ag level was below the Type 3 VWD criteria of 5 IU/dL. The measurable amount of VWFpp indicates that these patients do synthesize VWF, but the mature VWF is probably cleared so fast that the VWF:Ag level remains very low. Indeed in most of these patients mutations were found that are known to cause accelerated clearance such as the p.Arg1205His and p.Ser1285Pro mutations [26]. For the clinical therapy of these patients this will not be of that much importance, since patients will receive the same VWF concentrates, but it might be important for genetic counseling and as a diagnostic tool the VWFpp can discriminate between ‘true’ type 3 VWD and Type 1 VWD in which the VWF is cleared very fast.

**Part 3: Aspects of VWF beyond hemostasis**

Besides playing an important role in hemostasis, VWF also seems to be a negative regulator of angiogenesis [27], the formation of new blood vessels from preexisting ones. VWF is stored in WPBs in endothelial cells. But besides VWF, also other proteins are stored in the WPB. Several of these proteins are known to be involved in angiogenesis, like angiopoietin-2 (Ang-2), galectin-3 (Gal-3) and osteoprotegerin (OPG). Since VWF is essential for the formation of WPBs, these factors rely on VWF for their storage in the WPB and secretion into the circulation. We therefore speculated in Chapter 7 that disturbed WPB formation and/or exocytosis due to VWF mutations, would lead to a dysregulated secretion of angiogenic mediators. Using the WiN cohort (see Chapter 6) plasma samples of types 1, 2 and 3 VWD patients were analyzed for their angiopoietin-1 (Ang-1), Ang-2, Gal-3, OPG and vascular endothelial growth factor (VEGF) levels. Compared with controls, VWD patients had higher Ang-1 and VEGF levels and lower Ang-2 levels. Type 1 and 2 patients had lower Ang-2 levels compared with controls, while type 3 VWD patients had higher OPG levels compared with controls. VEGF levels were strongly dependent upon VWF levels in plasma, and were the highest in patients with type 3 VWD. Patients with VWD have thus various disturbances in angiogenic markers in plasma. However, defects in VWF, leading to impaired or absent formation of WPB in VWD patients, do not consistently lead to dysregulated release of other WPB components. The variations in angiogenic mediators may contribute to the development of vascular malformations, such as angiodysplasia that can occur in VWD.

From the literature it was shown that endothelial VWF regulates in vitro angiogenesis [27]. BOECs isolated from type 1 and 2 VWD patients showed enhanced angiogenesis compared with BOECs isolated from healthy individuals. Whether this also holds for type 3 VWD BOECs is unknown, since successful isolation has been reported to a limited extent. Characterization of the angiogenic capacity of BOECs from VWD patients is still limited and differences between the different types of VWD have not been investigated in detail. We therefore examined in Chapter 8 the angiogenic capacity of BOECs from patients diagnosed with type 1 (n=4), type 2 (n=6) and type 3 (n=1) VWD to further explore the possibility of using BOECs as an endothelial cell model to study
the pathogenic role of VWF mutations in regulating angiogenesis. Considering all 11 VWD patients, migration velocity and total tube formation was similar between VWD patients and controls. However, BOECs from the type 3 VWD patient and one type 2B patient showed enhanced angiogenic capacity. Directional migration was impaired in 9 out of 11 VWD patients. We also observed that the angiogenic response of BOECs from VWD patients was limited to early passage numbers. While BOECs from VWD patients can be a useful tool for \textit{ex vivo} assessment of endothelial cell function in patients with different types of VWD, limitations (such as earlier loss of angiogenic capacity) should be taken into account when studying BOECs from VWD patients.

**Future perspectives**

In recent years it has become evident that VWF is much more than just a coagulation factor which supports platelet adhesion and protecting coagulation factor VIII from premature clearance, and has been implicated in multiple pathological processes ranging from inflammatory diseases to cancer. The studies presented in this thesis explored several aspects of VWF from synthesis, secretion and clearance of the protein to functions beyond hemostasis.

Although the general assumption is that VWF plasma levels in individuals with blood group O is lower as a result of enhanced clearance of the protein, our data described in \textit{Chapter 5} suggest that this is not entirely true. Our hypothesis that the blood group O individuals clear the VWF protein faster but that this is independent of the blood group antigens on VWF, is substantiated by preliminary results obtained from pharmacokinetic data of VWF concentrate infused in VWD patients. Faster clearance of the VWF protein in blood group O individuals suggests that their clearance mechanism might be different than in individuals with blood group A, B or AB. One of the limitations in our study was the fact that all three VWD patients had blood group O. We cannot exclude the possibility that endogenous antibodies against A and B antigens interacted with the A and B antigens on VWF. Studies on infusions with recombinant VWF products, which are now used in clinical trials [28], in type 3 VWD patients with different blood groups should be carried out to exclude the possible interaction of naturally occurring Anti-A and Anti-B antibodies present in the circulation of individuals with antigens present on VWF. Experiments regarding uptake and endocytosis of VWF by macrophages or other cells involved in VWF clearance isolated from different blood group donors might give more insight whether VWF is more efficiently cleared by macrophages from blood group O donors than non-O donors. Those studies will advance our knowledge about the mechanism(s) of VWF clearance, which might lead to a more personalized treatment of VWD patients in the future.

VWF has been described as a ‘molecular bus’, carrying other proteins in the circulation. Besides FVIII also other ligands have been shown to be associated with VWF including but not limited to ADAMTS-13, P-selectin, insulin-like growth factor binding protein-7, osteoprotegerin, angiopoietin-2, galectin-1 and galectin-3. With regard to
the WPB components galectin-3, angiopoietin-2 and osteoprotegerin, the functional consequences of their binding to VWF have been investigated to a limited extent, if at all. Being in complex with VWF may prevent the interaction with their natural ligand via sterical hindrance, as is the case for FVIII. However, knowledge is scarce on this issue, and it would be interesting to investigate the shared functional effects between VWF and its binding partners. Since endothelial cells contain several other VWF-binding proteins with pro- and anti-angiogenic properties that we did not study in Chapter 7, such as galectin-1, connective tissue growth factor and insulin-like growth factor binding protein-7, it is likely that VWF plays an exceedingly complex role in regulating angiogenesis at different levels. It remains to be determined whether the effect of VWF on angiogenesis is qualitative or quantitative, but it seems likely that its role is somehow linked to angiodysplasia seen in a subset of VWF patients. Angiodysplasia may result from vascular malformations resulting from the dysregulated release in VWD patients. Patients with VWD demonstrated elevated levels of VEGF and high levels of VEGF can lead to the formation of fragile capillaries, with disrupted structure reminiscent of angiodysplasia. The elevated VEGF and Ang-1 levels, may therefore contribute to vascular complications such as angiodysplasia that occur frequently in VWD patients. Angiodysplasia is most frequently observed in patients lacking the high molecular weight (HMW) VWF multimers (types 2A, 2B and 3), but so far no evidence has been described for a specific role of HMWM in the cellular models of angiogenesis. However, extracellular pathways have been identified by which VWF controls angiogenesis, and thus it is possible that HMW multimers may affect the interaction of VWF with endothelial cells. In Chapter 8 we characterized the angiogenic capacity of BOECs isolated from VWD patients, including type 2 and 3 VWD. Although our group of patients was small, our results suggests that BOECs from type 2 VWD patients and the type 3 patient have enhanced angiogenic capacity compared with BOECs from type 1 VWD patients. Future studies will be required to determine the exact role of VWF multimers in angiogenesis. Three-dimensional microvessel systems have been developed to study angiogenesis in vitro. The recent development of microfluidic vascular networks, allows us to study many aspects of microvascular function [29,30]. Seeding BOECs, isolated from VWD patients, into these synthetic vessels would permit us to study the angiogenic response of endothelium of a VWD patient and the role of (functionally) defective VWF in regulating angiogenesis in different types of VWD in much more detail. Furthermore, BOECs can for example be seeded into these microvessels with and without perivascular cells to investigate local interactions with these cells, which is an important step in angiogenesis. In-depth characterization of the molecular pathways through which VWF regulates angiogenesis will provide novel therapeutic targets for the treatment of angiodysplastic gastrointestinal bleedings in VWD patients where VWF concentrates are not sufficient.

Since the discovery of VWF, significant progress has been made in our fundamental knowledge of both the disease and the protein. But it appears that we only have seen the tip of the iceberg when it comes to the functional diversity of this resourceful
protein. Even its classic role in hemostasis is not completely understood. Therapeu-
tic inhibitors of VWF-mediated platelet adhesion and activation may inhibit the pro-
gression of inflammatory diseases like atherosclerosis, hepatosteatosis and ischemic
stroke. With regard to the non-hemostasic functions of VWF, many opportunities also
remain to be explored. The combination of data obtained from both clinical and fund-
damental research on VWF will no doubt contribute in increasing our knowledge of
this versatile protein and VWF-related research has a bright future ahead. The studies
described in this thesis and the suggested future research will advance our knowledge
about the biogenesis of VWF and its functions beyond hemostasis. This knowledge
might eventually lead to a personalized treatment for VWD and its complications in
the future. Back in 1926, Dr. von Willebrand certainly did not fully grasp what great
implications his report on a bleeding disease would have decades later.
References


