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Dafna Groeneveld

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Dafna Groeneveld



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On the miscellaneous aspects of von Willebrand factor

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CHAPTER

1

Introduction and Outline

Introduction

History

In 1926, almost 90 years ago, the first case of what is now known as von Willebrand disease (VWD), was published in the Finnish scientific journal *Finnish society of Medicine* [1,2]. Prof. dr. Erik von Willebrand described in this journal his first case of an inheritable bleeding disorder that he had observed in several members of a family from the Åland islands. The dominating symptoms of the disorder consisted of nose bleeding, bleeding from the gums, bleeding from the female genital tract and bleeding from trivial wounds. His first case was that of a young girl, Hjördis. Already at a young age the girl had experienced several severe bleeding episodes. At the age of 13, she bled to death during her fourth menstrual period. Von Willebrand concluded that the family suffered from a previously unknown hemophilia condition, which in contrast to hemophilia affected both sexes, and called it hereditary pseudohemophilia. In 1957 Nilsson and co-workers concluded that the impaired clot formation was due to the lack of a plasma factor, the von Willebrand factor (VWF), which was present in both normal and in hemophilia A plasma [3,4].

Von Willebrand Disease

A decreased concentration or abnormal function of VWF is responsible for VWD, the most common inherited bleeding disorder with an estimated prevalence ranging from 0.035% to 1.3% of the population [5-7]. Prevalence based on the number of patients registered at specialized centers is much lower than estimates based on population screenings. Patients registered at hemostasis centers probably have a more symptomatic phenotype, while individuals identified during screenings only have a mild form of the disease [6,8]. VWD is a heterogeneous disorder, which resulted in the initial description of more than 20 subtypes. The current classification merged these into a total of 6 subgroups (Table 1) [9]. A reduced concentration of structurally normal VWF is classified as type 1 VWD. Qualitatively abnormal variants of VWF are classified as type 2 VWD (with subgroups 2A, 2B, 2M and 2N). Complete deficiency of

Table 1. VWD Classification

VWD Subtype	Description*
Type 1	Partial quantitative deficiency of VWF.
Type 2A	Qualitative VWF defect resulting in a reduction of VWF-dependent platelet adhesion.
Type 2B	Qualitative VWF defect resulting in increased VWF-dependent platelet adhesion.
Type 2M	Qualitative VWF defect associated with specific defects in platelet/VWF interaction but with a normal range of multimers.
Type 2N	Qualitative VWF defect resulting from defective VWF binding to factor VIII (FVIII) and consequently low levels of circulating FVIII.
Type 3	Clinically severe quantitative disorder resulting from a markedly reduced or absent platelet and plasma VWF (less than 5U/dL). Consequently, FVIII activity is also reduced.

*From the ISTH-SSC VWF Online Database [10] and the latest classification update [9].

the VWF protein is classified as type 3 VWD. Treatment of VWD is based on increasing plasma levels of functional VWF. This may be achieved by infusion of exogenous VWF concentrate or by releasing endogenous VWF from the endothelial storage organelles Weibel-Palade bodies (WPB), via administration of DDAVP. Therefore treatment with DDAVP is only useful in type 1 VWD and in some cases of type 2 VWD.

Von Willebrand factor

VWF is a glycoprotein circulating in plasma as large multimers. When activated upon vascular damage VWF serves as an adhesion molecule for platelets thereby initiating platelet plug formation. VWF is also the carrier protein of coagulation factor VIII (FVIII) (Figure 1). VWF synthesis is restricted to endothelial cells and megakaryocytes [11,12]. Upon translocation to the endoplasmic reticulum, VWF dimerizes through the formation of C-terminal disulfide bonds at the CK-domains of the VWF monomers [13]. In the endoplasmic reticulum N-linked glycosylation is initiated. The dimers are transported to the Golgi apparatus where the N-linked glycosylation is completed and O-linked sugars are added [14]. In the trans Golgi network dimers form multimers via N-terminal disulfide bonds at the D'D3 domain and the VWF propeptide (VWFpp) is proteolytically removed [15] (Figure 2). Part of the synthesized VWF multimers is se-

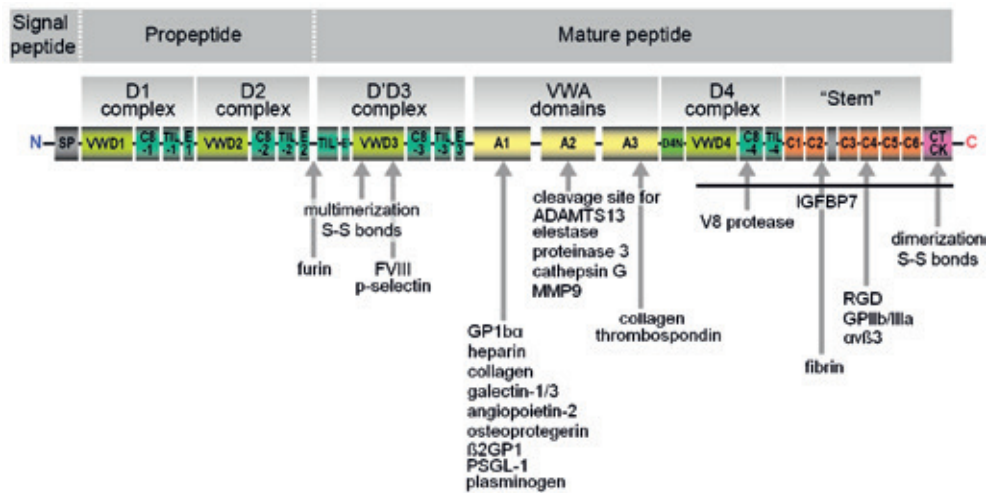


Figure 1. Schematic representation of the VWF monomer structure. The domain structure [32] and functional binding sites of VWF. The different types of structural domains are indicated by different colours. The signal peptide (SP) and propeptide are cleaved upon translocation into the endoplasmic reticulum and in the Golgi apparatus respectively. The locations of the intersubunit disulfide bonds (S-S) are shown in the CK and D3 domains, which are important for the formation of VWF dimers and multimers, respectively. Locations of known binding sites are shown for VWF ligands as well as the cleavages sites for VWF proteases. FVIII, factor VIII; GP, glycoprotein; PSGL-1, P-selectin glycoprotein ligand-1; ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13; MMP9, matrix metalloproteinase 9; IGFBP7, insulin-like growth factor-binding protein 7.

creted constitutively into the plasma, where it has a variable half-life of about 12 hours [16]. The remaining VWF is stored in cell-specific organelles; WPB in endothelial cells or α -granules in megakaryocytes. The highest molecular weight VWF multimers are stored and thus are released at sites of vascular damage in response to secretion stimuli like thrombin, stress, vasopressin or its synthetic analogue desmopressin (DDAVP) [17,18] (Figure 2).

After secretion into the plasma the large VWF multimers are proteolytically cleaved by ADAMTS13 (A Disintegrin and Metalloproteinase with a Thrombospondin type 1 motif). The ADAMTS13 cleavage site in the A2 domain of VWF is accessible only after partial unfolding of VWF, which is most likely induced by the shear stress exerted on VWF after binding to the endothelial surface [19] or exposed collagen [20]. The mechanisms involved in clearance and removal of VWF are not yet fully understood.

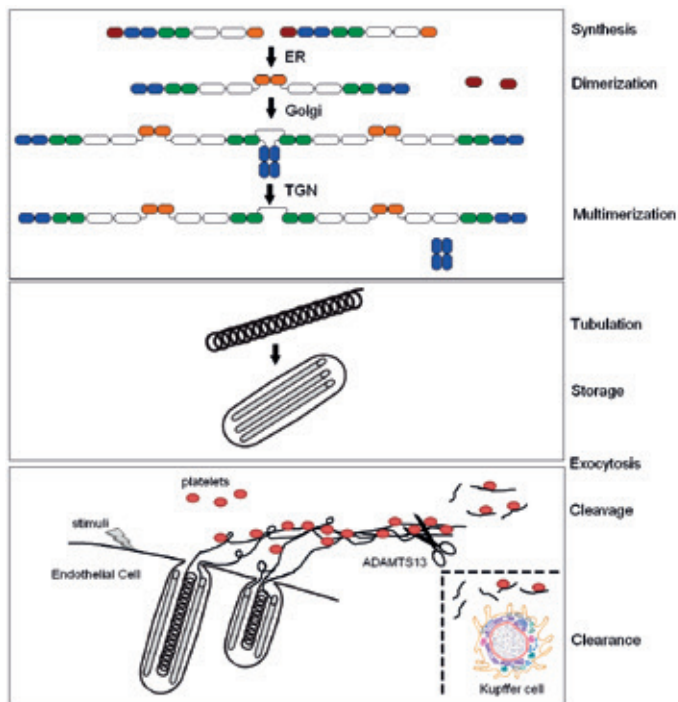


Figure 2. Schematic illustration of VWF metabolism. The structure of precursor VWF is indicated by different colors; red for signal peptide, blue for VWFpp (D1-D2 domains), green for D'D3 domains, white for A1-C6 domains and orange for CK domain. Important steps in VWF synthesis are removal of the signal peptide, dimerization and multimerization of proVWF and cleavage of the VWFpp resulting in mature multimers lacking VWFpp. Multimers are assembled in tubules and packed into WPB. WPB fuse with the plasma membrane upon stimulation of endothelial cells, allowing secretion of the ultra-large (UL) VWF strings. Under shear stress ULVWF can self-associate, leading to enormous VWF strands. Blood platelets (red circles) adhere with high affinity to ULVWF. ADAMTS13 cleaves ULVWF, giving rise to plasma VWF. Proteolyzed VWF is then (mainly) cleared by specific liver-macrophages, the Kupffer cells. ER, endoplasmic reticulum; TGN, trans Golgi network.

Intravenous injection of VWF in VWF deficient mice led to accumulation of the bulk of VWF in the liver. However, it was also found in a number of other organs, like the spleen, kidneys and lungs. When taking the size of the organs into account, VWF is taken up relatively efficient by both liver and spleen in particular [21]. Macrophages in both liver and spleen seem to contribute to the removal of VWF from the circulation and several receptors are now identified which are capable of binding VWF, for example C-type lectin domain family 4 member M (CLEC4M), Sialic acid-binding immunoglobulin-type lectins 5 (Siglec-5), Low density lipoprotein receptor-related protein 1 (LRP1) and Asialoglycoprotein receptor (ASGPR). These ligands could therefore mediate the removal of VWF from the circulation [22](Figure 2).

VWF is capable of inducing cell-signalling pathways beyond hemostasis and is associated with several other pathophysiological processes. For example, VWF seems to actively participate in the inflammatory response. Platelet-decorated VWF strings released from endothelial cells can recruit leukocytes under conditions of high shear stress [23] and VWF promotes leukocyte extravasation [24]. VWF seems to negatively regulate angiogenesis [25], the process of formation of new blood vessels from pre-existing vessels. VWF seems to regulate angiogenesis via multiple intracellular and extracellular pathways involving the VWF ligands $\alpha\beta 3$ and angiopoietin-2. But other pathways may also be involved since galectin-3, osteoprotegerin and insulin-like growth factor binding protein 7 (IGFBP7), are also found to be involved in angiogenesis and are associated with VWF inside and outside the endothelium [26-28]. While VWF limits the Vascular Endothelial Growth Factor (VEGF)-induced proliferation of endothelial cells [25], the opposite effect is observed for smooth muscle cells [29], suggesting that VWF could play a role in the thickening of the vascular wall. Besides modulating proliferation of cells, VWF can also induce apoptosis. The interaction of VWF with the platelet receptor GPIIb induces apoptotic events in platelets [30]. This pro-apoptotic property of VWF seems not restricted to platelets only. Tumor cells have a higher metastatic potential in the absence of VWF. The inhibitory effect of VWF on tumor cell survival is probably via the interaction with the integrin $\alpha\beta 3$. Certain tumor cells have armed themselves against VWF via the production of ADAM28, a protease that cleaves VWF in a shear dependent manner [31]. So, apart from its role in hemostasis, it has recently become clear that VWF is a much more complex multifaceted protein than initially thought. Several aspects of the metabolism and function of VWF were studied in the different chapters of this thesis.

Outline of the Thesis

Part 1: Aspects of synthesis and secretion of VWF

Chapter 2 gives an overview of factors that influence VWF plasma levels. Besides mutations within the VWF gene that can lead to impaired synthesis, secretion or clearance, also other factors influencing plasma VWF levels are described, showing that VWD is a complex genetic disorder. In **Chapter 3** we compared the effects of VWF

variants with impaired intrachain (p.Cys1130Phe and p.Cys2671Tyr) and interchain (p.Cys2773Ser) disulfide bonds on the biogenesis and exocytosis of WPB. We showed that natural mutations of cysteines involved in the formation of interchain disulfide bonds do not affect either the storage in WPB or secretion of von Willebrand factor, whereas mutations of cysteines forming intrachain disulfide bonds lead to reduced von Willebrand factor storage and secretion because the von Willebrand factor is retained in the endoplasmic reticulum. Expression studies for storage and secretion of VWF with mutations in A domains are limited, we therefore studied in **Chapter 4** the storage and secretion properties of 6 different mutant VWF molecules characterized by mutations within the A domains of VWF. Three mutations in the A1 domain (p.Ser1285Pro, p.Leu1307Pro and p.Arg1374His), two mutations in the A2 domain (p.Arg1583Trp and p.Tyr1584Cys) and one mutation in the A3 domain of VWF (p.Val1822Gly) were transiently transfected into HEK293 cells to analyze the biogenesis and exocytosis of WPB.

Part 2: Aspects of clearance of VWF

Individuals with blood group O have approximately 25% lower plasma VWF levels. The general assumption is that VWF covered with blood group O antigens (H-antigens), is cleared faster, via yet unknown mechanisms, compared to VWF with A or B antigens present. We therefore studied in **Chapter 5** the clearance rates of VWF obtained from donors with either blood group A, B or O. However, we could not demonstrate the assumed faster clearance of O-VWF, instead we showed that within one individual there is no difference in clearance between the different blood groups. We therefore think that the blood group O individuals clear VWF faster compared to non-O individuals, but that this faster clearance is independent of the blood group antigens present 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 are released in equimolar amounts in the plasma, but the half-lives differ. The ratio between these two parameters can therefore be used to discriminate between normal and accelerated VWF clearance. In **Chapter 6**, by using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios, we aimed to define the pathophysiology of 658 type 1, 2 and 3 VWD patients included in the Willebrand in the Netherlands (WiN) cohort.

Part 3: Aspects of VWF beyond hemostasis

It has recently been shown that VWF can act as a negative regulator of angiogenesis. VWF is stored in endothelial cells in WPB together with angiopoietin-2, osteoprotegerin and galectin-3. These proteins are known to mediate angiogenesis. We hypothesized in **Chapter 7** that defects in VWF, potentially leading to impaired or loss of WPB formation, result in dysregulated release of angiogenic mediators in the circulation. Using the WiN cohort (see **Chapter 6**) plasma samples of 655 type 1, 2 and 3 VWD patients were analyzed for their angiopoietin-1, angiopoietin-2, galectin-3, osteoprotegerin and Vascular Endothelial Growth Factor (VEGF) levels.

Blood Outgrowth Endothelial Cells (BOECs) isolated from type 1 and 2 von Willebrand disease (VWD) patients showed enhanced *in vitro* angiogenesis compared with BOECs from healthy individuals. Characterisation of the angiogenic capacity of BOECs from VWD patients is however, 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 11 patients diagnosed with type 1, type 2 and type 3 VWD. All the studies described in this thesis are summarized and discussed in **Chapter 9**.

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Part 1

Aspects of synthesis and secretion of VWF

CHAPTER

2

Von Willebrand Disease: Influences on Von Willebrand Factor plasma levels

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Adapted and updated from Hematology Education 2011; 5: 76-83

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Abstract

Von Willebrand Disease (VWD) is a bleeding disorder characterized by reduced plasma von Willebrand factor (VWF) levels (VWD types 1 and 3) or functionally abnormal VWF (VWD type 2). Type 1 VWD is the most frequent form and leads to mild or moderate bleeding tendencies. Low plasma levels in type 1 VWD patients are the result from mutations in the VWF gene, leading to decreased synthesis, impaired secretion, increased clearance of VWF or a combination of these conditions. Several studies have shown that genetic changes within the VWF gene are common and are highly penetrant in the more severe type 1 VWD cases. However, in approximately 30 to 40% of the index cases no (causative) mutations were found, suggesting that other factors outside the VWF gene can determine VWF plasma levels. Factors such as ABO blood group, platelet $\alpha_2\beta_1$ polymorphisms, age, and hormonal alterations have major influences on VWF levels and/or bleeding phenotype. 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 difficult because of the many factors influencing VWF levels.

Introduction

Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as large multimers. When activated upon vascular damage VWF serves as an adhesion molecule for platelets thereby initiating platelet plug formation. VWF is also the carrier protein of coagulation factor VIII (FVIII). VWF synthesis is restricted to endothelial cells and megakaryocytes [1,2]. Upon translocation to the endoplasmic reticulum (ER), VWF dimerizes through the formation of C-terminal disulfide bonds at the CK-domains of the VWF monomers [3]. In the ER N-linked glycosylation is initiated. The dimers are transported to the Golgi apparatus where the N-linked glycosylation is completed and O-linked sugars are added [4]. In the trans Golgi network dimers form multimers via N-terminal disulfide bonds at the D'D3 domain and the propeptide is proteolytically removed [5]. Part of the synthesized VWF multimers is secreted constitutively into the plasma, where it has a variable half-life of about 12 hours. The remaining VWF is stored in cell-specific organelles; the Weibel-Palade bodies (WPB) in endothelial cells or α -granules in megakaryocytes. The highest molecular weight VWF multimers are stored and thus are released at sites of vascular damage in response to secretion stimuli like thrombin, stress, vasopressin or its synthetic analogue desmopressin (DDAVP). After secretion into the plasma the large VWF multimers are proteolytically cleaved by ADAMTS13 (A Disintegrin and Metalloproteinase with Thrombospondin motifs). The ADAMTS13 cleavage site in the A2 domain is accessible only after partial unfolding of VWF, which is most likely induced by the shear stress exerted on VWF after binding to the endothelial surface [6]. The mechanisms involved in clearance of VWF are not yet fully understood. Intravenous injection of VWF in VWF deficient

mice led to accumulation of the bulk of VWF in the liver [7]. However, it was also found in a number of other organs, like the spleen, kidneys and lungs. When taking the size of the organs into account, VWF is taken up relatively efficient by both liver and spleen in particular. Recently it was shown that the Ashwell receptor on hepatocytes is capable of binding VWF which lacks its sialic acid group [8]. Macrophages in both liver and spleen seem to contribute to the removal of VWF from the circulation [7] and several receptors are now identified which are capable of binding VWF, for example CLEC4M, Siglec-5, LRP1 and ASGPR [9]. These ligands could therefore mediate the removal of VWF from the circulation.

Von Willebrand Disease

A decreased concentration or an abnormal function of the VWF protein is responsible for von Willebrand Disease (VWD), the most common inherited bleeding disorder with an estimated prevalence ranging from 3-4 per 100.000 to 1.3% of the population [10]. Prevalences based on the number of patients registered at specialized centers are much lower than estimates based on population screenings. Patients registered at hemostasis centers probably have a more symptomatic phenotype, while people identified during screening only have a mild form of the disease [11,12.] VWD is a heterogeneous disorder, which resulted in initial description of more than 20 subtypes. The current classification merged these to a total of 6 subgroups (Table 1) [13].

A reduced concentration of structurally normal VWF is classified as type 1 VWD. Qualitatively abnormal variants of VWF are classified as type 2 VWD (with subgroups 2A, 2B, 2M and 2N). Complete deficiency of the VWF protein is classified as type 3 VWD.¹³ For an update on type 3 VWD see Leebeek and De Wee [14].

Treatment of VWD is based on increasing functional plasma VWF levels. This may be achieved by infusion of exogenous VWF concentrate or by releasing endogenous VWF,

Table 1. VWD Classification	
VWD Subtype	Description*
Type 1	Partial quantitative deficiency of VWF. Multimers may be abnormal, but the proportion of large multimers is not significantly decreased. Typically autosomal dominant in inheritance although diagnosis is complicated by reduced penetrance and variable expressivity.
Type 2A	Qualitative VWF defect resulting in a reduction of VWF-dependent platelet adhesion. Associated with absence of the largest multimers. Generally autosomal dominant.
Type 2B	Qualitative VWF defect resulting in increased VWF-dependent platelet adhesion. Associated with (usually) reduced high molecular weight multimers and often reduced platelet counts. Inheritance is autosomal dominant.
Type 2M	Qualitative VWF defect associated with specific defects in platelet/VWF interaction but with a normal range of multimers. Inheritance is autosomal dominant.
Type 2N	Qualitative VWF defect resulting from defective VWF binding to FVIII and consequently low levels of circulating FVIII. Inheritance is autosomal recessive.
Type 3	Clinically severe quantitative disorder resulting from a markedly reduced or absent platelet and plasma VWF (less than 5U/dL). Consequently, FVIII activity is also reduced. Inheritance is autosomal recessive.

*From the ISTH-SSC VWF Online Database [10] and the latest classification update [9].

stored in WPBs, via administration of DDAVP. Therefore treatment with DDAVP is only useful in type 1 VWD and in some cases of type 2 VWD. Responsiveness to DDAVP varies per person, but is rather stable intra-individually. Normally it will give a 2 to 4-fold rise in VWF and FVIII levels. Clinical effectiveness to prevent or control bleeding symptoms depends therefore (largely) on the plasma VWF levels achieved after administration, which in turn depends primarily on the basal levels in the patient. DDAVP is mainly effective in patients with baseline VWF ristocetin cofactor activity (VWF:Rco) and FVIII coagulant activity levels higher than 10U/dL [15].

Type 1 Von Willebrand Disease

While type 3 is rare (1 in 1 million), type 1 VWD is the most common form of the disease (approximately 50-75% of all VWD cases). VWD type 1 is characterized by reductions in VWF antigen (VWF:Ag), VWF:Rco as well as FVIII, resulting in mild to moderate bleeding tendency. The distribution pattern of the VWF multimers is normal. Diagnosis of type 1 VWD can be difficult, especially in cases with mild symptoms, as it is hard to distinguish them from healthy individuals who have VWF levels at the lower end of the normal distribution.

Low plasma levels in these VWD patients are the result from mutations, leading to decreased synthesis, impaired secretion, increased clearance or a combination of these conditions. Unlike type 3 patients, who are usually homozygous or compound heterozygous for VWF gene mutations in both alleles, type 1 patients usually have a single mutated allele. Since type 1 VWD is a quantitative defect, one would expect that these patients are carriers of type 3 mutations, and that the normal allele accounts for the reduced, but functionally normal VWF levels found in the plasma. However, mutations identified in type 1 VWD are predominantly missense mutations and only 15% of the mutations lead to null alleles [16]. This is in sharp contrast to type 3 VWD, where approximately 85% of the mutations are predicted to result in null alleles. The majority of type 1 VWD patients thus do not appear to be just carriers of type 3 mutations [17]. This difference in the genetic basis between the two quantitative defects is further supported by the fact that the average VWF level in obligatory type 3 carriers is significantly higher compared to obligatory type 1 carriers. This is also reflected by differences in bleeding symptoms; 40 % of the type 3 obligatory carriers have at least one bleeding symptom, compared to 89% of the type 1 obligatory carriers [18].

Three multicenter studies in Europe [19], Canada [20] and the UK [21] have recently investigated the molecular pathogenesis of type 1 VWD in over 300 patients. Despite the difference in recruitment criteria between the studies, the seven most common mutations found in Index Cases (IC) were both identified in Europe and Canada (Table 2). These studies further showed that genetic changes within the VWF gene are common and are highly penetrant in the more severe type 1 VWD cases. In the European “Molecular and Clinical Markers for the Diagnosis and Management of Type 1 VWD (MCMDM-1VWD)” study, no mutation was found in 30% (45 out of 150) of the

Table 2. Most common missense mutations found in the three multicenter studies.

Candidate Mutation*	UK study (32 IC) n (%)	Canadian study (123 IC) n (%)	European study (150 IC) n (%)	Total (305 IC) n (%)
Y1584C	8 (25)	18 (14.6)	13 (8.7)	39 (12.8)
R1205H	4 (12.5)	3 (2.4)	10 (6.7)	17 (5.6)
R924Q	3 (9.4)	8 (6.5)	4 (2.7)	15 (4.9)
R854Q	-	3 (2.4)	5 (3.3)	8 (2.6)
R2464C	-	3 (2.4)	3 (2)	6 (2)
P1266L	-	4 (3.3)	1 (0.7)	5 (1.6)
P2063S	-	3 (2.4)	1 (0.7)	4 (1.3)
R1315C	-	-	4 (2.7)	4 (1.3)
R1374C	-	-	4 (2.7)	4 (1.3)
V1279I	-	4 (3.3)	-	4 (1.3)
C1130F	-	-	3 (2)	3 (1)
C1130R	-	-	3 (2)	3 (1)
M740I	-	-	3 (2)	3 (1)
N1231T	-	3 (2.4)	-	3 (1)
R1325C	-	3 (2.4)	-	3 (1)
V1229G	-	3 (2.4)	-	3 (1)
C2304Y	-	-	2 (1.3)	2 (0.7)
P1413L	-	1 (0.8)	1 (0.7)	2 (0.7)
G1415D	-	-	2 (1.3)	2 (0.7)
R1374H	-	-	2 (1.3)	2 (0.7)
R1668S	-	2 (1.6)	-	2 (0.7)
Y1146C	-	1 (0.8)	1 (0.7)	2 (0.7)

*Listed mutations were found in at least two or more IC, unless the mutation was also found in another study. In bold the mutations found in at least two out of the three studies. Note that the mutations that were only found in the European Study are all associated with an abnormal multimer pattern (figure 1). IC with these mutations could therefore have been excluded from the two other studies.

IC. Similar results were found in studies performed in the UK (47%) and Canada (37%) [19-21]. The lack of finding a mutation in approximately 35-40% of the IC indicate that also other (environmental) factors outside the VWF gene can influence VWF levels in patients diagnosed with VWD type 1. In the next paragraphs several phenotypes and genotypes of patients diagnosed with type 1 VWD will be discussed to understand the large variability seen in phenotypes of patients with type 1 VWD.

Multimer patterns

Although one of the criteria for diagnosis of type 1 VWD is having a normal multimer pattern, several IC in the multicenter studies showed abnormal multimers in the plasma. In the UK and Canadian studies individuals with abnormal multimers were excluded. In the MCMDM-1VWD study 38% (57 out of 150) of the IC had abnormal multimer patterns and may formally not fit into the accepted criteria for type 1 VWD. However, when evaluated against the 2006 classification criteria [13] that state that the proportion of high molecular weight (HMW) multimers should not be decreased

significantly, 22 of 57 IC (39%) with abnormal multimers could be reclassified as type 2, whereas 35 of 57 (61%) still fitted classification as type 1 VWD [22]. Furthermore, those abnormal multimers reflect only very subtle abnormalities and are different from the abnormal multimer pattern characteristic for type 2A or 2B VWD. Interestingly, in all those IC with abnormal multimers mutations were found [19,23]. In contrast, in only 55 % of the IC with a normal multimer profile a mutation was identified. All mutations found in the group of abnormal multimers are located carboxyl to the D' domain, except for a single occurrence of the R854Q mutation. None were found in the propeptide region (Figure 1). Three mutations were found in both the normal and abnormal multimer groups (R854Q, R1205H and C2304Y), indicating that a specific group of mutations is responsible for the abnormal multimer profile (Figure 1) [19]. In the Canadian study recent analysis of multimer patterns, using the same technique as in the European study, identified abnormal multimers in 39% of the IC, similar to the European study [24]. Several of those mutations leading to abnormal multimer profiles were expressed in COS-7 cells. Mutations located in the D4-CK domain (L2207P, C2257S, G2441C and C2477Y) all showed marked intracellular retention and impaired secretion of VWF. Also major loss of the HMW multimers and anodic shifts of multimeric bands was observed in single transfections of the mutants. Cotransfections with wild-type VWF (wt-VWF), mimicking heterozygosity, largely corrected the decrease and anodic shifts [25]. The R2464C and Q2520P mutations, which showed smeary multimer patterns with faster running oligomer bands in patients plasma

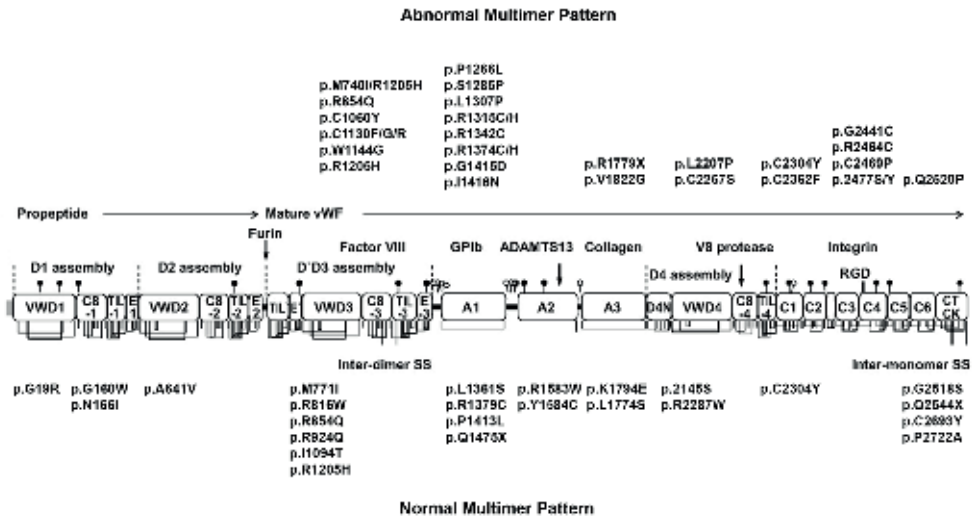


Figure 1. Missense mutations found in index cases with normal and abnormal multimer patterns. The mutations listed are placed at their specific position in the VWF protein. This figure is largely based on the results of the MCMDM-1VWD study, with some additions and deletions due to new insights [19]. Cysteines are shown as vertical lines and are connected for chemically defined disulfide bonds. N- and O-linked glycans are shown as closed and open lollipops, respectively. Boundaries of D assemblies are shown as vertical dashed lines. Image adapted from Zhou et al. [70].

samples [22], showed abnormal anodic migration in single transfections. This was shifted towards (nearly) normal in cotransfections with wt-VWF [25]. Also other mutations which showed a small loss or a relative decrease of the largest multimers in patients carrying those mutations [22] have been investigated in expression studies. For example C1130F/R showed impaired secretion, intracellular retention and abnormal multimer pattern in cotransfections with wt-VWF [26,27]. In multimer patterns from other patients, the oligomers showed barely visible outer sub-bands with smears around the central band. This pattern is indicative of reduced proteolytic processing by ADAMTS13. Although the differences in multimers are minor, the group of abnormal multimers is clearly distinguishable from the group with normal multimers: the average VWF:Ag level is much lower [19], linkage to VWF is much stronger [28] and the chance of finding a mutation is much higher in the group with abnormal multimers (100%) than normal multimers (55%) [19,23].

Impaired synthesis and secretion

Decreased levels of VWF can be due to several mechanisms. Decreased synthesis of the VWF protein is the cause for reduced plasma levels in patients carrying a mutation leading to a null allele. In the European cohort the average VWF levels in 8 IC who had a single mutation predicted to lead to a non-expressed allele, was 39 IU/dL [19]. Impaired secretion and increased intracellular retention of synthesized VWF protein seems to be the major mechanism involved in patients with missense mutations. We have expressed 14 mutations found in the MCMDM-1VWD study to evaluate their contribution to the phenotype observed in the patients. We have established that 7 mutants, located throughout the VWF gene, contribute to the phenotype on the basis of increased intracellular retention and impaired secretion of VWF compared to wt-VWF, while 4 mutants are probably causative mutations based on the mild reduction of secreted VWF [25]. Patients with mutations G160W, N166I or M771I showed a normal VWF propeptide (pp)/VWF:Ag ratio and normal multimer structure. However the VWFpp levels were below the normal range. Reduced levels of VWFpp and VWF:Ag with normal multimer structure suggests that these mutations are responsible for reduced synthesis, rather than increased intracellular retention [29]. Several mutations that were expressed are involved in the loss or gain of a cysteine residue. Four out of five (C2257S, C2304Y, G2441C, and C2477Y) caused marked intracellular retention and loss of HMW multimers in combination with faster migration of multimeric bands. Upon cotransfection with wt-VWF the defect was largely restored. Tjernberg *et al.* [30] have described similar results for another mutation (C2362F) involving cysteine residues, located in the same region. Although cysteines in these regions are not directly involved in the dimerization and multimerization process of VWF, the mutations do result in slightly abnormal multimer patterns. This is probably due to the fact that the unpaired cysteines lead to changes in the three-dimensional structure [26,31]. Impaired secretion of VWF may also be due to a defect in the WPB formation. Indeed Michaux *et al.* showed that 3 type 1 VWD mutations had a delay in the formation of WPB and also showed a reduction in the length and number of WPB [32].

We have recently also identified impaired formation and reduced numbers of WPB in several VWF mutations (Chapter 3 and 4 of this thesis). Furthermore, we showed that impaired synthesis is frequently observed in type 1 VWD patients (Chapter 6 of this thesis).

Accelerated clearance

Another mechanism that could explain the low VWF plasma levels in type 1 VWD is increased clearance of VWF. Some clearly causative mutations show significantly reduced VWF levels upon expression, however in co-transfections with wt-VWF the expression seems normal. This led to the hypothesis that mutations may be involved in faster clearance of the protein. The first report on increased clearance described the rapid disappearance of VWF upon DDAVP infusion in 7 patients carrying the combined M740I/R1205H mutations (Type Vicenza) [33]. Haberichter *et al.* [29] identified seven patients with reduced VWF survival predicted by a markedly increased VWFpp/VWF:Ag ratio. In all these seven patients mutations were identified that have been previously reported with reduced VWF survival, including R1205H, C1130G/F/R and W1144G. The majority of the cases with decreased VWF survival is characterized by significantly reduced steady-state VWF:Ag levels, usually below 30 IU/dl [34,35]. The half-life of VWF:Ag is also markedly reduced after DDAVP infusion. In the MCMDM-1VWD cohort it became evident that the extent of increased clearance was not the same for all missense mutations, and the highest VWFpp/VWF:Ag ratios clustered in the VWF D3 and A1 domains (Figure 2) [36]. The increased VWFpp/VWF:Ag ratio was particularly raised in patients with abnormal multimers and mutations.

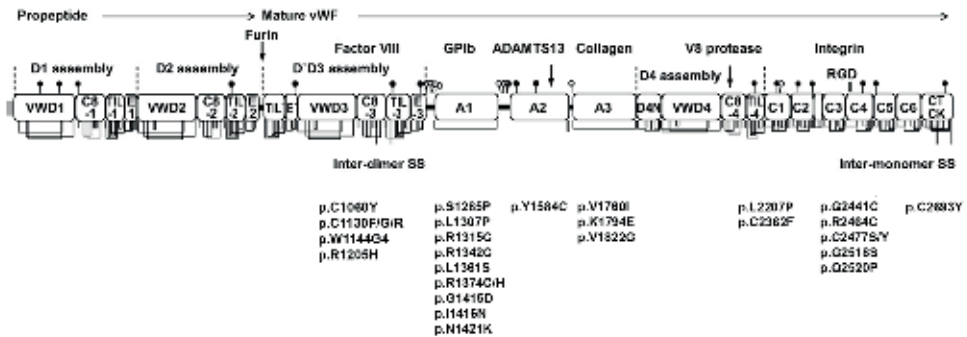


Figure 2. Location of VWF mutations associated with increased VWF clearance in type 1 VWD. The mutations listed are placed at their specific position in the VWF protein. This figure is based on the results of the MCMDM-1VWD study [36]. Patients with listed mutations had a median VWFpp/VWF:Ag ratio above the upper limit of normal reference range (>2.2). Cysteines are shown as vertical lines and are connected for chemically defined disulfide bonds. N- and O-linked glycans are shown as closed and open lollipops, respectively. Boundaries of D assemblies are shown as vertical dashed lines. Image adapted from Zhou *et al.* [70].

The possible contribution of ADAMTS13 proteolysis to the rapid clearance has been postulated. Indeed one mutation, the Y1584C, seems to be linked with increased susceptibility of VWF to ADAMTS13 proteolysis [37]. But detailed analysis of mutations C1130F and C1149R revealed that increased clearance in these cases was not due to increased proteolysis by ADAMTS13 [34]. Sialic acid residues on the VWF glycans are known to promote ADAMTS13-mediated proteolysis and also seem to play a role in the clearance of the protein. Asialo-VWF is cleared more rapidly than sialylated VWF in animal models [38]. The Ashwell receptor, an asialoglycoprotein receptor on hepatocytes, is able to bind asialo-VWF [7], thereby removing it from the circulation.

Recent data suggest that the receptor might also be able to bind sialylated glycoproteins [39], but whether this is the case for VWF remains unknown. The VWFpp/VWF:Ag ratio measured in healthy blood group O subjects indicate that they have a faster VWF clearance than non-O subjects [40]. This suggests that changes in carbohydrate structure of VWF may affect clearance and therefore VWF levels. For the clinical practice increased clearance might be an important phenotype to be considered. Insight into the survival of VWF after DDAVP infusion is relevant for the treatment of type 1 VWD patients. If the half-life of endogenous circulating VWF after DDAVP treatment is too short, it might be preferable to treat the patient with a VWF concentrate instead of DDAVP infusion.

Factors outside the VWF gene

The lack of finding a mutation in approximately 35-40% of the IC in the multicenter studies, indicate that also other (environmental) factors outside the VWF gene can influence VWF levels in patients diagnosed with VWD type 1. Also the variability of responses to DDAVP treatment between patients with the same mutation suggests that other factors contribute to the VWF levels [41]. This notion is also reflected in the 2006 VWD classification that no longer restricts the diagnosis VWD to mutations in the VWF gene [13].

Blood groups. It has been shown that 66% of the variation in VWF levels is genetically determined and that the ABO blood group locus accounts for 30% of that variation [42]. VWF is one of the few plasma proteins that contain the ABO blood group sugars. The ABH antigens are only attached to the N-linked oligosaccharide chains of VWF. Almost 30 years ago it was recognized that the ABO blood groups are associated with VWF plasma levels [43], and this has been confirmed in several other studies [44]. Individuals with blood group O have approximately 25% lower VWF plasma levels compared to non-O individuals. Furthermore, blood group O is overrepresented among VWD type 1 patients. The mechanisms how ABO blood groups are responsible for VWF levels remain unknown. A direct mechanism seems likely as 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 [45]. Furthermore, the VWF levels correlate with the amounts of A and B antigens present on the VWF mol-

ecule [46,47]. The ABH antigens may have an effect on biosynthesis, secretion, clearance of VWF or a combination thereof. The ABH antigens on VWF do not seem play a role in synthesis and secretion as the plasma propeptide levels are independent of the ABO blood group [48]. Platelet VWF levels are independent of ABO blood group, which is probably explained by the fact that platelet VWF does not express the ABH antigens [49]. Another study showed that the rise in VWF level after DDAVP treatment appears to be similar between patients with type 1 VWD with different blood groups, suggesting that secretion efficiency is not affected by the blood group antigens [50]. Nossent *et al.* [48] estimated that the half-life of VWF in controls with blood group O is almost 2 hours shorter compared to non-O blood group controls and it was shown that in healthy individuals the VWF half-life after DDAVP administration was shorter in blood group O individuals [40]. Furthermore, the ratio between propeptide and mature VWF, which are released in an equimolar ratio, is increased in individuals that have blood group O compared to non-O individuals [35,48]. These observations suggest that the ABH antigens on VWF determine its clearance rate, where blood group O-VWF might be cleared faster compared to non-O VWF. However, this supposition is not yet challenged in a direct comparison, nor have the molecular mechanisms been elucidated. New insights into the mechanism how ABO blood group influences VWF plasma levels have been described in Chapter 5 of this thesis. One VWF mutation seems to be linked with the ABO blood group. In the UK study the Y1584C mutation was found together with blood group O in 95% of the VWD cases [21], and in another study 11 out of the 12 IC had blood group O [51], a higher proportion than the overall 77% prevalence of group O reported among type 1 VWD [52].

Age. In preterm neonates the VWF levels are moderately low, but at birth the levels rise above adult norms. Stress in the neonate during the delivery might be the cause of this rise, since it's known that stress or exercise can induce significant release of VWF [53]. Several other reports have shown that with age the VWF levels slowly increase, with a rate of approximately 10 IU/dl per decade [52,54]. This increase during life has an influence on the diagnosis in relation to age. Diagnosis of VWD at a young age might be questioned later in life.

Genetic modifiers. Besides the ABO locus, a number of other loci have been identified as determinants for VWF plasma levels. Genome-wide association studies identified more than 20 SNPs in over 10 genes such as STXBP5, SCARA5, STAB2, STX2, CLEC4M [55] that are associated with VWF levels. Among type 1 VWD patients the frequency of the integrin $\alpha_2\beta_1$ 807C allele, which is associated with low receptor density, was found significantly higher than in the normal population [56]. Low density of the integrin $\alpha_2\beta_1$ might result in less efficient binding of platelets to collagen and may explain the variability between bleeding symptoms in patients with the same VWF levels. Indeed Kunicki *et al.* [57] found that this haplotype was associated with an increased bleeding score. The integrin α_{11b} haplotype 1 was also associated with an increased bleeding score [57]. Further evidence of locus heterogeneity was provided by

Daly *et al.* [58] who identified a heterozygous mutation in the P2Y₁₂ ADP receptor gene in a patient with mild type 1 VWD and a VWF defect. Platelets from this patient showed reduced and reversible aggregation in response to ADP. So the defect in the P2Y₁₂ gene could contribute to the bleeding tendency in type 1 VWD patients [58]. We have found a polymorphism in the arginine vasopressin 2 receptor (V2R) gene, which was associated higher VWF propeptide, VWF and FVIII levels [59]. Recently, Rydz *et al.* [60], have shown that polymorphisms in the *CLEC4M* gene, a mannose specific receptor on endothelial cells, contribute to variable plasma levels of VWF probably by differences in VWF binding and internalization. Some modifier genes for plasma VWF levels have been identified in mice, but human homologues remain to be identified [61,62].

Hormonal influences. Data obtained from several studies on the relationship between menstrual cycle and VWF level are contradictory. One study showed no change in VWF:Ag and VWF:RCo levels during the normal menstrual cycle [63]. A similar study however found lower levels for both parameters in the follicular phase [54], while another study showed significantly lower levels of VWF:Ag during menses compared with the follicular phase [64]. Women investigated in these studies were healthy women; no data are available about the possible change in VWF levels during the menstrual cycle in VWD type 1 patients. The use of oral contraceptives causes a small rise in levels of both VWF:Ag and VWF:RCo, but again no data are available for women who have VWD [65]. During pregnancy the level of VWF increases 3 to 5-fold in women without VWD, and in most women with VWD type 1 the levels will reach normal range at the end of pregnancy. Castaman *et al.* [66] studied 23 women with VWD during pregnancy. Women with R854Q, R1374H, V1665E, V1822G and C2362F mutations showed complete normalization of VWF levels during their pregnancy. Women with R1205H and C1130F mutations had only a slight increase of VWF during pregnancy [66]. This correction during pregnancy might have clinical consequences for the therapy, although postpartum hemorrhage may occur due to a rapid fall of VWF after delivery [67].

Conclusions

In the large multicenter studies on type 1 VWD many mutations were found, located throughout the entire VWF gene. The majority of these mutations are missense mutations and only a few IC had mutations that were predicted to lead to null alleles. An interesting finding in the two large European and Canadian cohorts was the fact that although VWD type 1 is a quantitative defect, some patients showed slightly abnormal multimer patterns. This observation asks for reconsideration of the criteria for VWD type 1. The mechanisms by which these mutations cause the observed clinical manifestations are slowly revealed. For some mutations it has been shown in expression studies that they cause impaired secretion by intracellular retention or degradation. This seems to be the more general mechanism for the reduced levels found in the plasma of type 1 VWD patients with missense mutations. However, several mutations

were found to (also) have an association with increased clearance, which may have therapeutic implications.

Besides mutations in VWF other factors have an influence on VWF levels or bleeding phenotype. The ABO blood group is one of the major determinants of plasma levels. Blood group O individuals have approximately 25% lower levels and are overrepresented among patients with type 1 VWD. Other environmental factors such as age, stress and hormonal influences also alter plasma levels, making the diagnosis of type 1 VWD even more complicated. Recently some polymorphisms in platelet integrins were found to be associated with increased severity bleeding scores and polymorphisms in the V2R and CLEC4M are associated with VWF levels. All these genetic variants, along with genetic modifiers that have yet to be identified, show that VWD type 1 is a multifactorial disorder, where all these factors contribute to the highly variable genotype and phenotype of this bleeding disorder. With all the knowledge now available on the variety of mutations found in type 1 VWD, the question arises whether we should screen patients with possible VWD for VWF gene mutations (for a recent debate see Peake [68] and Favaloro [69]). When laboratory testing shows abnormal multimer patterns screening for mutations seems rather pointless, because a mutation will be found in 100% of the cases as evidenced in the MCMDM-1VWD study. Finding a mutation in these cases will not add any information that will change the diagnosis or treatment of the patient. One could argue that screening may be useful in cases where the mutation leads to accelerated clearance, for example the R1205H and C1130F mutations, since this might change the therapy in case of bleedings. But genetic testing is time-consuming and costly, while more accurate information on clearance will be available from a DDAVP test infusion when blood samples are taken at later time points. And, as shown in the multicenter studies, genetic testing will fail to find a causative mutation in approximately one third of the patients. As discussed earlier, several other factors beside a VWF gene mutation can influence plasma levels and ultimately the clinical manifestation. So this only leaves a very small group of patients with type 1 VWD where screening for a mutation might be more informative. For clinical purposes therefore, genetic testing in type 1 VWD seems for now unwarranted.

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CHAPTER

3

Biogenesis of Weibel-Palade bodies in von Willebrand disease variants with impaired von Willebrand factor intrachain or interchain disulfide bond formation

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Abstract

Background: Mutations of cysteine residues in von Willebrand factor are known to reduce the storage and secretion of this factor, thus leading to reduced antigen levels. However, one cysteine mutation, p.Cys2773Ser, has been found in patients with type 2A von Willebrand disease who have normal plasma levels of von Willebrand factor. We hypothesize that disruption of either intra- or interchain disulfide bonds by cysteine mutations in von Willebrand factor has different effects on the biogenesis of Weibel-Palade bodies. **Design and Methods:** The effect of specific cysteine mutations that either disrupt intrachain (p.Cys1130Phe and p.Cys2671Tyr) or interchain (p.Cys2773Ser) disulfide bonds on storage and secretion of von Willebrand factor was studied by transient transfection of human embryonic kidney cell line 293. Upon expression of von Willebrand factor these cells formed endothelial Weibel-Palade body-like organelles called pseudo-Weibel-Palade bodies. Storage of von Willebrand factor was analyzed with both confocal immunofluorescence and electron microscopy. Regulated secretion of von Willebrand factor was induced by phorbol 12-myristate 13-acetate. **Results:** p.Cys1130Phe and p.Cys2671Tyr reduced the storage of von Willebrand factor into pseudo-Weibel-Palade bodies with notable retention of von Willebrand factor in the endoplasmic reticulum, whereas p.Cys2773Ser-von Willebrand factor was stored normally. As expected, wild-type von Willebrand factor formed proteinaceous tubules that were seen under electron microscopy as longitudinal striations in pseudo-Weibel-Palade bodies. p.Cys2773Ser caused severe defects in von Willebrand factor multimerization but the factor formed normal tubules. Furthermore, the basal and regulated secretion of von Willebrand factor was drastically impaired by p.Cys1130Phe and p.Cys2671Tyr, but not by p.Cys2773Ser. **Conclusions:** We postulate that natural mutations of cysteines involved in the formation of interchain disulfide bonds do not affect either the storage in Weibel-Palade bodies or secretion of von Willebrand factor, whereas mutations of cysteines forming intrachain disulfide bonds lead to reduced von Willebrand factor storage and secretion because the von Willebrand factor is retained in the endoplasmic reticulum.

Introduction

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that facilitates platelet adhesion to an injured vessel wall and carries coagulation factor VIII in the circulation [1]. Aberrations in the function or concentration of VWF may lead to the most common human inherited bleeding disorder von Willebrand disease (VWD).

The main source of plasma VWF is the endothelial cell which stores VWF in a unique organelle called the Weibel-Palade body (WPB). VWF is released from WPB by basal and regulated mechanisms [2,3]. During its biosynthesis, VWF undergoes extensive post-translational modifications that include formation of intrachain and interchain disulfide bonds. The interchain disulfide bonds bridge the cysteine knot domains

(“tail-to-tail”) and D3 domains (“head-to-head”) of VWF to form dimers and multimers of up to 80 monomeric subunits [1,4,5]. Such interchain disulfide bond formation has recently been shown to be facilitated by the low pH seen in the *trans* Golgi and WPB [6,7]. Through a process called tubulation, VWF is organized at the *trans* Golgi into proteinaceous tubules that are stored in the WPB. Under an electron microscope VWF tubules appear as striations (if viewed longitudinally) or as hollow rings (if viewed in cross-section) [4,5].

VWF contains numerous cysteines [8] and a large number of mutations of cysteines have been identified in VWD patients with either quantitative (VWD types 1 and 3) or qualitative (VWD type 2) defects of VWF (ISTH-SSC VWF mutation database (www.vwf.group.shef.ac.uk/). The mechanisms by which specific cysteine mutations lead to the different clinical phenotypes of VWD are largely unknown. Based on the expression data of cysteine mutations in VWF, we and others have shown that intracellular retention of VWF is a common mechanism underlying VWD with quantitative deficiencies of VWF [9-12]. Recently we showed that impaired WPB formation and reduced regulated secretion of VWF contribute to this intracellular retention [13]. However, cysteines p.Cys2771 and p.Cys2773, which are involved in interchain disulfide bond formation at the cysteine knot domain, [14] have been implicated in type 2A(IID) VWD in patients with normal plasma VWF antigen level [15-17] suggesting normal storage and secretion. We, therefore, hypothesized that disruption of interchain disulfide bonds may not hamper VWF induced biogenesis of WPB and secretion into the circulation whereas disruption of intrachain disulfide bonds does.

To address this hypothesis, the intracellular storage and secretion of p.Cys2773Ser-VWF mutant was compared with that of two other VWF mutants, p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF, in which intrachain disulfide bonds are disrupted. We found that disruption of intrachain disulfide bonds in VWF by mutations p.Cys1130Phe and p.Cys2671Tyr impairs WPB biogenesis whereas disruption of an interchain disulfide bond by p.Cys2773Ser does not affect WPB formation. Our findings also confirmed that VWF tubulation and storage in WPB are not dependent on C-terminal dimerization.

Design and Methods

Patients and mutations

The mutations investigated in this study had all been identified earlier in VWD patients. p.Cys1130Phe (c.3389G>T) was identified in a heterozygous state in patients originally diagnosed with type 1 VWD with moderately severe bleeding tendencies [18,19]. The phenotype of patients carrying p.Cys1130Phe has more recently also been classified as 2A(IIE) [20,21]. p.Cys2671Tyr (c.8012G>A) was described in a compound heterozygous type 3 VWD patient with a deletion of the second allele [22]. p.Cys2773Ser (c.8318G>C) was identified in heterozygous type 2A(IID) patients [17].

Plasmid constructs

Recombinant pSVH expression plasmids containing full length cDNA encoding either the wild-type human VWF (WT-VWF) or the p.Cys2671Tyr and p.Cys2773Ser VWF variants have been described before [9]. The full-length VWF cDNA fragments, obtained by EcoRI restriction of these pSVH-VWF plasmids, were cloned into the pCI-neo mammalian expression vector (Promega, Madison, WI, USA). Mutation p.Cys1130Phe was introduced into pCI-neo WT-VWF plasmid with the QuikChange XL SiteDirected Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and verified by sequencing.

Cell culture and transfection

Cells of the human embryonic kidney cell line 293 (HEK293) were purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured in minimum essential medium Eagle alpha modification (MEM- α , Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μ g/mL gentamicin (Invitrogen, Carlsbad, CA, USA). Using the FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany), 0.66 μ g/mL plasmid constructs (final concentration in the medium; 0.33 μ g/mL WT-VWF and mutant VWF plasmids for co-transfections) were transiently transfected into HEK293 cells according to the manufacturer's instruction.

Immunofluorescent staining and antibodies

Transfected cells were stained with immunofluorescent antibodies and analyzed with a Leica SL confocal laser scanning microscope with a 63X/1.40 NA oil objective, as previously described [13]. Monoclonal antibody CLB-RAg35 and polyclonal rabbit anti-human protein disulfide isomerase (PDI) antibody A66 (obtained from Prof. I. Braakman, Department of Chemistry, Utrecht University, Utrecht, The Netherlands) were used as primary antibodies against VWF and the endoplasmic reticulum (ER) marker PDI, respectively [23,24]. Alexa 488- and Alexa 594-conjugated secondary antibodies were purchased from Invitrogen. To quantify the extent of retention of VWF in the ER, we determined the percentage of cells expressing VWF which also showed retention of VWF in the ER by co-staining the VWF with PDI.

Transmission electron microscopy

Transfected cells grown on 35-mm Petri dishes were fixed and prepared for transmission electron microscopy as previously described [13,25]. Briefly, cells treated with osmium tetroxide and tannic acid were serially dehydrated with ethanol and embedded in Epon. The samples cut into thin sections of 70-100 nm were stained with uranyl acetate and lead citrate, and then analyzed with a Tecnai 12 at 120 kV equipped with a 4kx4k CCD camera (Model Eagle, Fei Company, The Netherlands).

Structural analysis of von Willebrand factor

Seventy-two hours after transfection cells were incubated with release medium [OPTIMEM1 medium, 10 mM HEPES (N-2hydroxyethylpiperazine-N'-2-ethanesulfon-

ic acid), 0.2% bovine serum albumin, pH 7.4] for 24 h. The conditioned media were electrophoresed through 1.6% agarose gel containing 1% sodium dodecyl sulfate for multimer analysis. VWF multimers were visualized by western blotting as described elsewhere [9]. Electrophoresis and blotting were performed under non-reducing conditions. To analyze the processing of VWF, reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting were performed for the conditioned media and cell lysates. Cell lysates were prepared with Passive Lysis Buffer (Promega) supplemented with 100 μ M phenylmethylsulfonyl fluoride (Roche) and the protease inhibitor cocktail Complete™ with EDTA (Roche). Samples were reduced using 20 mM dithiothreitol, separated by Novex 6% Tris-Glycine gel electrophoresis (Invitrogen), immunostained with polyclonal rabbit anti-human VWF conjugated to horseradish peroxidase (DAKO) and visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

Basal and regulated secretion of von Willebrand factor

In the absence of stimulation, VWF is mainly secreted via the basal secretory pathway from WPB [3]. HEK293 cells are known to form pseudo-WPB that are similar to the WPB in endothelial cells [13]. Basal and regulated secretion of VWF was determined as previously described [13]. Briefly, 24 h after transfection, cells were incubated with fresh culture medium for 48 h and then the conditioned media and cell lysates were collected to determine basal secretion of VWF. To analyze the regulated secretion of VWF, 72 h after transfection cells were incubated for 60 min with release medium containing 160 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) or vehicle (dimethylsulfoxide). VWF:Ag in the media and cell lysates was determined by an enzyme-linked immunosorbent assay. For the calculation of regulated secretion, secreted VWF was expressed as a fraction of total VWF (amount in the medium plus lysate). Statistical analyses were performed with Student's t-test using GraphPad Prism software (version 4.02).

Results

Retention of von Willebrand factor in the endoplasmic reticulum

As shown in Figure 1, WT-VWF formed numerous elongated pseudo-WPB organelles. Each of the VWF mutants was able to form pseudo-WPB although these organelles were relatively short or more granular compared with those formed by WT-VWF. As indicated by the co-localization of VWF and the ER marker PDI, both p.Cys1130Phe and p.Cys2671Tyr led to considerable retention of synthesized VWF in the ER. In contrast, most of the p.Cys2773Ser-VWF was stored in pseudo-WPB similar to those observed for WT-VWF. Quantification of VWF storage showed that 60-80% of cells expressing p.Cys1130Phe or p.Cys2671Tyr displayed retention of VWF in the ER, whereas this was less than 10% for WT-VWF or p.Cys2773Ser-VWF (Figure 1). The retention of p.Cys1130Phe-VWF in the ER was reduced by co-transfection with WT-VWF. In this case the proportion of cells showing ER retention of VWF decreased from 83% to 26%.

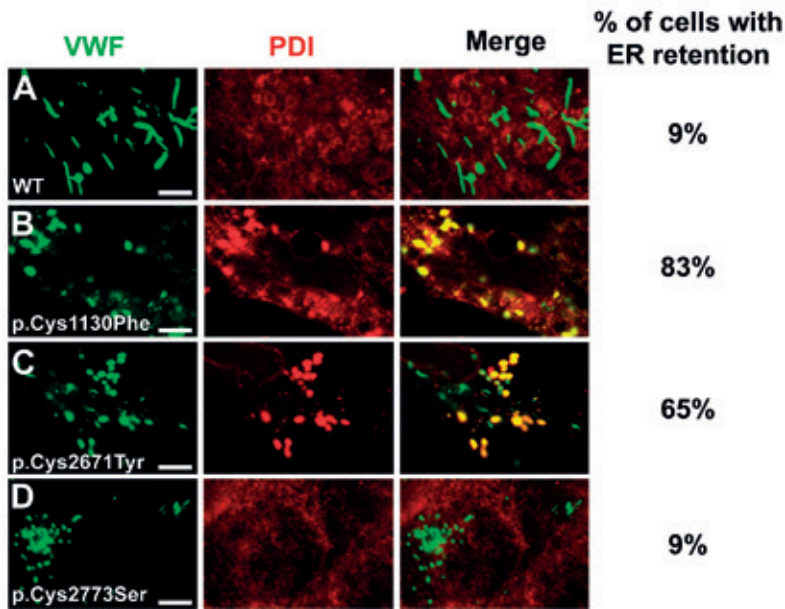


Figure 1. Intracellular localization of VWF variants in transfected HEK293 cells. HEK293 cells were transiently transfected with WT-VWF or VWF mutants as indicated. Fixed cells were stained for VWF (left panel, green) and for PDI (the ER marker, middle panel, red). In the right panel (merge of green and red channels), the pseudo-WPB show up in green (VWF staining only), and the ER containing VWF shows up in yellow as a result of double staining for VWF and PDI. Scale bar = 5 μ m. 175–370 cells expressing each of VWF variants from two independent experiments were analyzed and the percentage of cells that showed retention of VWF in the ER (cells displaying yellow) is indicated on the right.

There was no reduction in ER retention of p.Cys2671Tyr-VWF upon co-transfection with WT-VWF. The different efficiencies in storage of VWF variants were also reflected by PMA-induced pseudo-WPB exocytosis (see section on *Secretion of VWF*).

Analysis of the cell ultrastructure with transmission electron microscopy revealed pseudo-WPB containing VWF tubules in cells expressing WT-VWF as well as each of the mutants (Figure 2A-E). The tubules formed by the three VWF mutants appeared normal compared to those formed by WT-VWF (Figure 2A) [25]. This indicates that all three mutants were able to form pseudo-WPB although p.Cys1130Phe-VWF did so rarely. Furthermore, grossly dilated ER was frequently observed in cells expressing p.Cys1130Phe (Figure 2F) or p.Cys2671Tyr (Figure 2G). The ER morphology in cells expressing p.Cys2773Ser was normal (Figure 2H).

The retention of VWF mutants in the ER was further confirmed, using western blotting under reducing conditions, by evaluating the extent to which VWF was processed into its mature form. Secreted VWF consisted mainly of fully processed mature VWF subunits (Figure 3A). In the cell lysates, both mature subunits and uncleaved pro-VWF

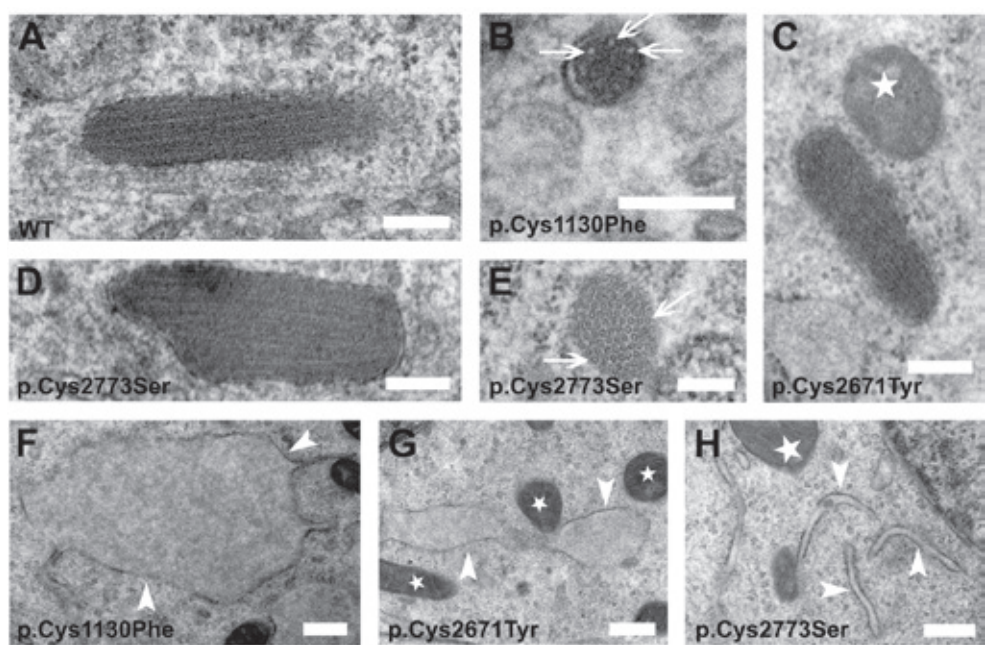


Figure 2. Ultrastructure of transfected HEK293 cells visualized by TEM. HEK293 cells were transiently transfected as indicated. Pseudo-WPB displaying VWF tubules were formed by WT-VWF (A) and the three mutants (B-E). Dilated ER was observed in cells expressing p.Cys1130Phe (F) or p.Cys2671Tyr (G) but not in cells expressing p.Cys2773Ser (H) or WT-VWF (not shown). Note that the VWF tubules were clearly visualized in the cross sections of pseudo-WPB (indicated by the arrows in panels B and E). The ER (indicated by the arrowheads) shown in panels F, G and H were imaged respectively in the same cells shown in panels B, C and D. The stars in panels C, G and H indicate mitochondria. In panel A-E, scale bar = 200 nm; in panels F-H, scale bar = 500 nm.

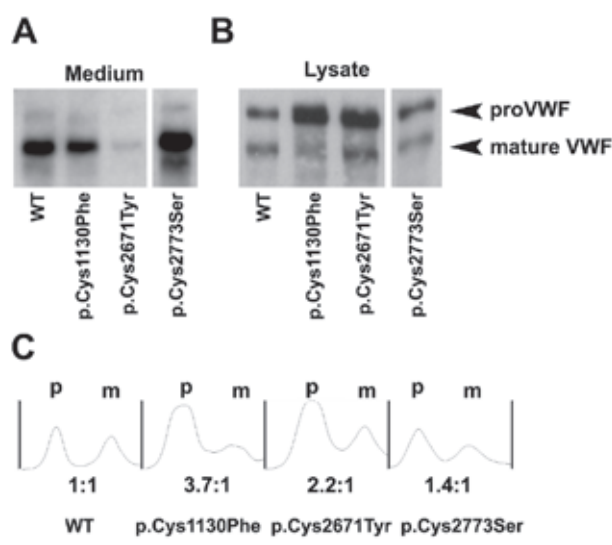


Figure 3. Subunit composition of VWF under reducing conditions. HEK293 cells were transiently transfected with WT-VWF or VWF mutants as indicated. VWF secreted into the medium (panel A: Medium) or VWF retained in lysate (panel B: Lysate) was reduced with DTT and analyzed with SDSPAGE and Western blot. In panel C, the ratio of proVWF (p) and mature VWF subunit (m) in the cell lysate was analyzed with Image J (NIH software, version 1.44P). Note that in panels A and B the parts separated by the space between p.Cys2671Tyr and p.Cys2773Ser are from the same Western blot.

were detectable for all VWF variants (Figure 3B). However, p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF showed relatively much more uncleaved pro-VWF compared to WT-VWF and p.Cys2773Ser-VWF (Figure 3C), indicating that p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF were retained in the ER, and did not reach the Golgi apparatus where furin cleaves pro-VWF [26-28].

Secretion of von Willebrand factor

WPB contributes to both the basal and regulated secretion of VWF [3]. To determine the effect of pseudo-WPB formation on the secretion of VWF mutants, both secretory pathways were examined. Basal secretion of VWF was defined as the secreted VWF:Ag level in the conditioned medium over 48 h (Table 1). Compared to WT-VWF, both p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF showed significantly reduced basal secretion, which was apparent from a decrease in VWF level in the medium as well as from the decreased ratio of VWF:Ag in medium over lysate. In contrast, the basal secretion of the p.Cys2773Ser variant of VWF was normal, with a similar medium/lysate ratio as that of WT-VWF. To mimic the heterozygous state of the mutations in carriers, cells were co-transfected with VWF mutants and WT-VWF at a 1:1 ratio. After this co-transfection with WT-VWF and mutant VWF, the basal secretion of VWF:Ag into medium was similar for all three mutants. However, the decreased ratio of VWF:Ag in medium over lysate observed for p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF indicates that the secretion of these VWF variants remained compromised in the heterozygous state (Table 1). Furthermore, the absolute amount of VWF protein produced by WT-VWF or the three variants was similar (total VWF:Ag in the medium and lysates; about 100 mU VWF per 7×10^5 cells) for both single transfections and co-transfections, confirming that the effects on WPB formation and ER retention of VWF (Figure 1) could not be explained by the expression level of the mutants. The secretion and total amount (medium plus lysate) of p.Cys2773Ser-VWF was higher than that of WT-VWF, although the difference was not statistically significant (Table 1; 126.5 mU in single transfections and 117.7 mU in cotransfections *versus* 97.9 mU for WT-VWF per

Table 1. Expression of VWF variants in transfected HEK293 cells.

Variant	Transfection	VWF:Ag* Medium		VWF:Ag* Lysate		Ratio†
		mU	%	mU	%	
WT-VWF		74.1 ± 4.7	100	23.8 ± 3.2	100	3.2 ± 0.2
p.Cys1130Phe	Single transfection	41.3 ± 2.7	56.4 ± 6.4	59.2 ± 11.1	255.9 ± 56.7	0.7 ± 0.1‡
	Co-transfection	63.7 ± 4.8	86.3 ± 4.1	44.0 ± 5.8	186.3 ± 9.9	1.5 ± 0.1‡
p.Cys2671Tyr	Single transfection	55.2 ± 5.8	76.0 ± 12.7	45.5 ± 4.8	194.3 ± 18.2	1.2 ± 0.2‡
	Co-transfection	65.8 ± 4.4	89.0 ± 4.1	32.2 ± 3.2	136.5 ± 5.9	2.1 ± 0.1‡
p.Cys2773Ser	Single transfection	90.8 ± 10.7	124.0 ± 18.2	35.7 ± 2.1	154.1 ± 16.1	2.5 ± 0.2§
	Co-transfection	84.1 ± 11.6	114.7 ± 17.7	33.6 ± 4.1	142.1 ± 5.9	2.5 ± 0.4§

* VWF:Ag (mU) was produced by about 7×10^5 cells in the medium or lysate. In parallel, VWF:Ag is expressed as percentage relative to the amount of VWF:Ag in the medium and lysate of cells expressing WT-VWF. Each value represents the mean ± SEM of three independent experiments in duplicate. † Ratio of the absolute amount of VWF:Ag in medium to VWF:Ag in lysate. ‡ Compared with WT-VWF $p < 0.01$. § Compared with WT-VWF $p > 0.05$.

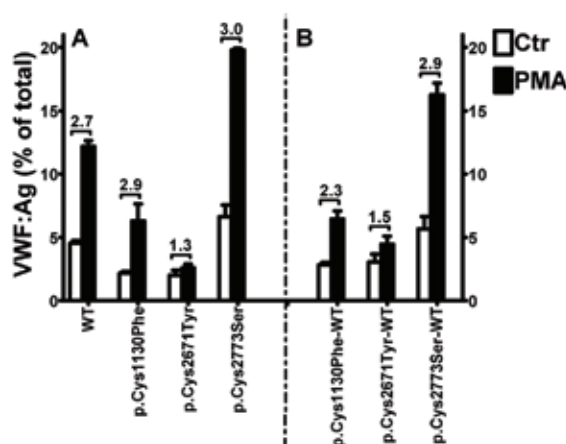


Figure 4. Regulated secretion of WT-VWF or VWF variants. HEK293 cells were transiently transfected with WT-VWF, p.Cys1130Phe, p.Cys2671Tyr and p.Cys2773Ser, respectively (Panel A). In panel B, p.Cys1130Phe, p.Cys2671Tyr or p.Cys2773Ser were co-transfected with WT-VWF at 1:1 ratio. Seventy-two hours post transfection HEK293 cells were rinsed twice with the release medium and incubated at 37°C for 60 minutes in the release medium without (Ctr) or with 160 nM PMA (PMA). Each bar represents VWF secreted into the release medium as a fraction of total VWF (medium plus lysate) times 100%. Mean and SEM are based on three independent experiments in duplicate. The numbers above the bars indicate the fold increase of secreted VWF comparing the stimulated (PMA) and control (Ctr) samples.

7×10^5 cells), consistent with the normal to high plasma levels of VWF in the patients [17]. Rapid clearance *in vivo* was shown to account for the more severe phenotypes of patients with p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF [29].

The regulated secretion of VWF was analyzed by using PMA, which efficiently induces exocytosis of pseudoWPB in HEK293 cells [13]. Incubation of HEK293 cells for 60 min with PMA increased the secretion of WT-VWF by 2.7-fold (12.2% of total VWF was secreted by PMA-stimulated cells *versus* 4.5% by control cells, Figure 4). Both p.Cys1130Phe- and p.Cys2773Ser-transfected cells showed good response to PMA stimulation although the total secretion of p.Cys1130Phe-VWF was much lower. Only a minimal increase in the secretion of p.Cys2671Tyr-VWF was observed after stimulation. Cotransfections with p.Cys2773Ser or p.Cys1130Phe produced a regulated secretion of VWF comparable to that of WT-VWF if judged by the fold increases, but to a much lower absolute level in the case of p.Cys1130Phe. Cotransfection of p.Cys2671Tyr only slightly increased the secretion of VWF during stimulation. These *in vitro* data for p.Cys1130Phe (co-transfection mimicking the heterozygous state) and p.Cys2671Tyr (single transfection mimicking the compound heterozygous state) are consistent with the response to DDAVP infusion in the patients with these mutations [29,30]. One may speculate that patients with p.Cys2773Ser will show a normal response to DDAVP; however, no patient was available for direct testing.

Multimeric analysis of secreted von Willebrand factor

We performed multimer analysis of the conditioned media (Figure 5). A full range of VWF multimers was present in the conditioned medium for WT-VWF and p.Cys2671Tyr-VWF, while p.Cys1130Phe-VWF showed relative loss of large multimers

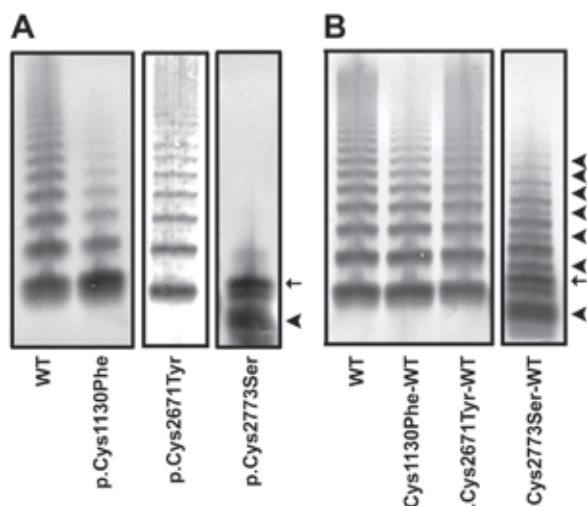


Figure 5. Multimer analysis of secreted VWF. Multimers are shown for single transfections (A) and cotransfections with WT-VWF at 1:1 ratio (B). The N-terminal dimers and odd-numbered multimers formed by VWF p.Cys2773Ser are indicated by the arrow and arrowheads, respectively. In panel A, the multimers of VWF p.Cys2671Tyr are from a different gel, the other separated parts are from one same gel. In panel B, the separated parts are from the same gel. The multimer patterns of secreted VWF were analyzed by SDS-agarose gel electrophoresis and Western blot under non-reducing conditions.

indicating that mutations close to the interchain disulfide bonds may have a minor but significant effect on VWF multimerization. p.Cys2773Ser-VWF mainly formed monomers and dimers. The appearance of double bands for the dimers was caused by the different mobility between C-terminal dimers and N-terminal dimers formed by p.Cys2773Ser [17]. The multimeric defects caused by p.Cys1130Phe and p.Cys2773Ser were only partly restored by co-transfection with WT-VWF. This is consistent with the multimer patterns in the patients [17,19,20]. Furthermore, the odd-numbered VWF multimers produced by co-transfection of p.Cys2773Ser with WT-VWF confirmed co-expression of both constructs in individual cells.

Discussion

Chemical modification and mutagenesis studies have demonstrated that cysteines p.Cys2771 and p.Cys2773 are involved in interchain disulfide bond formation and are equally essential for VWF dimerization [14]. Natural mutations in the two cysteines lead to essentially the same clinical phenotype [15-17]. In addition, extensive studies have indicated that p.Cys1130 at the N-terminal and p.Cys2671 at the C-terminal are not involved in the interchain disulfide bonds but rather are involved in the intrachain disulfide bonds [14,31,32]. We, therefore, selected the three mutations p.Cys2773Ser, p.Cys1130Phe and p.Cys2671Tyr that were identified in our center [17,18,22] as exemplary mutants to examine the differential effects of interchain *versus* intrachain disulfide bonds on WPB biogenesis. p.Cys2773SerVWF was normally stored in and secreted from transfected HEK293 cells despite strongly reduced dimerization and multimerization. In contrast, p.Cys1130Phe and p.Cys2671Tyr resulted in strong retention of VWF in the ER and less VWF stored in pseudo-WPB, which is consistent with previous reports for other cysteine mutations that disrupt intrachain disulfide bonds in

VWF [13,33]. Our data, therefore, suggest that VWF mutations that disrupt interchain disulfide bonds differ from mutations that disrupt intrachain disulfide bonds in the effect on WPB biogenesis.

The mutations that were analyzed in this study are located in different domains in VWF: p.Cys1130Phe in the D3 domain, p.Cys2671Tyr in the region immediately amino-terminal to the cysteine knot domain and p.Cys2773Ser in the cysteine knot domain itself. The different phenotypes of these mutations may be ascribed to the fact that they disrupt different domains of VWF but the differences in phenotypes may also be related to interchain *versus* intrachain defects. Several cysteine mutations, all involved in intrachain disulfide bonds, located in the D' (p.Cys788Arg), D3 (p.Cys1060Tyr, p.Cys1149Arg and p.Cys1225Gly) and cysteine knot (p.Cys2739Tyr and p.Cys2754Trp) domains showed very similar disruptive effects on the storage and secretion of VWF as p.Cys1130Phe and p.Cys2671Tyr [13,33]. This suggests that the effects are not domain-specific. Furthermore, p.Cys2739Tyr and p.Cys2754Trp in the cysteine knot domain are not stored properly, whereas p.Cys2773Ser in the same domain but involved in interchain disulfide bonds showed normal storage [13]. In addition, another mutation p.Cys1099Tyr in the D3 domain that disrupts interchain disulfide bonds between N-termini of VWF was identified in VWD type II C Miami patients

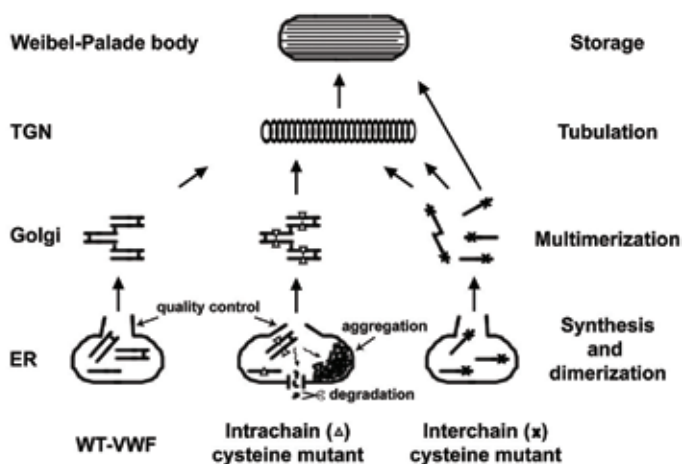


Figure 6. Schematic illustration of VWF intracellular trafficking. WT-VWF (left) is synthesized and dimerized in the ER, and passes the quality control to Golgi apparatus to multimerize and tubulize therein, and is eventually packed into WPB. Part of VWF mutants with impaired intrachain disulfide bond formation (middle) escape the quality control and are stored in WPB, the other part are retained in the ER undergoing aggregation or intracellular degradation. VWF mutants with impaired C-terminal interchain disulfide bond formation (right) fail to form C-terminal dimers in the ER. The monomers can get through the ER to the Golgi and partly form N-terminal dimers which are tubulized and stored into WPB. The remaining monomers are presumably stored into WPB, however, whether they are incorporated into VWF tubules is unknown. This hypothesis may also apply to the mutants lacking N-terminal interchain disulfide bonds.

characterized by normal plasma level of VWF [31,34,35]. Even stronger support for our hypothesis comes from the 2010 version of the ISTH-SSC VWF mutation database (www.vwf.group.shef.ac.uk/). It lists with 72 different mutations involving cysteine residues, 50 with loss of cysteines, 18 with gain of cysteines and 4 with premature stop codons (Table 2). Among these mutations, 42 that predict the loss of intrachain disulfide cysteines are associated with low plasma levels of VWF, whereas five (p.Cys1099Tyr, p.Cys2771Ser/Tyr and p.Cys2773Arg/Ser) that predict the loss of interchain disulfide cysteines are associated with normal to relatively high plasma levels of VWF [14-17,31,35]. We, therefore, believe that the differences are related to differential effects of mutations involved in intrachain *versus* interchain disulfide bonds.

We propose a model to explain the differential effects of intrachain *versus* interchain disulfide bonds on WPB formation (Figure 6). Disruption of intrachain disulfide bonds in VWF may mainly disturb the conformation of VWF such that the ER quality control system prevents trafficking to the Golgi apparatus [36]. Retained VWF molecules either aggregate in the ER to lead to ER dilation (Figure 2F-G) [13,33] or undergo intracellular degradation [13,37]. Some mutant VWF molecules appear to escape the quality control system and are able to reach the Golgi apparatus. The extent to which this escape takes place appears to depend on the nature of the defects. Once the mutants get to the *trans* Golgi, where further processes of VWF are highly regulated by pH, [6,7,38] their effects are minimal unless the defects cause severe changes in VWF conformation. In contrast, disruption of interchain disulfide bonds such as that in the VWF C-termini does not hamper trafficking to the Golgi apparatus and WPB thereafter. C-terminal dimerization of VWF is not, therefore, required for WPB formation. According to the notion that N-terminal dimerization (i.e. multimerization) of VWF is not required for WPB formation, [39] the mutations that disrupt interchain disulfides in VWF N-terminal may not disrupt the biogenesis of WPB as well. It is thus tempting to speculate that unpairing all the five interchain disulfide bonds (two at the N-terminal and three at the C-terminal) should have minimal, if any, effect on the storage and secretion of VWF.

Tubulation is a key step in the storage of VWF into WPB. Recent studies provide emerging evidence in support of a hypothesis that VWF tubulation is regulated by the low pH in *trans* Golgi and WPB [7,38,40,41]. In a cell-free system with low pH as in *trans* Golgi, Huang and colleagues showed that truncated N-terminal dimers of VWF D'D3 domains together with VWF propeptide formed tubular structures by self-assembly [38]. The present data confirmed this finding for full-length VWF in a cell system. The VWF p.Cys2773Ser that forms N-terminal dimers [17] and partly remains as monomers showed normal tubulation (Figure 2E). The persistence of monomers raises two questions: (i) whether (and how) the monomers are stored into pseudoWPB; and (ii) are these monomers able to form VWF tubules? As VWF is mainly secreted from WPB via basal and regulated pathways, the large amount of monomers that was detected in the medium of cells expressing p.Cys2773Ser (Figure 5A) suggests that VWF monomers

Table 2. VWF cysteine mutations identified in VWD*.

Loss of Cysteine				Gain of Cysteine			
Domain	Mutation	VWD type	VWF level	Domain	Mutation	VWD type	VWF level
D1	p.Cys57*	3	↓	D1	p.Trp377Cys	3	↓
D1	p.Cys263_E270del	3	↓	D2	p.Arg760Cys	2N	↓
D1	p.Cys275(Ser/Arg)†	1 or 3	↓	D'	p.Tyr795Cys	2N	↓
D2	p.Cys570Ser	2A	↓	D3	p.Tyr1107Cys	Unclassified	↓
D2	p.Cys623Trp	2A	↓	D3	p.Tyr1146Cys	1	↓
D'	p.Cys709Leufs*3	2A	↓	A1	p.Arg1308Cys	2B	n.a
D'	p.Cys788 (Arg/Tyr)	2N	↓	A1	p.Trp1313Cys	2B	n.a
D'	p.Cys804Phe	2N	↓	A1	p.Arg1315Cys	1 or 3	↓
D'	p.Cys858Phe	2N	↓	A1	p.Arg1342Cys	1	↓
D3	p.Cys996Gly	1	↓	A1	p.arg1374Cys	1 or 2A or 2M	↓
D3	p.Cys1060(Arg/Tyr)	1 or 2N	↓	A1	p.Arg1379Cys	1 or Unclassified	↓
D3	p.Cys1071Phe	3	↓	A1	p.Arg1399Cys	Unclassified	n.a
D3	p.Cys1099Tyr‡§	IIC Miami	=/↑	A2	p.Phe1514Cys	2A	n.a
D3	p.Cys1101(Arg/Trp)	Unclassified	n.a	A2	p.Tyr1584Cys	1	↓
D3	p.Cys1111Tyr	1	↓	A3	p.Trp1745Cys	2M	↓
D3	p.Cys1130(Phe/Gly/Arg)	1	↓	B1-B3	p.Arg2379Cys	1	↓
D3	p.Cys1149Arg	1	↓	C2	p.Gly2441Cys	1	↓
D3	p.Cys1157Phe	Unclassified	↓	C2	p.Arg2464Cys	1 or Unclassified	↓
D3	p.Cys1190Arg	1	↓				
D3	p.Cys1196Arg	2M or 3	↓				
D3	p.Cys1225Gly	2N	↓				
D3	p.Cys1227Arg	1	↓				
D3	p.Cys1234Trp	Unclassified	↓				
A1	p.Cys1272(Phe/Gly/Arg/Ser)	2A	↓				
A1	p.Cys1458Tyr	2A	n.a				
D4	p.Cys2174Gly	3	↓				
D4	p.Cys2257Ser	1	↓				
B1-B3	p.Cys2304Tyr	1	↓				
B1-B3	p.Cys2340Arg	1	↓				
B1-B3	p.Cys2362Phe	1 or 3	↓				
C2	p.Cys2477(Ser/Tyr)	1	↓				
C2	p.Cys2533*	Unclassified	↓				
C2	p.Cys2557Serfs*8	3	↓				
C2-CK¶	p.Cys2671Tyr	3	↓				
C2-CK¶	p.Cys2693Tyr	1	↓				
CK	p.Cys2739Tyr	3	↓				
CK	p.Cys2754Trp	3	↓				
CK	p.Cys2771(Ser/Tyr)‡	2A(IID)	=/↑				
CK	p.Cys2773(Arg/Ser)‡	2A(IID)	=/↑				
CK	p.Cys2804(Tyr/fs)	1 or 3	↓				
CK	p.Cys2806Arg	Unclassified	↓				

* Summarized from the ISTH-SSC VWF mutation database up to 2010 (www.vwf.group.shef.ac.uk/). †When there are multiple mutations reported at a position the various amino acid substitutions are indicated in brackets. ‡ Cysteines involved in the interchain disulfide bonds in VWF; n.a., data not available. § Not yet in the ISTH-SSC VWF mutation database [35]. ¶ The connecting area between C2 and CK domain.

are probably stored in the pseudo-WPB. Furthermore, immunofluorescent staining showed that very little VWF p.Cys2773Ser was localized in the ER (Figure 1D), indicating that the excessive monomers found in the lysate (*data not shown*) are indeed stored in the pseudo-WPB. It is unknown whether those monomers are able to form tubules in the *trans* Golgi network as it has been suggested that N-terminal dimerization is required for the tubulation of VWF in a cell-free system [38]. Strikingly, Zhou and colleagues observed a dimeric bouquet-like VWF complex formed at the acidic pH of the *trans* Golgi and WPB [7]. The C-terminal dimer of VWF was zipped up by the acidic pH from the cysteine knot to B1 domain (six domains) into a dimeric stem. This process was proposed to facilitate VWF tubulation [7]. As such, unpairing the interchain disulfide bonds at the cysteine knot domain, for example, by mutation p.Cys2773Ser, would unlock the start point of the zip. It is unknown whether the stability and/or conformation of the dimeric complex would be affected. As formation of the dimeric complex is highly pH-dependent, the p.Cys2773Ser monomers are likely to form the bouquet-like complex and, therefore, to incorporate into VWF tubules. In contrast, VWF mutations p.Cys2739Tyr and p.Cys2754Trp which cause severe conformational changes by defects in intrachain disulfide bonds led to disorganized tubular storage of VWF [13]. This suggests that the conformational changes in VWF may be more disruptive to VWF tubulation than no interchain pairing.

In conclusion, we postulate that natural mutations of cysteines involved in the formation of interchain disulfide bonds, p.Cys2771, p.Cys2773 and p.Cys2811 in the cysteine knot domain and p.Cys1099 and p.Cys1142 in the D3 domain, do not affect the storage and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds lead to reduced VWF storage and secretion due to ER retention.

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CHAPTER

4

Storage and secretion of naturally occurring von Willebrand factor A domain variants

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Abstract

Von Willebrand disease (VWD) is a bleeding disorder characterized by reduced plasma von Willebrand factor (VWF) levels or functionally abnormal VWF. 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, expression studies of variants located in the A domains of VWF are limited. We therefore characterized the biosynthesis of VWF mutations, located in the VWF A1-A3 domains, which were found in families diagnosed with VWD. Human Embryonic Kidney 293 (HEK293) cells were transiently transfected with plasmids encoding full-length wild-type VWF or mutant VWF. Six mutations in the A1-A3 domains were expressed. We found that all mutants, except one, showed impaired formation of elongated pseudo-Weibel-Palade bodies (WPB). In addition, two mutations also showed reduced numbers of pseudo-WPB, even in the heterozygous state, and increased endoplasmic reticulum retention, which is in accordance with the impaired regulated secretion seen in patients and. 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.

Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein. Upon vascular damage VWF serves as an adhesion molecule for platelets thereby initiating platelet plug formation. VWF is also the carrier protein of coagulation factor VIII (FVIII). VWF is synthesized in endothelial cells and megakaryocytes and is either secreted constitutively into plasma or stored in specific organelles; Weibel-Palade bodies (WPB) in endothelial cells or α -granules in megakaryocytes and platelets. The highest molecular weight VWF multimers are stored and are released from endothelial cells in response to agonists like thrombin, epinephrine, and vasopressin or its synthetic analogue desmopressin (DDAVP) [1,2]

A decreased plasma level or abnormal function of VWF is responsible for von Willebrand disease (VWD), the most common inherited bleeding disorder with an estimated prevalence ranging from 0.035% to 1.3% of the population [1,3]. Mutations in the VWF gene may lead to decreased VWF plasma levels via decreased synthesis, impaired secretion, increased clearance or a combination of these conditions. A subset of VWD patients shows a reduced response to DDAVP, a specific agonist for release of WPB, which is clinically applied to raise VWF plasma levels [4]. The lack of response to DDAVP suggests the absence of recruitable WPB. The VWF propeptide (D1-D2 domains) and D'D3 domains are involved in the tubular assembly of VWF, which drives WPB formation [5,6]. Michaux *et al.* [6] showed that for elongation of the WPB at least the propeptide and the D'-A1 domains are necessary. Interestingly, the reduced or

absent response to DDAVP was mainly found among patients with mutations in the D3-A3 domain of VWF [4]. This suggests that determinants for biogenesis of recruitable WPB are also located outside of the D1-A1 domains. However, most expression studies on the storage and secretion of VWF are focussed on VWF variants located in the D1-D3 or D4-CK domains of VWF [7-10]. Currently 364 different mutations are registered in the VWF database [11]; 161 (44%) of these mutations are located in the A1-A3 domains of VWF. However, expression studies on variants located in the A domains of VWF are limited, especially for variants which are not identified as apparent type 2A or 2B VWD. We therefore aimed to investigate and characterize the synthesis and secretion of VWF variants in the VWF A1-A3 domain.

Materials and Methods

Patients and Mutations

The mutations (Tables 1 and 2) that we have selected were originally identified in patients historically diagnosed with VWD type 1 [12-15]. However, subsequent detailed analysis of the multimeric patterns has revealed that some of those mutations were associated with minor multimer abnormalities [16]. Those multimer abnormalities were less outspoken than in classical type 2A patients; some may still be classified as type 1, whereas others should be better reclassified as type 2A (Table 1) [16,17].

Plasmid Constructs

Recombinant pCI-neo expression plasmid containing full-length wild-type human VWF (WT-VWF) cDNA has been described [7]. Mutations were introduced into pCI-neo WT-VWF plasmid with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). DNA was purified using the PureYield™ Plasmid Maxiprep System (Promega) and preparations were verified by sequencing.

Cell culture and transfections

HEK293 cells from ATCC (Rockville, USA) were cultured and transfected as described [7]. Briefly, using FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany) plasmid constructs were transiently transfected into HEK293 cells at a final concentration of 0.66 µg/mL (0.33 µg/mL WT-VWF and mutant VWF plasmids, respectively, for co-transfections). All data were collected 72 hours post-transfection.

Immunostaining

Cells were stained with immunofluorescent antibodies and analyzed by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective [7]. Polyclonal FITC-conjugated sheep anti-human VWF antibody (Abcam, Cambridge, UK) and polyclonal rabbit anti-human protein disulfide isomerase (PDI) antibody A66 (obtained from Prof. I. Braakman, Department of Chemistry, Utrecht University, Utrecht, The Netherlands) were used as primary antibodies against VWF and the endoplasmic reticulum (ER) marker PDI, respectively. Alexa 594-conjugated secondary antibody was

purchased from Invitrogen. Monoclonal mouse anti-LAMP1 (mAb BB6; kindly provided by Dr. S. Carlsson, Umeå, Sweden) was used to visualize lysosomes. The percentage of cells that showed ER retention of VWF was determined in two independent experiments for all VWF variants; in total 200 cells were analyzed per variant. To quantify pseudo-WPBs, 100 VWF-positive cells from two independent experiments were randomly selected and analyzed. We used similar morphological criteria as reported by Michaux *et al* [18]. Briefly, we categorized the pseudo-WPBs with a ratio of length over diameter <2 as “round pseudo-WPBs” and those with a ratio ≥ 2 as “elongated pseudo-WPBs.”

Structural analysis of VWF

VWF multimer analysis was performed as previously described [7]. Processing of VWF was analysed by reducing SDS-PAGE and Western blot analysis for the conditioned media and cell lysates. Samples were reduced using 20 mM dithiothreitol, separated by Novex 6% Tris-Glycine gel electrophoresis (Invitrogen), immunostained with polyclonal rabbit anti-human VWF conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark) and visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

Basal and regulated secretion of VWF

Basal secretion of VWF was determined in media and cell lysates collected 72 hours post-transfection as described previously [7]. Briefly, cells grown until 80% confluency, were transfected with plasmids and 24 hours after transfection medium was refreshed. 48 hours after refreshment medium was collected and cells were lysed. To analyze the regulated secretion of VWF, 72 hours post-transfection cells were incubated for 60 minutes with release medium containing 160 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) or vehicle (DMSO). VWF:Ag in media and cell lysates was determined by ELISA [19]. Regulated secretion is expressed as secreted VWF as a fraction of total VWF (medium plus lysate). Statistical analyses were performed by the Student's *t*-test using GraphPad Prism (version 4.02).

Transmission electron microscopy

Transfected cells grown on 35-mm petri dishes were fixed and prepared for transmission electron microscopy (TEM) as described [20]. Briefly, osmium tetroxide and tannic acid treated cells were serially dehydrated with ethanol and embedded in Epon. Samples were cut into thin sections of 70-100 nm, stained with 7% uranyl acetate and Reynolds lead citrate, and analyzed with a FEI company Tecnai 12 at 120 kV equipped with a 4kx4k CCD camera (Model Eagle, Fei Company, Eindhoven, The Netherlands).

Results

The mutations that we have selected were originally identified in patients historically diagnosed with VWD type 1 [12-15]. Characteristics of VWD patients carrying the selected mutations are given in Tables 1 and 2.

Pseudo Weibel-Palade body formation in VWF transfected cells

We analysed the formation and intracellular distribution of pseudo-WPB in HEK293 cells by transiently transfecting cells with VWF variants. Cells transfected with WT-VWF or VWF p.Arg1583Trp formed numerous elongated pseudo-WPB (Figure 1). p.Ser1285Pro, p.Leu1307Pro, p.Arg1374His, p.Tyr1584Cys and p.Val1822Gly induced pseudo-WPB that appeared to be shorter and rounder when compared to those observed for WT-VWF (Figure 1).

Table 1. Summary of patient characteristics obtained from the MCMDM-1VWD study.*

Variant	Domain	No. of ICs with this mutation	FVIII:C IU/dL	VWF:Rco IU/dL	VWF:Ag IU/dL	VWF:CB IU/dL	FVIII:C/VWF:Ag	Multimers	(Sub) type†
p.Ser1285Pro	A1	1	27	3	16	8	1.69	relative decrease of the large multimers, but normal triplet structure	1
p.Leu1307Pro	A1	1	33	7	16	6	2.06	relative decrease of the large multimers, but normal triplet structure	2A
p.Arg1374His	A1	2	48-79	10-16	29-74	22-23	1.07-1.66	classical 2A(IIIE) pattern	2A
p.Arg1583Trp	A2	1	56	54	52	57	1.07	normal	1
p.Tyr1584Cys	A2	10	21-182	36-93	21-80	20-115	0.70-2.32	normal	1
p.Val1822Gly	A3	1	35	26	19	45	1.84	2A(IIIE); relative decrease of the large multimers, barely visible outer sub-bands. Typical pattern but less pronounced	1

* Data previously obtained in the MCMDM-1VWD.

† (Sub) type designated by Budde *et al.* according to the 2006 classification.

Table 2. Summary of DDAVP characteristics.*

Variant	FVIII:C IU/dL			VWF:Rco IU/dL			VWF:Rco/Ag		Response‡
	Before	Peak	Fold increase	Before	Peak	Fold increase	Before	Peak	
p.Leu1307Pro	36	118	3.3	6	22	3.7	0.43	0.46	Partial
p.Arg1374His	73	213	2.9	18	34	1.9	0.22	0.18	Non-responder
p.Arg1583Trp	31	97	3.1	48	130	2.7	0.92	0.91	Complete
p.Tyr1584Cys	91	113	1.2	75	136	1.8	0.94	1.36	Complete
p.Val1822Gly	38	79	2.1	16	48	3.0	0.55	0.83	Partial

* Data reported by the MCMDM-1VWD study and own clinical data.

‡ Patients were defined as follows: complete responders: both VWF:Rco and FVIII:C were 50 IU/dL or higher after desmopressin; partial responders: VWF:Rco or FVIII:C were lower than 50 IU/dL but increased at least 3-fold; and nonresponders: neither criterion. Patients with VWF:Rco and/or FVIII:C 50 IU/dL or higher at baseline at time of testing were considered as complete responders if a level of 100 IU/dL or higher was reached for both measurements.

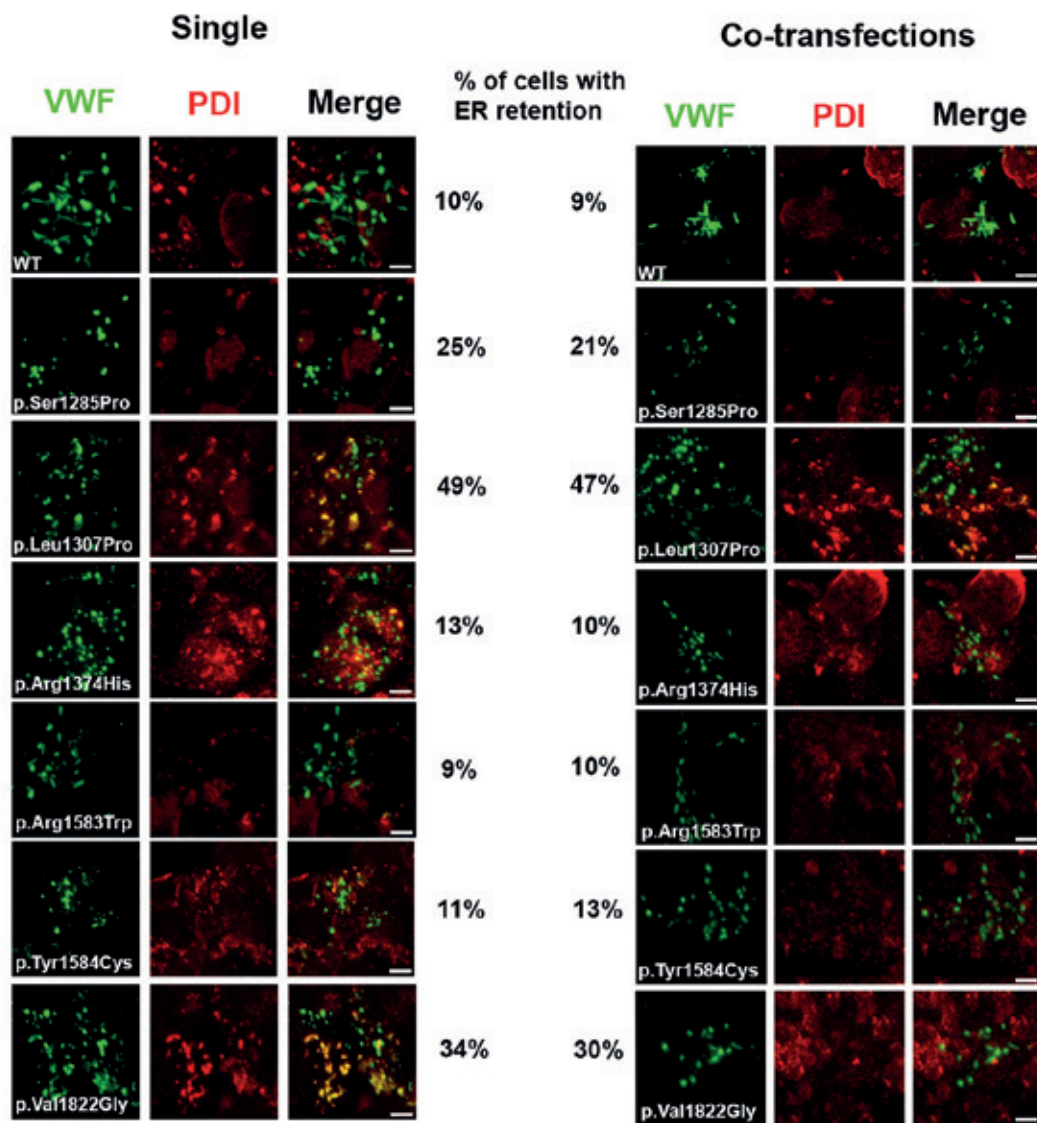


Figure 1. Intracellular localization of VWF in transfected HEK293 cells. HEK293 cells were transiently transfected with WT-VWF or mutant VWF (single transfections, left panel) or with WT- and mutant VWF (co-transfections, right panel). Cells were fixed for immunofluorescence imaging 72 hours post-transfection and stained for VWF (green channel) and PDI (an ER marker, red channel). In merged images, pseudo-WPB show up in green (VWF staining only), and VWF in the ER shows up as yellow (double staining of VWF and PDI). Scale bar = 5µm. The percentage of cells (indicated in the middle) that showed ER retention of VWF was determined in two independent experiments in 200-260 cells expressing each VWF variants in single and co-transfections with WT.

No VWF was detectable by immunofluorescent staining in lysosomes (*data not shown*), indicating that the round organelles are not VWF containing lysosomes. Increased retention of VWF in the ER, identified by the co-localization of VWF and PDI (yellow colour), was observed for p.Ser1285Pro, p.Leu1307Pro and p.Val1822Gly (Figure 1). Percentage of ER retention of p.Arg1374His, p.Arg1583Trp and p.Tyr1584Cys was similar to that of WT-VWF (Figure 1). To mimic the heterozygous state of patients, co-transfections of the variants with WT-VWF were performed. Upon co-transfection of mutant and WT-VWF (Figure 1, right panels) the percentage of transfected cells with ER retention was similar to that observed for the single transfections (Figure 1).

For quantification of the number and shape of pseudo-WPB we randomly selected confocal microscopy fields and analysed 100 cells from each sample. Pseudo-WPB were defined by a positive VWF staining, and the absence of co-staining with the ER marker PDI. For WT-VWF, 70% of the transfected cells contained more than 30 pseudo-WPB per cell. Similar numbers of pseudo-WPB were found for the VWF variants, except for the p.Leu1307Pro and p.Val1822Gly mutations where only 36% and 41% of the cells

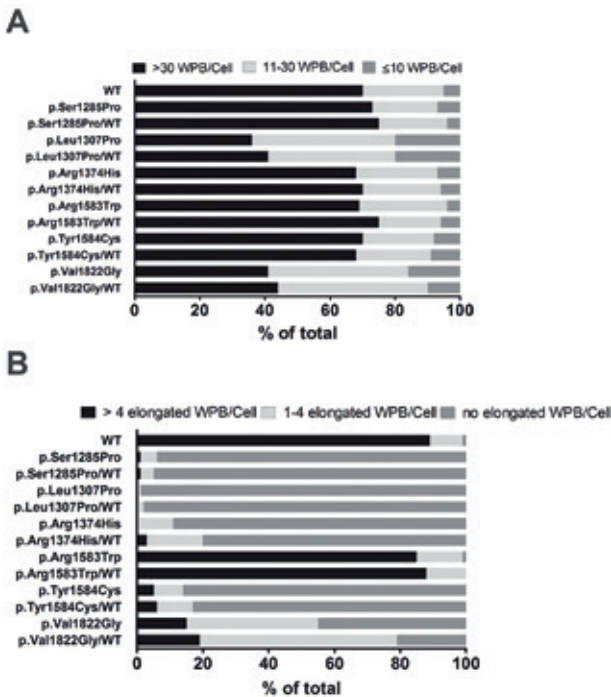


Figure 2. Defects in pseudo-WPB number and formation. HEK293 cells were transfected, fixed and stained as described in Figure 1. Images were taken by confocal microscopy and cells were analysed for quantitative analysis. Cells were divided into three categories according to the number of pseudo-WPB per cell (Panel A): more than 30; from 11-30; 10 or less per cell; or according to the number of elongated pseudo-WPB per cell (Panel B): more than 4; from 1 to 4; non elongated pseudo-WPB per cell. Per VWF variant, 100 cells expressing VWF were analysed for each bar. Each bar represents the mean of two independent experiments.

contained more than 30 pseudo-WPB respectively (Figure 2A). Co-transfection with WT-VWF only marginally increased the number of WPB formed by these variants (Figure 2A). When considering the shape of the pseudo-WPB, 89% of the WT-VWF transfected cells had 4 or more elongated (length over diameter >2) pseudo-WPB. For all variants, except the p.Arg1583Trp, a decrease in the number of elongated pseudo-WPBs per cell was observed. No elongated organelles could be found for the p.Leu1307Pro variant. Upon co-transfection with WT-VWF the percentage of cells displaying elongated pseudo-WPBs was not significantly improved for the VWF variants (Figure 2B).

Pseudo-WPB formation was further studied using TEM. In WT-VWF or p.Arg1583Trp transfected cells, elongated and electron dense pseudo-WPB were observed with the typical striations seen in endothelial cells WPB [21] (Figure 3A, E). Shorter and more

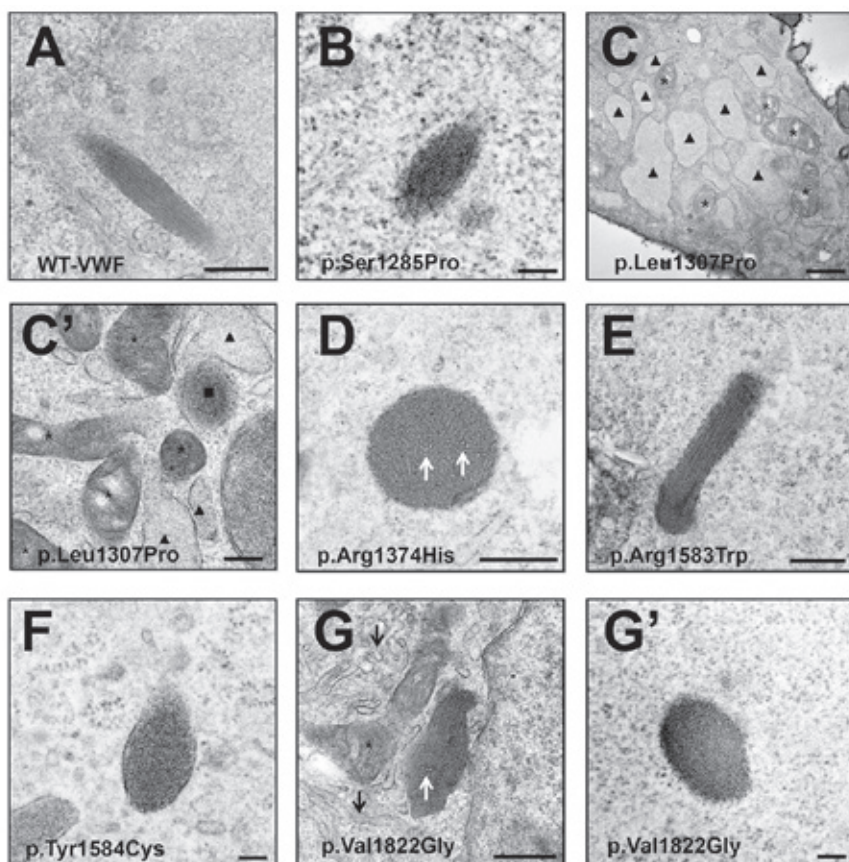


Figure 3. Ultrastructure of transfected HEK293 cells visualized by TEM. HEK293 cells were transiently transfected as indicated. Pseudo-WPB displaying VWF tubules were formed by WT-VWF (A) and the mutants (B, D-G'). White arrows indicate visible cross-sections of tubules (D,G). For the p.Leu1307Pro we were not able to visualize with certainty organelles with internal tubules (C', black square), however, dilated ER was predominantly observed (C, C'). The arrowheads, stars and black arrow indicate ER, mitochondria and Golgi, respectively. Scale bar panels A,C,E,G = 500nm; scale bar panels B,C',D,F,G' = 200nm.

round pseudo-WPB were found in cells expressing VWF p.Ser1285Pro, p.Arg1374His or p.Tyr1584Cys (Figure 3B, D, F). The p.Val1822Gly variant showed a few elongated pseudo-WPB, while round structures were found frequently (Figure 3G-G'), in concordance with the confocal images where most VWF-containing structures were short or round (Figure 2B). The round structures that we observed for the variants are recognizable as pseudo-WPB as they contain tubular structures resembling stored VWF (Figure 3B, D, F-G'). Grossly dilated ER surrounded by mitochondria was frequently observed in cells expressing the p.Leu1307Pro variant (Figure 3C), in accordance with the observed ~50% ER retention (Figure 1). We could not with certainty visualize organelles with internal tubules for p.Leu1307Pro, but round structures were observed (Figure 3C').

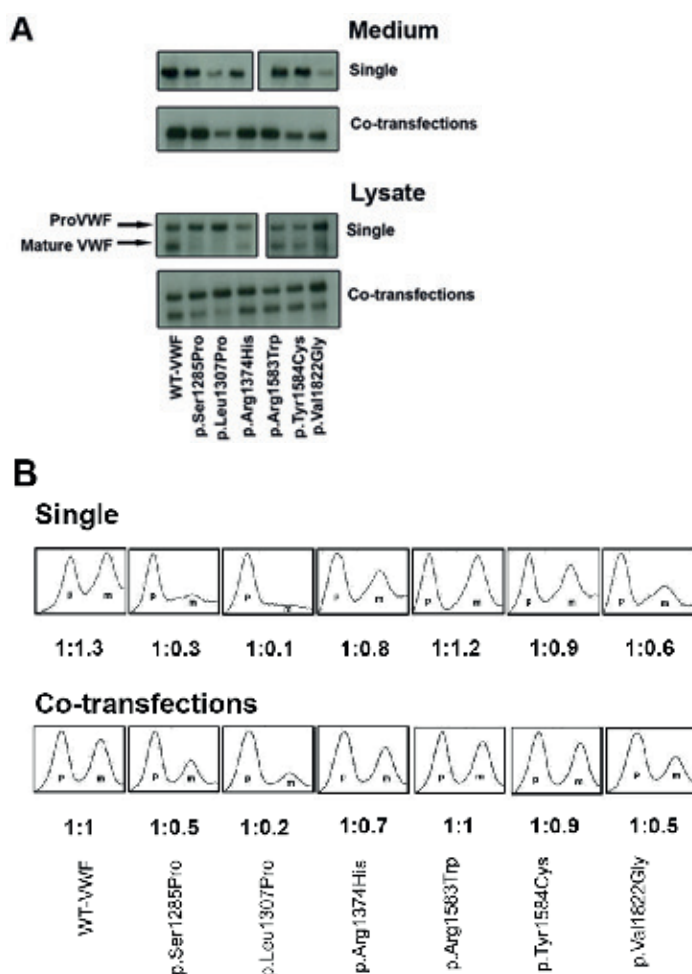


Figure 4. Subunit composition of VWF under reducing conditions. VWF secreted into medium or VWF retained in lysates was reduced with DTT and analyzed with SDS-PAGE and Western blot (A). In panel B, the ratio of proVWF (p) and mature VWF subunit (m) in the cell lysate was analyzed with Image J (NIH software, version 1.44P).

Retention of VWF was further investigated by evaluating the amount of processed VWF with Western blotting under reducing conditions. VWF secreted into the medium consisted of fully processed mature VWF subunits (Figure 4A). Cell lysates showed both mature VWF subunits and unprocessed proVWF. The p.Ser1285Pro, p.Leu1307Pro and p.Val1822Gly variants showed relatively more uncleaved proVWF compared to WT-VWF as indicated by the ratio between mature VWF and proVWF (Figure 4B, upper panel). This is in accordance with the increased ER retention measured in the transfected cells of these variants (Figure 1). Upon co-transfection with WT-VWF some increase in the relative amount of cleaved VWF was observed for p.Ser1285Pro and p.Leu1307Pro (Figure 4B, lower panel).

Multimeric structure of VWF

Compared to WT-VWF all variants, except p.Leu1307Pro, showed a similar and normal VWF multimer distribution in medium (Figure 5A) and lysate (Figure 5B). The p.Leu1307Pro mutation, however, led to a relative loss of the larger multimers in both medium and lysate. Upon co-transfection of p.Leu1307Pro with WT-VWF also larger multimers were observed (Figure 5). However, relatively more dimers and tetramers were observed in the multimer pattern of the p.Leu1307Pro/WT-VWF co-transfection when compared to the multimer pattern of WT-VWF alone. In the MCMDM-1VWD study subtle differences in multimer distribution were also observed in plasma samples of patients with mutations p.Ser1285Pro, p.Arg1374His and p.Val1822Gly (Table 1). The apparent discrepancy with the multimer analysis presented in this study may be explained by the fact that different techniques were used to visualize VWF multimers. Furthermore, in our study we analyzed multimer patterns of VWF released into medium whereas in the MCMDM-1VWD study plasma samples were used. Proteolysis of VWF by ADAMTS13 or clearance could therefore play a role in the observed minor multimer abnormalities.

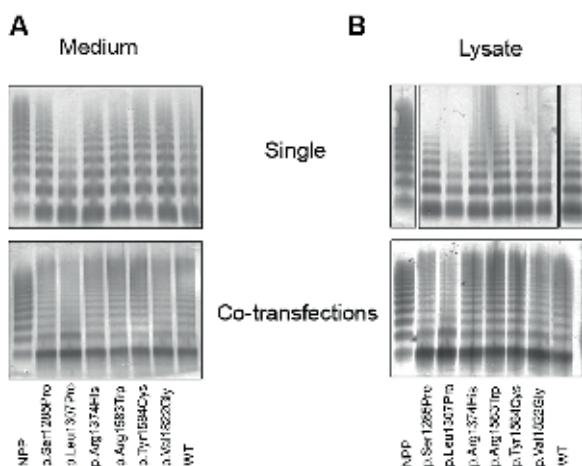


Figure 5. Multimeric analysis of recombinant VWF. Multimer patterns were analysed in conditioned media (A) and cell lysates (B). Multimers are shown for single- and co-transfections. The multimer patterns were analyzed by SDS-agarose gel electrophoresis and Western blot under non-reducing conditions. The separate lanes in panel B are from the same blot, but normal pool plasma (NPP) and wt-VWF (WT) were switched for consistency in the order of lanes.

Basal and regulated secretion of VWF

To investigate the effect of VWF mutants on the secretion of VWF, both basal and regulated secretion were studied. Basal secretion was defined as the amount of VWF:Ag in conditioned medium 72 hours post-transfection (Table 3 and Figure 6A).

p.Ser1285Pro, p.Arg1583Trp and p.Tyr1584Cys variants show normal basal secretion of VWF compared to WT-VWF (Figure 6A) in the single transfections. Furthermore, total VWF production (VWF in both medium and lysate) was similar for WT-VWF and these mutants (Table 3). In contrast, basal secretion of single transfected p.Leu1307Pro, p.Arg1374His and p.Val1822Gly mutations was markedly reduced when compared to WT-VWF. This was apparent from both the amount of VWF secreted into the medium as well as the reduced ratio between the absolute amount of VWF present in medium and lysate (Table 3). Also, the total amount of VWF (medium plus lysate) was significantly reduced (60-70mU VWF for the variants versus ± 100 mU VWF for WT-VWF) (Table 3).

Previously we showed that in co-transfection experiments both constructs are expressed in individual cells [8]. After co-transfection with WT-VWF basal secretion was restored for the p.Arg1374His mutation to almost normal (85 ± 2.4 relative to WT-VWF). The absolute amount of VWF produced by this mutant was also restored to normal levels upon co-expression of WT-VWF ($95\text{mU}\pm 4.8$ versus $102\text{mU}\pm 3.7$ for WT-VWF). The secretion defect observed for the variants p.Leu1307Pro and p.Val1822Gly was partly restored upon co-transfection with WT-VWF (51 ± 0.9 and 73 ± 0.6 respec-

Table 3. Expression of VWF variants in transfected HEK293 cells.

Variant	Transfection	VWF:Ag* medium		VWF:Ag* lysate		Total (medium + lysate)	Ratio medium/ lysate	Normalized Ratio§
		mU	%	mU	%			
WT-VWF		71 $\pm$ 2.3	100	32 $\pm$ 2.0	100	102 $\pm$ 3.7	2.2 $\pm$ 0.12	1
p.Ser1285Pro	Single transfection	73 $\pm$ 3.1	103 $\pm$ 4.5	39 $\pm$ 2.7	123 $\pm$ 1.2	112 $\pm$ 5.8	1.9 $\pm$ 0.05	0.9
	Co-transfection	71 $\pm$ 2.4	100 $\pm$ 1.2	35 $\pm$ 2.2	112 $\pm$ 2.1	105 $\pm$ 5.7	2.0 $\pm$ 0.12	0.9
p.Leu1307Pro	Single transfection	28 $\pm$ 1.2	40 $\pm$ 2.3	42 $\pm$ 3.3	132 $\pm$ 2.9	70 $\pm$ 4.4†	0.7 $\pm$ 0.03†	0.3
	Co-transfection	36 $\pm$ 1.0	51 $\pm$ 0.9	35 $\pm$ 2.0	112 $\pm$ 2.9	72 $\pm$ 2.6†	1.0 $\pm$ 0.06†	0.5
p.Arg1374His	Single transfection	43 $\pm$ 2.5	61 $\pm$ 2.8	25 $\pm$ 1.4	80 $\pm$ 2.3	68 $\pm$ 3.0†	1.7 $\pm$ 0.14†	0.8
	Co-transfection	60 $\pm$ 1.2	85 $\pm$ 2.4	35 $\pm$ 3.8	111 $\pm$ 6.4	95 $\pm$ 4.8	1.7 $\pm$ 0.14†	0.8
p.Arg1583Trp	Single transfection	69 $\pm$ 1.9	97 $\pm$ 3.5	27 $\pm$ 1.4	84 $\pm$ 2.1	95 $\pm$ 3.1	2.6 $\pm$ 0.09	1.2
	Co-transfection	72 $\pm$ 3.7	102 $\pm$ 4.3	31 $\pm$ 2.9	97 $\pm$ 3.8	103 $\pm$ 2.5	2.4 $\pm$ 0.3	1.1
p.Tyr1584Cys	Single transfection	72 $\pm$ 5.2	101 $\pm$ 4.0	27 $\pm$ 2.8	87 $\pm$ 9.0	99 $\pm$ 8.1	2.6 $\pm$ 0.08	1.2
	Co-transfection	71 $\pm$ 3.9	101 $\pm$ 4.2	32 $\pm$ 2.1	102 $\pm$ 2.2	104 $\pm$ 6.0	2.2 $\pm$ 0.03	1
p.Val1822Gly	Single transfection	36 $\pm$ 2.0	51 $\pm$ 3.2	21 $\pm$ 2.2	50 $\pm$ 8.7	57 $\pm$ 4.1†	1.8 $\pm$ 0.12†	0.8
	Co-transfection	52 $\pm$ 1.5	73 $\pm$ 0.6	33 $\pm$ 2.7	105 $\pm$ 5.7	85 $\pm$ 4.2†	1.6 $\pm$ 0.09†	0.7

*VWF:Ag (mU) was produced by about 7×10^5 cells in medium or lysate. VWF:Ag is also expressed as percentage relative to the amount of VWF:Ag in WT-VWF transfected cells. Each value represents the mean $\pm$ SEM of three independent experiments performed in duplicate.

† Compared with WT-VWF $p<0.05$ (student's-t test)

§ Ratio for WT-VWF is set at 1, ratios of variants are then normalized against WT-VWF.

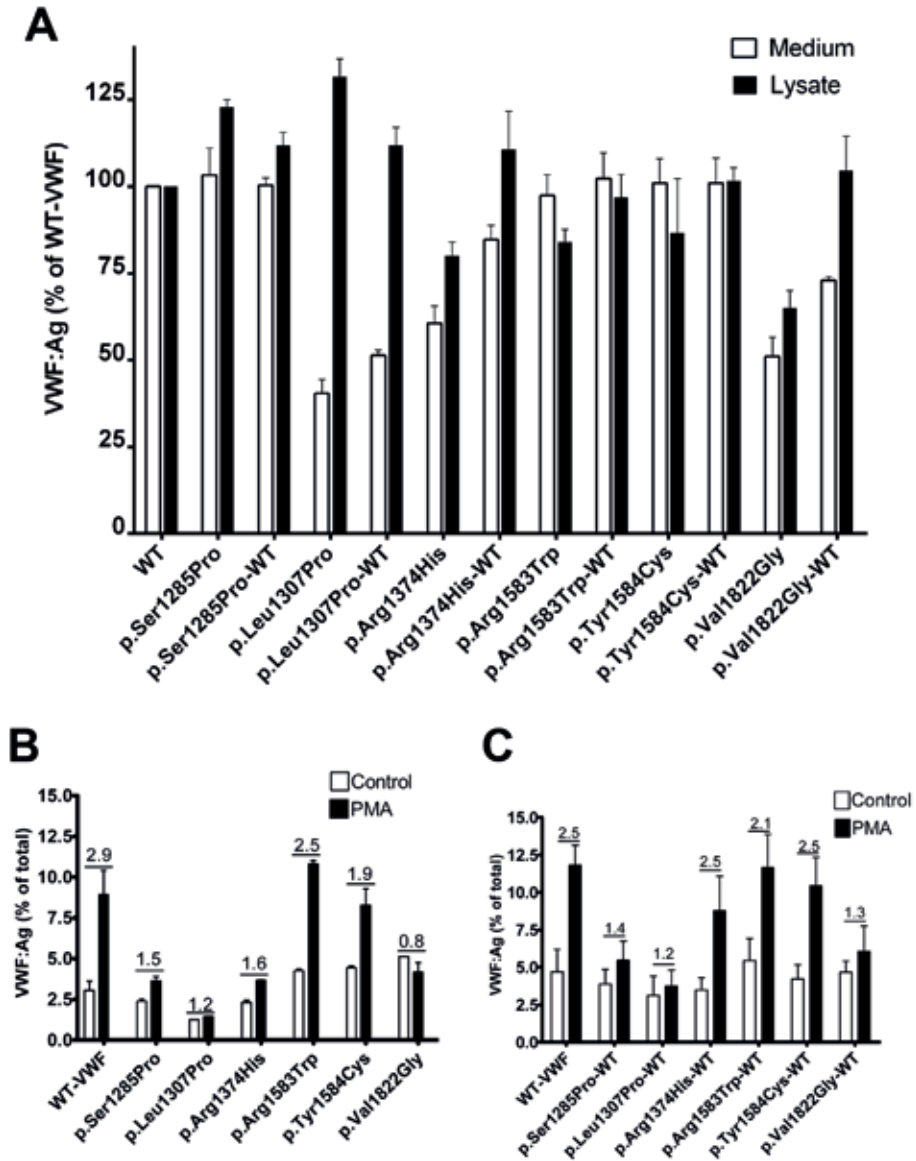


Figure 6. Basal and regulated secretion of VWF. HEK293 cells were transiently transfected with WT-VWF or mutants, or the mutants were co-transfected with WT-VWF at 1:1 ratio. (A) 72 hours post-transfection conditioned media and cell lysates were collected to determine basal secretion of VWF. WT-VWF is set at 100%. (B, C) Regulated secretion of VWF was analyzed as follows: 72 hours post-transfection HEK293 cells were rinsed and incubated at 37°C for 60 minutes in release medium with DMSO (Control) or with 160 nM PMA (PMA). Panel B shows regulated secretion of single transfections, panel C shows secretion of co-transfections. Each bar represents VWF secreted into the medium as a fraction of total VWF (secreted plus intracellular). Mean and SEM are based on three independent experiments in duplicate. Numbers above the bars indicate the fold increase of secreted VWF upon stimulation.

tively); the total VWF amount (medium plus lysate) remained reduced ($72\text{mU}\pm 2.6$, and $85\text{mU}\pm 4.2$, respectively, compared to $102\text{mU}\pm 3.7$ for WT-VWF). When compared with patients (Table 1), secreted levels of VWF in transfected cells were in concordance with plasma levels measured in patients with the corresponding mutations. Only the p.Ser1285Pro variant shows normal secretion in our system whereas the plasma level was significantly reduced in the patient (16 IU/dl). This difference is probably due to increased clearance of this VWF variant, as patients had increased VWFpp/VWF:Ag ratios [22].

The regulated secretion pathway of VWF was analyzed using PMA, which has been shown to efficiently induce exocytosis of pseudo-WPBs from transfected HEK293 cells and HUVECs [7,18,23]. After stimulation with PMA for 60 minutes, secretion of WT-VWF was increased almost 3-fold (9% of total VWF was secreted by cells stimulated with PMA versus 3% by non-stimulated cells, Figure 6B). Compared to WT-VWF transfected cells, p.Arg1583Trp and p.Tyr1584Cys transfected cells showed similar responses to PMA stimulation in both single and co-transfections (Figure 6B-C). This is also in line with the adequate response found in patients carrying these mutations after DDAVP infusion (2.7-fold and 1.8-fold increase of VWF:RCo) (Table 2) [4]. Cells expressing p.Ser1285Pro, p.Leu1307Pro, p.Arg1374His and p.Val1822Gly responded less well to PMA stimulation (0.8 to 1.6 fold increase following PMA stimulation) (Figure 6B-C). Co-transfection with WT-VWF corrected the response for p.Arg1374His and partially increased the response for p.Val1822Gly, but did not increase the response for p.Ser1285Pro and p.Leu1307Pro (Figure 6C).

Discussion

In this study we have expressed six VWF variants located within the A domains of VWF that were previously identified in patients diagnosed with VWD [12-15]. The p.Ser1285Pro (Figure 7A), p.Leu1307Pro (Figure 7B) and p.Arg1374His (Figure 7C) mutations are located within the A1 domain, the p.Arg1583Trp (Figure 7D) and p.Tyr1584Cys (Figure 7E) are located in the A2 domain and the p.Val1822Gly (Figure 7F) mutation is located in the A3 domain of VWF. We studied the ability of these VWD variants to induce the formation of pseudo-WPBs in HEK293 cells and we assessed whether PMA-induced WPBs release was impaired for these variants (Table 3).

Of the six mutations studied, the p.Arg1583Trp and p.Tyr1584Cys variants had normal basal VWF levels and responded adequately to PMA stimulation in the heterozygous state (Figure 6). However, the other four variants led to decreased basal secretion (p.Arg1374His), impaired regulated secretion (p.Ser1285Pro) or a combination thereof (p.Leu1307Pro and p.Val1822Gly) in the heterozygous state. A summary of the observed defects is given in Table 4.

Substitutions in the VWF gene may lead to misfolding of the VWF protein. ER localized molecular chaperones recognize misfolded proteins and promote their retention and

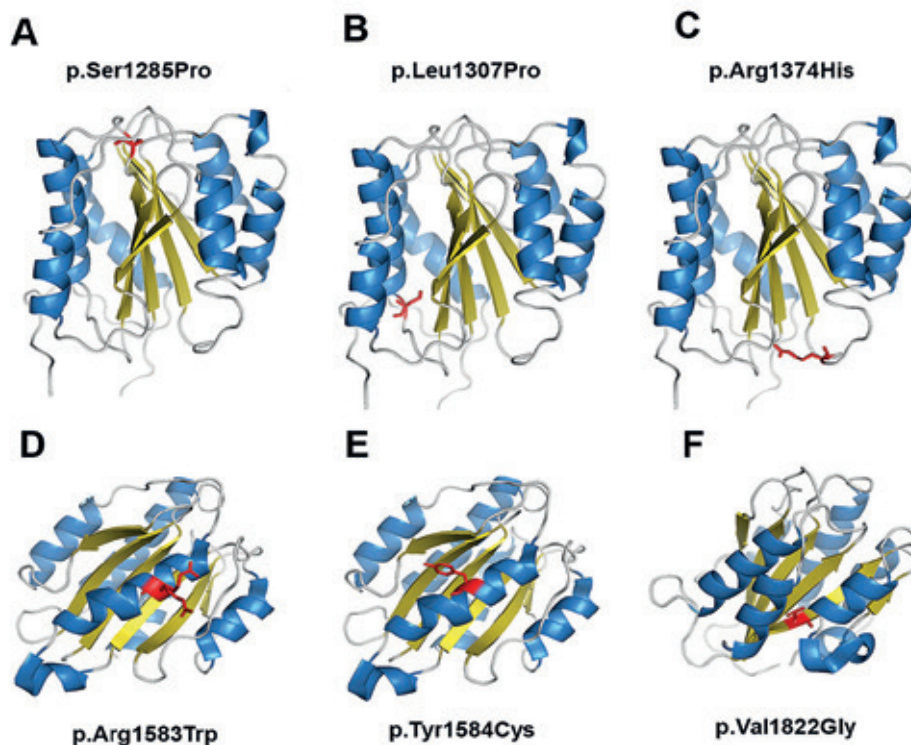


Figure 7. Secondary structure of the VWF A domains. Secondary structures of the A1, A2 and A3 domain of VWF were prepared using Pymol version 1.3. The positions of the mutations are indicated in red and visualised as “sticks”. Panel A shows the mutations that are located within the A1 domain, panel B the mutations that are located in the A2 domain and panel C shows the mutation located in the A3 domain of VWF

degradation [24–26]. Inspection of the A1–A3 substitutions analyzed in this study support a role for misfolding in ER retention. The serine at position 1285 is part of the glycosylation motif Asp-X-Ser [27] (Figure 7A). Disruption of this motif by substituting aspartic acid at position 1283 by alanine attenuated binding of the protein to glycoprotein Ib, probably by relaxation of the A1 domain fold [27]. Whether relaxation causes the defects seen in this variant remains to be determined. The leucine at position 1307 is located in the hydrophobic pocket at the end of the $\alpha 1$ helix of the A1 domain (Figure 7B). Proline has a poor helix forming propensity because it cannot donate an amide hydrogen bond and because its side chain interferes sterically with the backbone of the preceding turn [28]. Prediction software suggested that the substitution to a proline will lead to loss of stability of the protein (SIFT [29] and PolyPhen2 [30], *data not shown*), and this could therefore be the cause of increased ER retention of this VWF variant. The arginine at position 1374 makes important interactions in the $\alpha 3\beta 4$ loop of the A1 domain [31]. Within the crystal structure of the A1 domain the arginine is buried (Figure 7C) and its replacement by a histidine will probably destabilize the $\alpha 3\beta 4$ loop of the A1 domain [32]. The p.Val1822Gly substitution is located in the $\beta 5$

Table 4. Summary of defects caused by single transfection of mutations in the A domain of VWF compared to WT-VWF.

Domain	Variant	ER retention	Pseudo WPB formation ‡		Secretion of VWF		Secreted multimers
			No.	Elongated shape	Basal	Stimulated	
A1	p.Ser1285Pro	↑	N	↓↓	N	↓	N
A1	p.Leu1307Pro	↑↑	↓	↓↓↓	↓↓↓	↓↓	Lack of HMW multimers
A1	p.Arg1374His	=	N	↓↓	↓↓	↓	N
A2	p.Arg1583Trp	=	N	N	N	N	N
A2	p.Tyr1584Cys	=	N	↓↓	N	N	N
A3	p.Val1822Gly	↑	↓	↓	↓↓	↓↓	N

‡Based on immunofluorescent images (Figure 2). N, normal; =, similar to WT-VWF.

strand of the A3 domain and is buried in a hydrophobic pocket (Figure 7F). Substitution of a valine for a glycine will affect interactions with neighbouring hydrophobic residues, possibly resulting in destabilization of the A1 domain.

Except for the p.Arg1583Trp variant, all mutations formed round pseudo-WPB as evidenced by both confocal imaging and electron microscopy (Figures 1,3). The p.Ser1285Pro variant led to the formation of round pseudo-WPB and showed ER retention (Figures 1,2), which was supported by the relative increase of unprocessed proVWF (Figure 4). The impaired synthesis we observed is supported by the increased FVIII/VWF ratio found in patients (1.69, Table 1), which also suggests impaired synthesis of the VWF protein. Blood outgrowth endothelial cells (BOECs) from a patient carrying this variant also showed mainly short, round WPB and ER retention [33]. The increased VWFpp/VWF:Ag ratio measured in patients with this p.Ser1285Pro mutation suggests that this VWF variant is also cleared faster [22]. This may explain the difference between plasma levels of the patients and levels measured in our experimental system where clearance is not involved. The response to DDAVP was unfortunately not tested for patients with the p.Ser1285Pro mutation. However, one might speculate that patients will show a poor to partial response to DDAVP infusion based on our *in vitro* data and data obtained from BOECs of a VWD patient [33]. We anticipate that the regulated secretion in patients carrying this mutation might be quantitatively impaired due to retention of VWF in the ER.

The p.Leu1307Pro variant showed severe retention of the protein in the ER, a reduced number of WPB and produced only round storage organelles. Basal and regulated secretion of VWF were impaired as well. This phenotype was not improved when WT-VWF was co-transfected. This is in line with data obtained from BOECs of a VWD patient heterozygous for this mutation, in which round WPB and increased ER retention were observed [33] and the increased FVIII:C/VWF:Ag ratio of 2.06 (Table 1). DDAVP

response obtained from a patient carrying a p.Leu1307Pro substitution in our clinic would define this patient as a partial responder as the VWF:RCo is lower than 50 IU/dl after desmopressin (6 to 22 IU/dl after infusion, Table 2). This is in line with the low response to PMA in transfected HEK293 cells.

The p.Arg1374His variant showed a good response to stimulation with PMA in co-transfection experiments (Figure 6). The data on response to DDAVP infusion in patients heterozygous for this mutation are conflicting [4;34]. One out of 4 patients responded to DDAVP in a study by Federici *et al.* [34]; we have previously shown a good response to DDAVP, raising VWF:Ag levels more than 3-fold in three family members with this mutation [13], while in the MCMDM-1VWD study the patient did not respond (Table 2). Such a variation in response among patients carrying the same mutation suggests that the response to DDAVP is not exclusively determined by mutations in the VWF gene.

The p.Arg1583Trp mutation was the only variant that showed normal basal and stimulated secretion of VWF as well as normal formation of elongated pseudo-WPB (Table 4). In line with this observation, patients carrying this mutation have a mild phenotype and respond well to DDAVP infusion [4] (Tables 1 and 2). Alignment of the VWF amino acid sequence (Figure 8) shows that the arginine at position 1583 (Figure 7D) is poorly conserved among different vertebrate species, suggesting that the amino acid at this position is not critical and that substitution by tryptophan does not influence the structure or function of the protein. Since the expression data of p.Arg1583Trp are indistinguishable from WT-VWF, one might wonder whether this amino acid substitution is really pathogenic, also in the light of the fact that the VWF plasma level is close to normal (52 U/dl) and the FVIII/VWF ratio is approximately one, suggesting normal synthesis. The VWFpp/VWF:Ag ratio does not suggest increased clearance of the protein either [22].

The p.Tyr1584Cys variant (Figure 7E) has been described as a functional mutation as well as a polymorphism based on its prevalence [35-37]. This variant was found frequently in index cases in both the Canadian type 1 VWD study as well as in the European MCMDM-1VWD study, and often in association with blood group O, a known modifier of VWF plasma levels [12,15,38]. The p.Tyr1584Cys variant has been expressed before in COS-7, HEK293T and AtT-20 cells, where the total protein expression reached levels of 75% to 90% compared to WT-VWF transfected cells [15,39]. These levels are slightly lower than what we observed in the current study (Table 3). However, VWF:Ag plasma levels also vary a lot between patients (21-80 IU/dL, Table 1). Despite the formation of mostly round pseudo-WPB (Figure 2B), there was no ER retention of the protein and the number of storage organelles as well as basal and stimulated release of VWF was similar to that observed for WT-VWF (Figures 2A and 6). This is in line with patient data, which shows good response to DDAVP (Table 2).

		p.Ser1285Pro	p.Leu1307Pro	
H. sapiens	1275	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1302
E. chalcidodonta	1275	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1302
T. trutta	1275	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1302
E. teleostus	1266	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1295
X. laevis	1275	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1302
R. norvegicus	1272	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1301
A. g. g.	1261	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1300
E. ferus	1280	SPKADYFALGKSHLSKAEKSTENAEVDEHL	ELDGKHPINSGQVET	1306

		p.Arg1374His	
H. sapiens	1375	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1402
E. chalcidodonta	1375	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1402
T. trutta	1375	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1402
E. teleostus	1365	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1398
X. laevis	1375	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1402
R. norvegicus	1372	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1401
C. gallus	1361	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1398
E. ferus	1380	TSFQSRITLLTVGQFFGQENRTRFVQGLKQKMTFTVWATPRHL	1406

		p.Arg1583Trp	p.Tyr1584Cys	
H. sapiens	1543	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1566
E. chalcidodonta	1543	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1566
T. trutta	1543	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1566
E. teleostus	1542	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1565
X. laevis	1543	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1566
R. norvegicus	1545	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1568
C. gallus	1531	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1566
E. ferus	1552	QVETPPEFQKQKATLQVETSTVQGGKHTFNSTAL	GLNLSFTVQGGV	1567

		p.Val1822Gly	
H. sapiens	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
E. chalcidodonta	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
T. trutta	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
E. teleostus	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
X. laevis	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
R. norvegicus	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
C. gallus	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733
E. ferus	1702	QVHGKFGASGRVFLINDSLESVDIAEAASLHVA	1733

Figure 8: Conservation of amino acids in different vertebrate species. The VWF gene was aligned using the HomoloGene database. Alignments of regions surrounding the studied mutations with corresponding VWF sequences from different vertebrate species are shown in detail. Residues highlighted in black indicate fully conserved amino acids, whereas grey residues are highly conserved.

In single transfection the p.Val1822Gly did not respond to PMA stimulation. However, PMA did induce some release of VWF in the cells expressing p.Val1822Gly upon co-expression with WT-VWF (1.3 fold increase). This is in accordance with the marginal response to DDAVP infusion observed in the patients (Table 2). The defects in regulated secretion of VWF may be explained by the marked decrease in WPB formation by p.Val1822Gly (Figure 2A).

In summary, we have shown that naturally occurring VWD variants within the A domains of VWF can cause defects in both WPB formation and regulated secretion of VWF. Previous data showed that the D1-D2-D'-D3-A1 subunits are required for the formation of elongated WPBs [6]. In our study, we show that two mutations located within the A2 and A3 domain (p.Tyr1584Cys and p.Val1822Gly) also interfere with the formation of elongated WPB. Our findings show that mutations in the A2 and A3 domain can also modulate the elongation of WPBs, as is previously suggested for the p.Ala1716Pro variant [10]. Interestingly, we observed that PMA-induced release of VWF from spherical WPBs (as observed in cells expressing pArg1374His and pTyr1584Cys) was not impaired. This suggests that round WPBs are equally well released when compared to elongated WPB. This is in line with previous observations that

WPBs containing FVIII, leading to the formation of spherical WPBs, can still release their cargo [40,41]. Spherical WPBs in canine endothelial cells bearing the p.Tyr87Ser mutation, are rapidly releasing their cargo upon stimulation with appropriate secretagogues [42].

Overall our findings suggest that the regulated secretion upon PMA stimulation of transfected cells reproduces the *in vivo* situation (Tables 2& 4). Recent reports have demonstrated that BOECs isolated from VWD patients can be used to assess whether a patient responds to treatment with DDAVP [33,43]. However, successful isolation of BOECs seems to be donor-dependent and patient material may not always be available [33,44]. Our results indicate that HEK293 cells expressing VWF variants found in patients with VWD can be used to properly assess possible defects in regulated secretion of VWF.

Addendum

D.J. Groeneveld designed the study, performed the research and data analysis and wrote the manuscript; J.W. Wang designed the experiments, contributed to data analysis and writing of the manuscript; M. Mourik and R.J. Dirven performed part of the research; K.M. Valentijn reviewed the manuscript; J. Voorberg provided antibodies, interpreted the data and reviewed the manuscript; P.H. Reitsma designed the study, discussed and analyzed the data, and contributed to writing of the manuscript; J. Eikenboom designed the study, interpreted the data, and contributed to writing of the manuscript. All authors approved the final version of the manuscript.

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Part 2

Aspects of clearance of VWF

CHAPTER

5

No evidence for a direct effect of von Willebrand factor's ABH blood group antigens on von Willebrand factor clearance

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Abstract

Background: One of the major determinants of von Willebrand factor (VWF) plasma levels are the ABO blood groups, and individuals with blood group O have approximately 25% lower plasma levels. The exact mechanism behind this relationship remains unknown, although effects on clearance have been postulated. **Objectives:** Whether clearance of VWF is directly dependent on the presence of ABH antigens on VWF. **Methods:** Three type 3 von Willebrand disease (VWD) patients were infused with Haemate-P® and the relative loading of VWF with ABH antigens at different time points was measured. VWF-deficient mice were injected with purified plasma-derived human VWF obtained from donors with either blood group A, B or O. **Results:** In mice we found no difference in clearance rate between plasma derived A-, B- or O-VWF. Faster clearance of the blood group O VWF present in Haemate-P® infused in type 3 VWD patients would have resulted in a relative increase in the loading of VWF with A and B antigens over time. However, we observed a 2-fold decrease of A and B antigens in 2 out of 3 patients and stable loading in the third patient. **Conclusion:** There is no direct effect of ABH antigens on VWF in VWF clearance. We demonstrate that in a direct comparison within one individual O-VWF is not cleared faster compared to A- or B-VWF. Clearance differences in blood group O versus non-O individuals may therefore be related to the blood group status of the individual rather than the ABH antigen loading on VWF itself.

Introduction

When activated upon vascular damage, von Willebrand factor (VWF) supports platelet adhesion and activation and initiates platelet plug formation [1]. VWF is synthesized in endothelial cells and megakaryocytes and is stored in cell-specific organelles, respectively Weibel-Palade bodies (WPB) and α -granules [2,3]. Part of the synthesized VWF is secreted constitutively into the blood, where it has a half-life of about 12 hours [4].

The variation in plasma VWF levels is 66% genetically determined and the ABO blood group locus accounts for 30% of that variation [5]. VWF is decorated with ABO blood group sugars and those ABH antigens are attached to the N-linked oligosaccharide chains [6]. Several studies have shown that ABO blood groups are associated with VWF plasma levels [7;8]. Individuals with blood group O have approximately 25% lower VWF plasma levels. Individuals with the Bombay blood group, lacking expression of ABH antigens, even have lower VWF levels [9]. How ABO blood groups influence VWF levels exactly remains unknown.

The VWF levels correlate with the amounts of A and B antigens present on the VWF molecule [10,11]. The ABH antigens could have an effect on biosynthesis, secretion,

and clearance of VWF or a combination thereof. However, ABH antigens on VWF do not play a role in synthesis and secretion as the plasma VWF propeptide levels are independent of ABO blood group [12,13]. In endothelial cells derived from different blood group donors, the constitutive VWF secretion showed no difference, however those cells did not express ABH antigens on their surface nor on VWF [14]. Another study showed that the rise in VWF level after desmopressin treatment appears to be similar in patients with type 1 von Willebrand disease (VWD) with different blood groups, suggesting that secretion efficiency is not affected by blood group [15].

Nossent *et al.* [12] estimated, that the half-life of endogenous VWF in healthy controls with blood group O is almost 2 hours shorter compared with blood group non-O controls. In healthy individuals the VWF half-life after DDAVP administration was also shorter in blood group O individuals [16]. Furthermore, the ratio in plasma between VWF propeptide and mature VWF, which are released in equimolar amounts, is increased in individuals with blood group O compared with non-O individuals. This suggests faster clearance of mature VWF in blood group O individuals [12,13,17]. These observations clearly show that VWF plasma levels in individuals with blood group O are lower due to faster clearance of VWF. However, this supposition is not yet challenged in a direct comparison. All studies investigated the clearance of O-VWF in blood group O individuals. The question remains whether clearance differences are to be attributed to the blood group antigens on the VWF molecule itself or to the ABO blood group status of the individual that clears the VWF protein. The present study is the first investigating simultaneous VWF clearance of the different blood groups in a direct comparison.

Methods

Mice

VWF-deficient (VWF^{-/-}) female mice on a C57BL/6J background (embryos originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) were between 8 to 12 weeks old. Mice were housed under a 12-h light/dark cycle. Standard diet and drinking water were provided *ad libitum*. All procedures were approved by the animal welfare committee of the Leiden University (under registration #10037).

Patients

After written informed consent, VWF concentrate (Haemate-P®, CSL Behring, Marburg, Germany) was infused in three type 3 VWD patients (2 females and 1 male, all blood group O) in a dosage of 50 units FVIII per kg body weight. Blood samples were collected before administration of Haemate-P®, and at 10 min, 1, 2, 4, 6, 8, and 24 hours thereafter. The study was approved by the local ethical review committee of the Leiden University Medical Center.

VWF purification

Fresh frozen plasma from donors with blood groups A (n=3), B (n=3) or O (n=3) (Sanquin, Leiden, The Netherlands), was cryoprecipitated and precipitates were dissolved in elution buffer (25mM TEA, 15mM sodium citrate, 150mM NaCl, pH 7.5). Plasma VWF was separated by gel filtration chromatography on a Sepharose 4B column (GE Healthcare, Little Chalfont, UK). Collected fractions were measured for VWF antigen (VWF:Ag) [18]. VWF purified from donors with blood group O, containing only the H-antigen, is indicated as O-VWF. VWF purified from A and B donors is indicated as A-VWF (A-antigen present) and B-VWF (B-antigen present).

Clearance of plasma derived human VWF in mice

Female VWF^{-/-} mice were subcutaneously injected with 50 μ l Temgesic (0.1 mg/kg) for analgesia one hour prior to experiments. Three different human blood donors were used for each blood group, and five mice were used per human blood donor, so a total of 15 mice were used for each blood group. Fortyfive mice were injected into the tail vein with 200 μ l of 2U/ml purified plasma derived (pd)-VWF. At 1, 15, 30, 60, 120, 240 and 360 minutes after injection, 50 μ L of citrated-blood (final concentration of 0.32%) was collected via tail transection and tails were subsequently cauterized. After centrifugation (2500g for 15min), plasma was collected and human VWF:Ag was measured [18].

Macrophage depletion in mice

Female VWF^{-/-} mice (n=4 per group) were injected intravenously with either 200 μ l/mouse clodronate liposomes (gift of Roche Diagnostics GmbH, Mannheim, Germany) or saline (200 μ l/mouse) as a control. Two days after treatment, mice were injected via tail injection with 200 μ l Haemate-P[®] (5U/ml VWF:Ag) and citrated-blood was collected at several time points for determination of plasma VWF:Ag levels and clearance rates. Macrophage depletion was confirmed by staining liver and spleen cryostat sections with an antibody to a specific mouse macrophage marker F4/80 (AbD Serotec, Oxford, UK).

Measurement of A, B and H antigen expression on VWF

The amount of loading on VWF with A and B antigens was quantified as described [11]. Immobilized anti-VWF IgG (A0082, DAKO, Glostrup, Denmark) was saturated with VWF from the sample. Next, immobilized VWF is saturated with murine monoclonal anti-A or anti-B antibody (Immu Clone, Immucor, Rodermark, Germany). The amount of bound anti-A or anti-B antibody is quantified using an HRP-labelled goat anti-mouse IgM (Nordic Immunological laboratories, Tilburg, The Netherlands). The peroxidase activity of a sample was normalized against the peroxidase activity obtained with an A1 or B reference plasma sample, and the results were expressed as normalized ratios (nA-ratio or nB-ratio) [11]. The amount of H antigens expressed on

VWF was measured similar to O'Donnell *et al.* [19]. Plates were coated with anti-VWF Ig (DAKO), washed and incubated for 2 hours with plasma samples (1:20, 1:40, 1:50 and 1:100 dilution). Plates were washed and incubated for 2 hours with Lectin from *Ulex europaeus*/UEA-I-HRP (1:150 dilution, AMS Biotechnology, Abingdon, UK) and peroxidase activity was measured. Blood group O plasma from a healthy control was used as standard curve and was assigned a value of 1 U/ml. To determine the percentage of H antigen expressed per unit VWF, the amount of H antigen detected was divided by the amount of VWF:Ag present in the ELISA well and multiplied by 100.

Detection of A, B or H antigen on VWF using Western Blotting

Plasma samples were reduced using 20 mM dithiothreitol, and separated by Novex 6% (A and B antigens) or Novex 4% (H-antigen) Tris-Glycine gel electrophoresis (Invitrogen, Carlsbad, CA, USA). Equal amounts of VWF protein were loaded. Gels were immunostained with polyclonal rabbit anti-human VWF antibody conjugated to HRP (DAKO). Duplicate lanes were immunostained with murine monoclonal anti-A or anti-B antibody (Immucor) (1:10 dilution) or with Lectin from *Ulex europaeus*/UEA-I-HRP (AMS Biotechnology) (5 µg/ml). Blots for detection of A and B antigens were subsequently incubated with a HRP-labelled goat anti-mouse IgM (Nordic). Staining was visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

Pharmacokinetic data of infused VWD patients

In a previous study [20], ten type 1 VWD, ten type 2A VWD and eight type 3 VWD patients received a single i.v. VWF concentrate infusion with a median of 79 IU/kg VWF:RCo (32 IU/kg FVIII:C). From that study we obtained the calculated half-lives of VWF:RCo and VWF:Ag [20]. Another six type 3 VWD patients received a single i.v. VWF concentrate infusion with a median of 45 (range 25-50) IU/kg FVIII:C. Plasma samples were drawn at different time points and assayed for VWF:Ag and VWF:RCo and half-lives were calculated.

Data analysis and statistics

Analysis of pharmacokinetic data was performed using GraphPad Prism (version 6.0, La Jolla, CA, USA). Clearance data from mice experiments were fitted to the two phase exponential decay equation $Y = A \times e^{(-k_1 \times t)} + B \times e^{(-k_2 \times t)} + \text{Plateau}$ to obtain A , k_1 , B , and k_2 . These parameters were used to calculate mean residence time (MRT) employing the equation; $MRT = (A/k_1^2 + B/k_2^2) / (A/k_1 + B/k_2)$ as previously described [21]. Clearance data from type 3 VWD patients were fitted to the one phase exponential decay equation $Y = A \times e^{(-kt)}$ to obtain k . Half-lives were calculated using the first order equation; $t_{1/2} = \ln 2 / k$. Quantification of western blots was performed using Fiji (Fiji Is Just Image, <http://fiji.sc>). Statistical analyses were performed by the Student's unpaired *t*-test or One-Way ANOVA followed by Tukey's multiple comparisons test.

Results

VWF clearance is not influenced by ABO blood group in mice

We first investigated whether sampling strategy has any influence on the clearance of VWF in VWF $-/-$ mice. As shown in Figure 1A, no difference in decay curves of injected Haemate-P[®] was observed between mice that were sampled only once (MRT $2.2\text{h} \pm 0.9$) and mice that were sampled multiple times (MRT $2.0\text{h} \pm 0.8$). Further experiments were therefore done using the multiple sampling approach. Furthermore, the amount of injected Haemate-P[®] did not affect clearance rates (low dose VWF, MRT $2.3\text{h} \pm 0.6$ versus high dose VWF, MRT $2.5\text{h} \pm 0.5$) (Figure 1B).

As shown in Figure 1C no significant difference was observed in clearance rate between A-VWF (MRT $1.8\text{h} \pm 0.3$), B-VWF (MRT $1.4\text{h} \pm 0.2$) or O-VWF (MRT $1.9\text{h} \pm 0.3$).

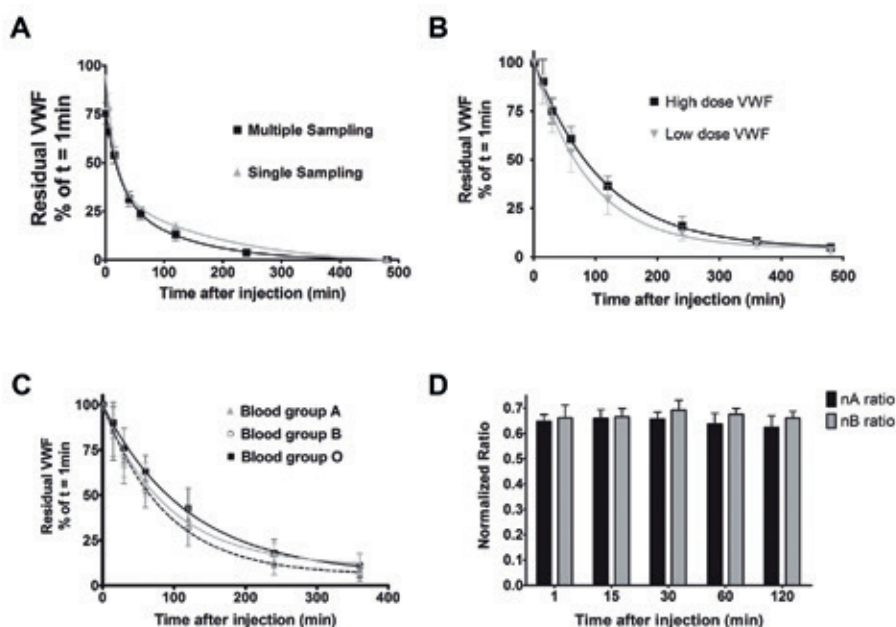


Figure 1. Clearance of pd-VWF from blood groups A, B and O donors in VWF $-/-$ mice. Panel A; In one group (single sampling, grey triangle) different mice (total $n=12$, $n=3$ per time point) were sampled at different time points after injection with $200\mu\text{l}$ Haemate-P[®] ($5\text{U}/\text{ml}$ VWF:Ag); each mouse was sampled only once. In the second group ($n=3$, multiple sampling, black square) each mouse was sampled several times at different time points. Panel B; two different dosages ($2.5\text{U}/\text{ml}$ and $50\text{U}/\text{ml}$) of Haemate-P[®] were injected into VWF $-/-$ mice ($n=10$ per dosage). Panel C; clearance profiles of human plasma derived-VWF from donors with either blood group A, B or O in VWF $-/-$ mice ($n=$ total of 15 per blood group, 5 per donor). Panel D; loading of VWF with A and B antigens in VWF $-/-$ mice after injection with $200\mu\text{l}$ ($10\text{U}/\text{ml}$ VWF:Ag) Haemate-P[®] [11]. Because VWF:Ag levels at the last two time points were below the threshold of the assay, loading was not measured for these time points. Data represent mean $\pm$ SD.

When clearance rates of A-VWF and B-VWF were combined into non O-VWF (MRT $1.6\text{h}\pm0.3$) there was also no differences in clearance compared with O-VWF (MRT $1.9\text{h}\pm0.3$, $p=0.2$). These results suggest that in mice VWF clearance rate is not influenced by ABH antigens present on VWF. In another approach, VWF^{-/-} mice were injected with Haemate-P®, a pd-VWF concentrate obtained from a pool of donors including all ABO blood groups. Injection of Haemate-P®, and subsequent measurement of the changes over time in the loading of A and B antigens on VWF, facilitates the simultaneous investigation of differential clearance of A-, B- and O-VWF in a single test animal. In case ABH antigens influence clearance of VWF, the proportions of O-VWF, A-VWF and B-VWF in the circulating pool of VWF will change over time. Thus, the normalized ratio of A and B antigen on VWF (expressed as nA and nB) in the pool of VWF should also change over time. The loading with A and B antigen should increase when O-VWF is cleared faster. The loading of VWF with A- and B antigens however, was not significantly affected over time (p for linear trend 0.06 and 0.79 for A- and B-antigen respectively, Figure 1D) indicating no effect of ABH antigens on VWF clearance. To investigate whether Kupffer cells mask a possible ABO dependant uptake of VWF by other cells in the liver, we depleted macrophages in VWF^{-/-} mice. Depletion of macrophages by clodronate liposomes lead to a significant decrease in clearance rate of VWF (MRT $3.1\text{h}\pm0.83$) compared with saline treated animals (MRT $2.1\text{h}\pm0.36$, $p<0.05$) (Figure 2A), confirming that Kupffer cells contribute to VWF clearance. However, after depletion of Kupffer cells there was still no ABO dependent clearance observed (Figure 2B-C).

Blood group O VWF is not cleared faster in VWD patients

Since our mouse model has its limitations due to possible differences in clearance mechanisms of VWF compared to humans, we also studied clearance of VWF in humans. As no blood group specific purified VWF is available for infusions in humans, we infused three type 3 VWD patients with Haemate-P® (Table 1 for patient characteristics) and measured the amount of A and B antigens present on VWF. This experimental set-up allows to measure simultaneous VWF clearance of the different blood

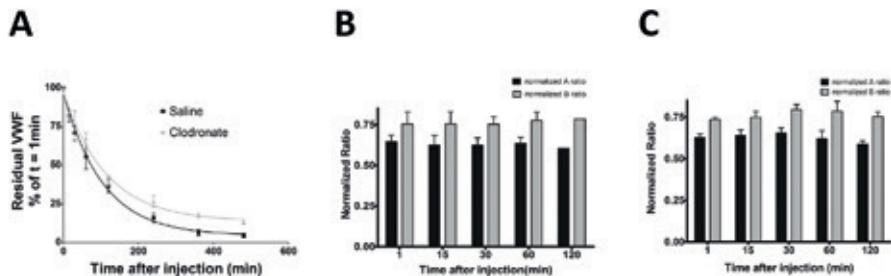


Figure 2. Clearance of Haemate-P® in VWF^{-/-} mice after macrophage depletion. Panel A; VWF clearance rate in saline (n=4, black squares) and Clodronate (n=4, grey triangles) treated animals. Panel B; loading of A (black bars) and B (grey bars) antigen on VWF in saline treated animals. Panel C; loading of A (black bars) and B (grey bars) antigen on VWF in Clodronate treated animals. Data represent mean $\pm$ SD

Table 1. Patient characteristics for every time point.

Patient number	Time (min)	FVIII:C (IU/mL)	VWF:Ag (IU/mL)	VWF:RCo (IU/mL)
1	0	0.08	<0.04	<0.20
	10	0.85	2.14	1.20
	90	0.73	1.61	1.05
	900	0.70	0.60	0.21
2	0	0.11	<0.04	<0.20
	10	1.17	3.20	2.63
	60	1.17	3.20	2.24
	120	1.11	2.65	2.01
	240	1.04	2.45	1.53
	360	1.01	2.18	1.27
	480	0.99	1.90	1.06
	1440	0.71	0.55	0.40
3	0	<0.03	<0.04	<0.20
	10	1.16	2.88	2.25
	60	1.16	2.74	1.58
	120	0.85	2.38	1.44
	240	0.84	1.84	0.99
	360	0.87	1.55	0.80
	480	0.85	1.30	0.69
	1440	0.70	0.18	<0.20

groups in a direct comparison in one individual. As described in the previous paragraph, faster clearance of O-VWF would lead to increased normalized ratios of A and B antigen on VWF in time. However, the opposite was observed for two patients; a 2-fold decrease in the amount of A and B antigen loading on VWF during infusion. Interestingly, in the third patient the loading of both A and B antigens on VWF remained steady over time (Figure 3A). The amount of loading with A and B antigens was also confirmed using the non-saturating assay as described by O'Donnell *et al* [10] (*data not shown*).

In principle, endogenous anti-A and anti-B antibodies could shield the antigens on VWF to the antibodies used in the ELISA. However, we could not detect direct antibody binding by adding an anti IgM antibody to captured VWF from the samples (*data not shown*). Furthermore, Western-blotting confirmed the decrease in the amount of antigen loading on VWF at the latest time point for patient 2, while the amount of antigen loading on VWF for patient 3 remained the same (Figure 3B). The decreased loading therefore reflects a true decrease in VWF loaded with A and B antigens. Also the loading of H-antigens on VWF was declining in time and analysis of the Western blots showed that the density of the bands was also decreasing in time (Figure 3C, D, Table 2). A- and B-VWF are thus not cleared faster in our patients, leading to the reduced normalized ratios (Figure 3A,B) since the relative amount of O-VWF present in the circulation would then increase over time. These results suggest that ABH antigens are removed from the VWF molecule.

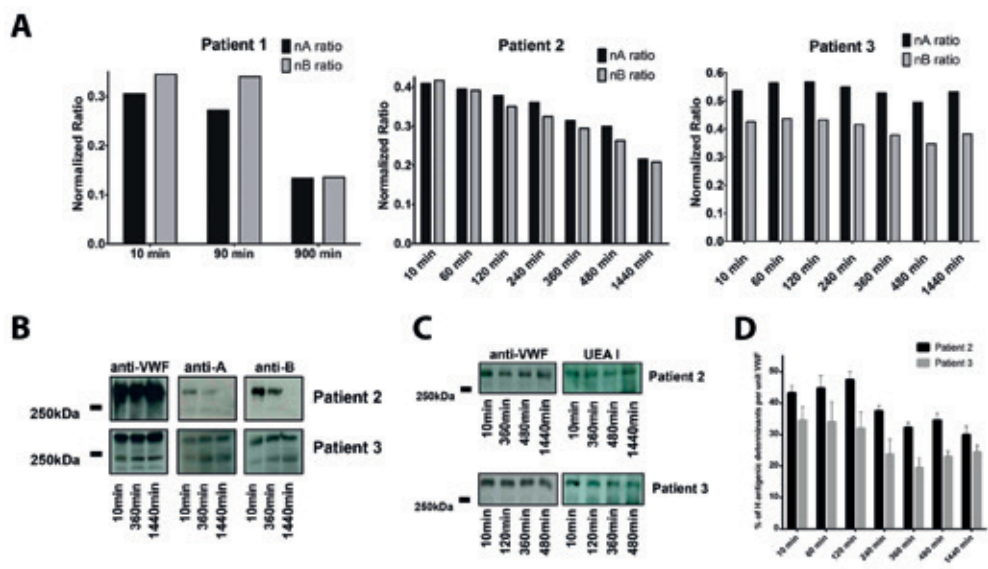


Figure 3. Clearance of Haemate-P® in VWD type 3 patients. Panel A; loading of VWF with A (black bars) and B (grey bars) antigens at different time points. Data represent the mean loading from duplicate wells. Panel B; Western blot of plasma samples after reducing conditions from time points 10 min, 6 hours and 24 hours after infusion of patients 2 and 3. Blots were stained for VWF, A-antigen and B-antigen. Panel C; Western blot of plasma samples after reducing conditions from time points 10 min, 2 hours (for patient 3), 6 hours, 8 hours and 24 hours (for patient 2) after infusion. Blots were incubated with anti-VWF and UEA-I lectin. Due to the low VWF:Ag level, the latest time point for patient 3 was excluded. Equal amounts of VWF:Ag were loaded per lane (see also Table 2 for quantification of the western blots). Panel D; loading of VWF with H antigen at different time points for patient 2 (black bars) and 3 (grey bars). Due to limited availability of plasma from patient 1, this patient was omitted. Data represent mean±SD from 2 independent dilutions measured in duplicate.

Table 2. Density of VWF and H-antigen bands on Western blot.								
Time point (min)		Area		Percentage		Relative density*		Corrected density†
		VWF	H-antigen	VWF	H-antigen	VWF	H-antigen	
Patient 2	10	25049806	20033434	25	30	1	1	1
	360	20702492	17463898	20	26	0.83	0.87	1.05
	480	23908271	15613522	24	24	0.95	0.78	0.82
	1440	31636454	13187108	31	20	1.26	0.66	0.52
Patient 3	10	32375999	24766806	29	35	1	1	1
	120	28513342	17474078	26	26	0.88	0.74	0.84
	360	27722856	10558492	25	21	0.86	0.59	0.69
	480	22476170	10691593	20	19	0.69	0.53	0.77

* Time point 10 minutes is set as a standard with a relative density of 1 for both VWF and H-antigen band. Rest of bands are relative to t=10min.
† Corrected density was calculated by dividing the relative density of H-antigen band by the relative density of VWF band.

Clearance of Haemate-P® in blood group O vs non-O VWD patients

To investigate whether the ABO blood group status of the individual influences the clearance rather than the ABH antigen loading of the VWF protein per se, we studied the clearance of Haemate-P® in VWD patients with blood group O and non-O. The half-lives of both VWF:Ag and VWF:RCo were shorter in type 3 VWD patients with blood group O compared to patients with non-O (8.4h, 95%CI 3.9-12.9 vs 9.6h, 95%CI 3.2-16.0 for VWF:Ag and 5.8h, 95%CI 3.0-8.5 vs 8.6h, 95%CI 4.3-13.0 for VWF:RCo, Figure 4A), however this difference was not statistically significant, probably due to the small group size. The same difference was also observed in type 1 (10.9h, 95%CI 6.1-15.8 versus 16.1h, 95%CI 10.9-21.3 for VWF:Ag and 5.7h, 95%CI 3.9-7.5 versus 10.5h, 95%CI 5.3-15.6 for VWF:RCo) and type 2A VWD patients (6.9h, 95%CI 2.0-11.8 versus 11.0h, 95%CI 3.6-18.3 for VWF:Ag and 3.8h, 95%CI 0.7-6.9 versus 9.0h, 95%CI 4.8-13.1 for VWF:RCo) (Figure 4B,C). These preliminary results support our hypothesis that blood group O individuals clear VWF faster than blood group A or B individuals independent of the blood group status of VWF.

Discussion

In this study we investigated the assumed faster clearance of VWF from individuals with blood group O. Since we cannot measure possible differences in clearance rates of human VWF from different donors simultaneously in humans, we first investigated whether we could detect a clearance difference in VWF -/- mice. This mouse model has advantages since it allowed us to 1) measure simultaneous VWF clearance of different blood groups in a direct comparison and 2) measure clearance profiles of VWF puri-

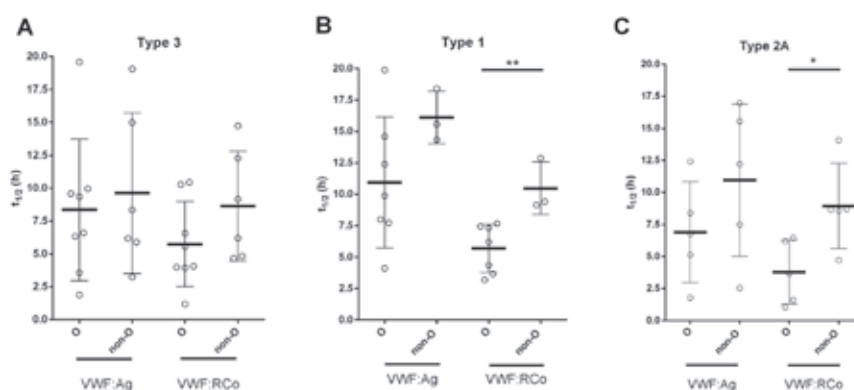


Figure 4. Half-lives of VWF:Ag and VWF:RCo in blood group O vs non-O VWD patients. Pharmacokinetic data obtained from patients with type 3 VWD (n=14, panel A), type 1 VWD (n=10, panel B) and type 2A VWD (n=10, panel C) who were previously infused with Haemate-P® were analysed for the VWF half-life. Patients were divided in blood group O or blood group non-O. Individual patients are plotted. Black bars indicate mean $\pm$ SD. * p < 0.05, ** p < 0.01.

fied from individuals with either blood group A, B or O. However, in VWF -/- mice we did not observe differences in the clearance of purified human A-, B- or O-VWF. We also did not observe the expected increase in the relative amount of A and B antigen loading on VWF in VWF -/- mice injected with Haemate-P®. In Haemate-P® infused type 3 VWD patients we could also not observe the expected increase.

Mice have genes homologous to the human A-, B- and H-transferase genes. However, the enzymatic activity is much lower than the human homologues [22]. The expression of the genes is limited to a few tissues [23]. Clearance of VWF in mice might therefore not be dependent on the ABH antigens present on VWF, either because the VWF clearance system is different than in humans or because mice might not recognize human ABH antigens on VWF. This could explain why we did not observe a faster clearance of O-VWF. However, although the enzymatic activity is lower, mice do express the transferase genes. It is therefore more likely that the VWF clearance system in mice is able to recognize human ABH antigens present on VWF. This is supported by results from a study of Wolofsky *et al.* who observed increased phagocytosis of infected human blood group O erythrocytes compared to infected blood group A and B erythrocytes in mice [24].

Mouse ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) does not cleave human VWF very efficiently [25], so whether proteolysis has an influence on ABO dependent VWF clearance could not be answered. Increased susceptibility to ADAMTS13 proteolysis of O-VWF has been postulated as a mechanism for increased clearance of O-VWF. However, basal VWF levels are similar in normal and ADAMTS13 -/- mice [26]. Additionally, wild type VWF and the p.Ser-1618Pro VWF variant, which results in more susceptibility to ADAMTS13 cleavage, had similar clearance rates in a rat model [27]. It is therefore unlikely that (potential) increased ADAMTS13 susceptibility of O-VWF would lead to an accelerated clearance.

Given the limitations of the mouse model, we also investigated the assumed faster clearance of O-VWF in patients with type 3 VWD. By infusion of Haemate-P® we were able to study clearance of VWF loaded with A, B and H antigens simultaneously in one individual, excluding any variability between subjects and variation over time. To our surprise, loading of VWF with A and B antigens decreased over time. This decrease could not be explained by simple shielding of antigens *in vivo*. Faster removal of A- and B-VWF compared to O-VWF would lead to a decreased ratio, although this would be contradictory to the assumed faster clearance of O-VWF. However, the amount of H-antigen present on VWF also decreased during clearance. The decrease in A, B and H-antigen loading indicates that either the A, B and H-antigens are removed from the VWF molecule or heavily glycosylated VWF is cleared faster, leading to the reduced amounts of loading. All three patients have blood group O, and whether interaction between endogenous Anti-A and Anti-B antibodies and the antigens on VWF could influence the clearance of VWF, by subsequent clearance of the whole complex or by removing ABH antigens on VWF, is as yet unknown. However, we could not detect direct

antibody binding to antigens on VWF in our plasma samples, nor could we demonstrate antibody binding to ABH antigens on VWF by incubating VWF deficient plasma from different blood group donors with Haemate-P®. The ideal experimental set-up to tackle this problem would be infusing a VWD type 3 patient with blood group AB, thereby excluding possible antibody-antigen interaction, however this type of patient is very rare [28], and was not available.

In 2 infused patients we observed a 2-fold decrease in A and B antigen loading, but in the other patient we did not observe such a strong decrease in antigen loading. We have no reasonable explanation for the discrepancy between patients 1 and 2 versus patient 3. There is no difference in the amount of A- and B-antibodies present, as antibody titers between the three patients are comparable, nor have the patients received different batches of Haemate-P®.

Taking together the results, we could not demonstrate the assumed faster clearance of O-VWF. It could be that the association between ABO blood group and plasma VWF levels is not just via an effect on VWF clearance but could include individual differences, for example receptor mediated uptake of VWF [29]. Mannucci *et al.* [30], found that their recombinant VWF product, which lacks the ABH antigens, had a similar pharmacokinetic profile compared with human pd-VWF in patients with VWD. This findings support our results that ABH antigens on VWF itself do not affect clearance.

Nevertheless, there is overwhelming evidence suggesting increased VWF clearance in individuals with blood group O [12,13,16,17]. However, all those studies investigated the clearance of O-VWF in a blood group O individual and A-VWF in a blood group A individual. Since we demonstrated that within one individual O-VWF is not cleared faster compared to A- or B-VWF, we postulate that the ABO blood group dependent differences in VWF clearance depend on the ABO blood group status of the individual and not on the ABH antigens on VWF per se. Since VWF is the carrier protein of factor VIII (FVIII) and clearance of FVIII is largely dependent on VWF, clearance of (infused) FVIII will most likely also be dependent of the blood group status of the individual. This hypothesis is supported by our preliminary results of a shorter half-life in blood group O individuals, independent of the ABO blood group of the circulating VWF. However more pharmacokinetic data is required to substantiate this assumption.

Authorship Contributions

D.J. Groeneveld designed the study, performed the research and data analysis and wrote the manuscript; T. van Bekkum., K. Cheung, and R.J. Dirven performed part of the research and reviewed the manuscript; G. Castaman performed part of the research and reviewed the manuscript. P.H. Reitsma discussed and interpreted the data, and reviewed the manuscript; B.van Vlijmen designed the study, performed part of the research, interpreted the data and reviewed the manuscript; J. Eikenboom designed the study, interpreted the data, and contributed to writing and reviewing of the manuscript. All authors approved the final version of the manuscript.

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Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER

6

Von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease

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Abstract

The ratios between von Willebrand factor propeptide (VWFpp) or FVIII coagulant activity (FVIII:C) and VWF antigen (VWF:Ag) reflect synthesis, secretion and clearance of VWF. We aimed to define the pathophysiology of 658 types 1, 2 and 3 von Willebrand disease (VWD) patients with VWF levels ≤ 30 U/dL from the “Willebrand in the Netherlands” (WiN-) study using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios. We also evaluated the use of VWFpp in the classification and diagnosis of VWD. Based on the ratios, reduced VWF synthesis was observed in 18% (67/380) of type 1 patients and in only 2% (5/240) of type 2 patients. A significant proportion of type 3 patients had detectable VWFpp (14/37, 41%). These patients had a lower bleeding score than type 3 patients with complete absence of both VWF:Ag and VWFpp: 14.0 vs. 19.5, $p=0.025$. The majority of these patients had missense mutations associated with rapid VWF clearance, whereas “true” type 3 patients were homozygous for null alleles. In conclusion, VWFpp identified severe type 1 VWD with very low VWF levels in patients who had previously been classified as type 3 VWD. This study underlines the clinical significance of the VWFpp assay in the diagnosis and classification of VWD.

Introduction

Von Willebrand factor (VWF) is a large multimeric glycoprotein that mediates platelet adhesion and aggregation at sites of vascular injury, and serves as the carrier of factor VIII (FVIII) to prevent its premature clearance [1,2]. Synthesis of VWF is restricted to endothelial cells and megakaryocytes [3], where it is formed as a precursor protein, pre-proVWF, with a signal peptide, a propeptide and a mature subunit [4]. After translocation to the endoplasmic reticulum the signal peptide is cleaved, and the pro-VWF undergoes extensive post-translational modifications, including dimerization in the endoplasmic reticulum and multimerization in the Golgi system [5,6]. The VWF propeptide (VWFpp) is subsequently removed from the mature VWF, however it stays noncovalently bound [7]. Part of VWF is secreted constitutively into the plasma and the remaining part is stored in Weibel-Palade bodies in the endothelium or α -granules in megakaryocytes [8,9]. After release into the circulation, the VWFpp and the mature VWF completely dissociate [8].

Plasma VWF concentrations represent a balance between synthesis, secretion and clearance from the circulation. VWF and VWFpp are secreted equimolarly, but are cleared independently with estimated half-lives of 8-12 hours for VWF and 2 hours for VWFpp [10,11]. The ratio between VWFpp and VWF antigen (VWF:Ag) can therefore be used to assess the rates of synthesis, secretion and clearance of VWF [10,12,13]. In addition to VWFpp, the ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag can also be used to assess VWF synthesis and clearance [14]. Since FVIII and VWF circulate in blood as a complex and are cleared together, their half-lives are strongly related [15]. VWF is deficient or defective in von Willebrand disease (VWD), the most com-

mon inherited bleeding disorder [16,17]. Low VWF levels are caused by reduced VWF synthesis or secretion, increased VWF clearance, or a combination thereof [18,19]. Recently, Eikenboom *et al.* have shown that the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios represent the pathophysiology of VWD and correspond to different VWF gene mutations in type 1 VWD [20]. However, it is still unknown whether these ratios can also provide insight into the pathophysiological mechanism in types 2 and 3 VWD.

VWD patients are classified as type 1, 2 or 3 VWD according to their remaining level of functional VWF [16,21]. Type 1 VWD is characterized by partially reduced VWF levels, while type 3 VWD show completely absent VWF plasma levels. In type 2 VWD, functionally abnormal variants of VWF are synthesized. Type 2 VWD is subclassified as 2A, 2B, 2M or 2N, based on the type of functional defect in VWF [16,21]. In some patients it is difficult to classify VWD correctly, as the available diagnostic laboratory tests do not provide adequate information to distinguish between VWD (sub)types. In addition, these laboratory assays are less sensitive in measuring extremely low VWF and FVIII levels and are therefore less reliable.

In this large nationwide cross-sectional Willebrand in the Netherlands (WiN)-study, we aimed to define the pathophysiology of type 1, 2 and 3 VWD using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios irrespective of the VWF gene mutation. In addition, we evaluated if the use of VWFpp can improve the diagnosis and classification of VWD.

Methods

Participants

In the nationwide cross-sectional “Willebrand in the Netherlands” (WiN)-study 804 VWD patients who had previously been diagnosed with types 1, 2 or 3 VWD, were included [23–25]. The inclusion criteria for the WiN-study were (1) hemorrhagic diathesis or a family history of VWD and (2) historically lowest VWF levels ≤ 30 U/dL (VWF:Ag and/or VWF:RCO) and/or FVIII:C levels ≤ 40 U/dL. Patients were excluded if they were known to have other hemostatic disorders resulting in hemorrhagic diathesis. The Medical Ethical Committees at all participating centers approved this study, and all participants gave informed consent.

For the current study, only patients from whom plasma was obtained were selected ($n=681$). Exclusion criteria were pregnancy ($n=7$) and the recent use of desmopressin or replacement therapy at the time of blood sampling ($n=16$). Therefore, a total of 658 VWD patients were included in the current study.

Assessment of bleeding symptoms

All patients completed a questionnaire regarding bleeding episodes, treatment of VWD, co-morbidity, and Quality of Life [24–27]. To calculate a bleeding score (BS), as

previously described by Tosetto [28], we used information on the severest life-time event of each of twelve specific bleeding symptoms. To avoid prophylaxis-bias, bleeding symptoms were not scored if patients had received prophylactic desmopressin or prophylactic replacement therapy before a surgical intervention, dental extraction or delivery [24,29]. However, if bleeding did occur despite prophylactic treatment, this bleeding was scored according to the Tosetto BS.

Laboratory measurements

Patients' plasma was obtained at inclusion in the study. Venous whole blood was collected in 0.105M sodium citrate tubes and centrifuged twice at 2200x g for 10 minutes at room temperature and stored at -80°C. VWF:Ag, VWF activity (VWF:Act), FVIII:C, VWF binding to FVIII (VWF:FVIIIIB), and VWF multimers were determined centrally at the Erasmus University Medical Center, Rotterdam, as described [24]. VWFpp was measured centrally at the Leiden University Medical Center, Leiden. This assay was also used in the previous paper from Eikenboom *et al.* [20], and was measured at the same center. VWFpp antigen was determined with an ELISA using antibodies from Sanquin (Amsterdam, the Netherlands) as described [10,20]. First microtiter plates were coated with antibody CLB-Pro 35 overnight at 4°C, then blocked with 1% Bovine Serum Albumin (BSA) at room temperature for 2 hours. Next the diluted samples were incubated for 2 hours at 37°C. VWFpp was detected with peroxidase-conjugated antibody CLB-Pro 14.3. Pooled normal plasma was used to create a standard curve. At the time of the study, no international standard was available for VWFpp and therefore the plasma pool was arbitrarily set at 100 U/dL. Details on the blood-sampling procedure and laboratory measurements at inclusion in the study have been described in more detail by de Wee *et al* [24].

Definitions

Determination of VWD type was based on the current ISTH guidelines [16], using centrally measured plasma concentrations of VWF:Ag, VWF:Act and FVIII:C; VWF multimer patterns; VWF:FVIIIIB assay; and locally performed ristocetin-induced platelet aggregation (RIPA) tests [24].

Type 1 VWD patients had a VWF:Act/VWF:Ag ratio ≥ 0.70 , whereas type 2 patients had a ratio < 0.70 . If type 2 patients had normal multimers they were classified as 2M. Type 2A or 2B VWD patients showed abnormal multimers. If the locally performed RIPA test showed increased VWF affinity for platelets, patients were classified as type 2B. Type 2N patients had a FVIII:C/VWF:Ag ratio < 0.70 , and VWF:FVIIIIB $< 60\%$. Type 3 VWD was defined as having both VWF:Ag and VWF:Act levels < 5 IU/dL, irrespective of FVIII:C level.

The normal range (2.5th to 97.5th percentile) used for VWFpp was 82-173 U/dL, for the VWFpp/VWF:Ag ratio 0.8-2.2, and for the FVIII:C/VWF:Ag ratio 0.6-1.9, as reported before by Eikenboom *et al.* [20].

Statistical methods

Descriptive statistics for categorical data are presented as numbers with percentages (n, %). As data were non-normally distributed, continuous variables are presented as median with 25-75% interquartile ranges [IQR]. For comparison of proportions, chi-squared tests were used. The Kruskal-Wallis test was used to test differences in VWF:pp, VWFpp/VWF:Ag ratio and FVIII:C/VWF:Ag ratio between types 1, 2A, 2B, 2M, 2N and 3 VWD. Mann-Whitney U-test were applied to detect differences between types and to compare bleedings score between groups. Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS Inc, Chicago, USA). A p-value <0.05 was considered statistically significant.

Results

A total of 658 VWD patients were included in the current analyses, 381 of whom had type 1 VWD, 240 type 2 VWD, and 37 type 3 VWD, according to the current ISTH guidelines [16]. Baseline characteristics are presented in table 1.

VWF parameters and ratios per type of VWD

In type 1 patients, median VWFpp was 91 U/dL [IQR 68-116], median VWFpp/VWF:Ag ratio 2.2 [IQR 1.7-3.1] and median FVIII:C/VWF:Ag ratio 1.8 [IQR 1.4-2.3] (Figure 1A and table 2). This is in accordance with previously published data by Eikenboom *et al.* [20]. In type 2 patients, median VWFpp was 104 U/dL [81-136], which was higher than in type 1 patients ($p < 0.001$). Median VWFpp/VWF:Ag ratio was 4.5 [3.2-6.0] and median

Table 1. Baseline characteristics

Characteristics	VWD Patients (n=658)
Age (years), median (range)	43.5 (1-83)
Male sex, n (%)	251 (38)
VWD type, n (%)	
1	380 (58)
2	241 (37)
2A	158
2B	42
2M	27
2N	14
3	37(6)
Blood group O, n (%)*	398(61)
VWF:Ag (IU/dL), median [IQR]	29[18-45]
VWF:Act (IU/dL), median [IQR]	22 [8-53]
FVIII:C (IU/dL), median [IQR]	51 [32-73]
Bleeding score, median [IQR] [†]	11 [6-16]

*4 missing, [†]32 missing

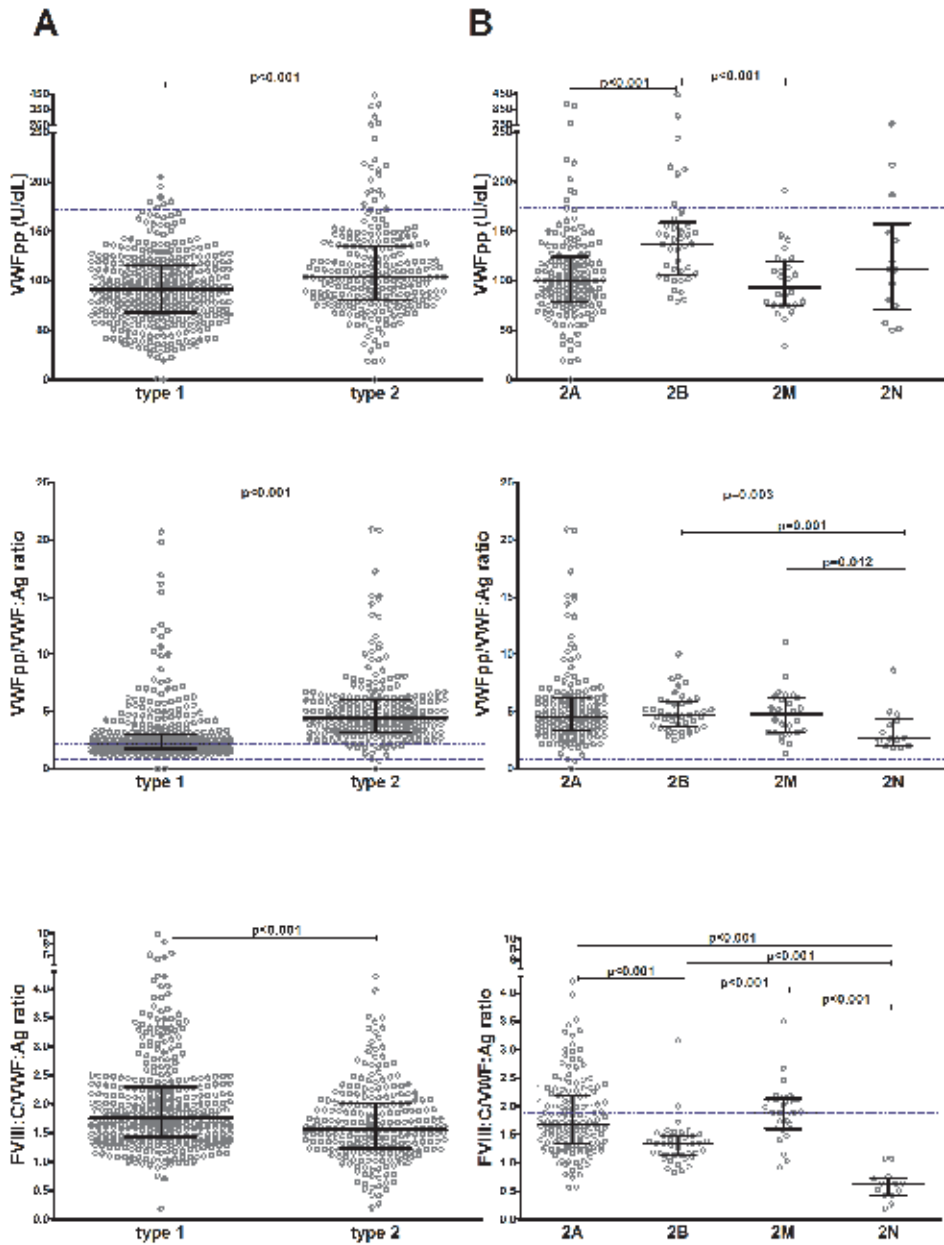


Figure 1: Ratio's per type of VWD A: VWFpp levels, VWFpp/VWF:Ag ratios and FVIII:C/VWF:Ag ratios are shown for type 1 VWD and type 2 VWD patients. For a better presentation of the graphic VWFpp/VWF:Ag ratio, one type 1 VWD patient with both a high VWFpp/Ag ratio (33.7) and a FVIII:C/VWF:Ag ratio of 3.4 was omitted. B: For all patients with type 2A, 2B, 2M and 2N VWD the VWFpp levels, VWFpp/VWF:Ag ratios and FVIII:C/VWF:Ag ratios are shown. The central horizontal line represents the median. The upper and lower horizontal lines show the 25-75% interquartile ranges. The dash lines illustrate the normal ranges (2.5th to 97.5th percentile) for VWFpp (82-173 U/dL), for VWFpp/VWF:Ag ratio (0.8-2.2), and for FVIII:C/VWF:Ag ratio (0.6-1.9).

Table 2. VWF parameters and ratios per type of VWD

Type of VWD	n	VWF:Ag (IU/dL) median [IQR]	VWF:Act (IU/dL) median [IQR]	FVIII:C (IU/dL) median [IQR]	VWFpp (U/dL) median [IQR]	VWF:Act/VWF:Ag ratio median [IQR]	VWFpp/VWF:Ag ratio median [IQR]	FVIII:C/VWF:Ag ratio median [IQR]
1*	381	38 [23-53]	46 [24-72]	67 [49-88]	91 [68-116]	1.2 [1.0-1.4]	2.2 [1.7-3.1]	1.8 [1.4-2.3]
2	240	24 [16-34]	8 [4-16]	37 [27-48]	104 [81-136]	0.4 [0.2-0.5]	4.5 [3.2-6.0]	1.6 [1.2-2.0]
2A	157	22 [14-32]	8 [3-15]	37 [27-47]	99 [79-123]	0.4 [0.2-0.5]	4.5 [3.3-6.2]	1.7 [1.3-2.2]
2B	42	28 [23-40]	8 [6-14]	39 [28-48]	137 [106-157]	0.3 [0.2-0.4]	4.6 [3.7-5.8]	1.3 [1.1-1.5]
2M	27	24 [16-30]	5 [3-13]	42 [31-52]	93 [75-119]	0.3 [0.1-0.4]	4.8 [3.1-6.2]	1.9 [1.6-2.1]
2N	14	34 [28-44]	45 [29-54]	19 [13-28]	111 [71-157]	1.2 [1.0-1.5]	2.7 [2.0-4.4]	0.6 [0.4-0.7]
3	37	1 [0-4]	0 [0-1]	3 [1-9]	0 [0-60]**	NA	NA	NA

*Median and 25-75% interquartile range (IQR) of VWFpp level and ratios in type 1 VWD patients are in accordance with the results from Eikenboom et al. [20] **VWFpp levels below the assay detection limit (<4 U/dL) were considered 0 (n=25). Abbreviations: VWF = von Willebrand factor, VWD = von Willebrand disease, n = number, VWF:Ag = VWF antigen, VWF:Act = VWF activity, FVIII:C = factor VIII coagulant activity, VWFpp = von Willebrand factor propeptide, IQR = 25-75% interquartile range, NA = not applicable

FVIII:C/VWF:Ag ratio 1.6 [1.2-2.0] in the type 2 patients. The VWFpp/VWF:Ag ratio was higher in type 2 VWD than in type 1 VWD ($p<0.001$) and the FVIII:C/VWF:Ag ratio was lower in type 2 VWD compared with type 1 VWD ($p<0.001$) (table 2 and Figure 1A). This difference in VWFpp, VWFpp/VWF:Ag and FVIII:C/VWF:Ag between type 1 and 2 VWD was also observed after exclusion of type 2N patients (all $p<0.001$): median VWFpp in type 2A, 2B and 2M VWD was 103 U/dL [81-135], median VWFpp/VWF:Ag was 4.6 [3.4-6.2], and median FVIII:C/VWF:Ag was 1.6 [1.3-2.1].

VWF parameters and ratios per subtype of type 2 VWD

In VWD 2B patients, median VWFpp was significantly higher than in 2A patients or 2M patients: 137 U/dL [IQR 106-157] vs. 99 U/dL [79-123] or 93 U/dL [IQR 75-119], both $p<0.001$ (Figure 1B and table 2). VWFpp/VWF:Ag ratio was increased in 2A, 2B and 2M patients based on the normal range (0.8-2.2)[20]; 4.5 [3.3-6.2] in 2A, 4.6 [3.7-5.8] in 2B, and 4.8 [3.1-6.2] in 2M VWD. Type 2B patients had significantly lower FVIII:C/VWF:Ag ratios than 2A or 2M patients (1.3 [1.1-1.5] vs. 1.7 [1.3-2.2] or 1.9 [1.6-2.1], both $p<0.001$). Median VWFpp and median VWFpp/VWF:Ag ratio were similar between type 2N and type 1 VWD. Type 2N patients had a significantly decreased FVIII:C/VWF:Ag ratio compared with type 1, 2A, 2B and 2M VWD (all $p<0.001$), as expected by the decreased FVIII binding affinity in type 2N.

Pathophysiological mechanisms in type 1 VWD

An increased VWFpp/VWF:Ag ratio (> 2.2) defines accelerated clearance of VWF and an increased FVIII:C/VWF:Ag ratio (>1.9) correlates with reduced VWF synthesis (Figure 2A). The pathophysiological mechanisms of type 1 VWD were variable with increased clearance of VWF in 23% of type 1 patients (89/380), reduced synthesis of VWF in 18% (67/380), and a combination of these in 23% (86/380). In 36% (138/380) of type 1 patients other pathophysiological mechanisms may play a role, as VWFpp/VWF:Ag and FVIII:C/VWF:Ag were within normal ranges (Figure 2).

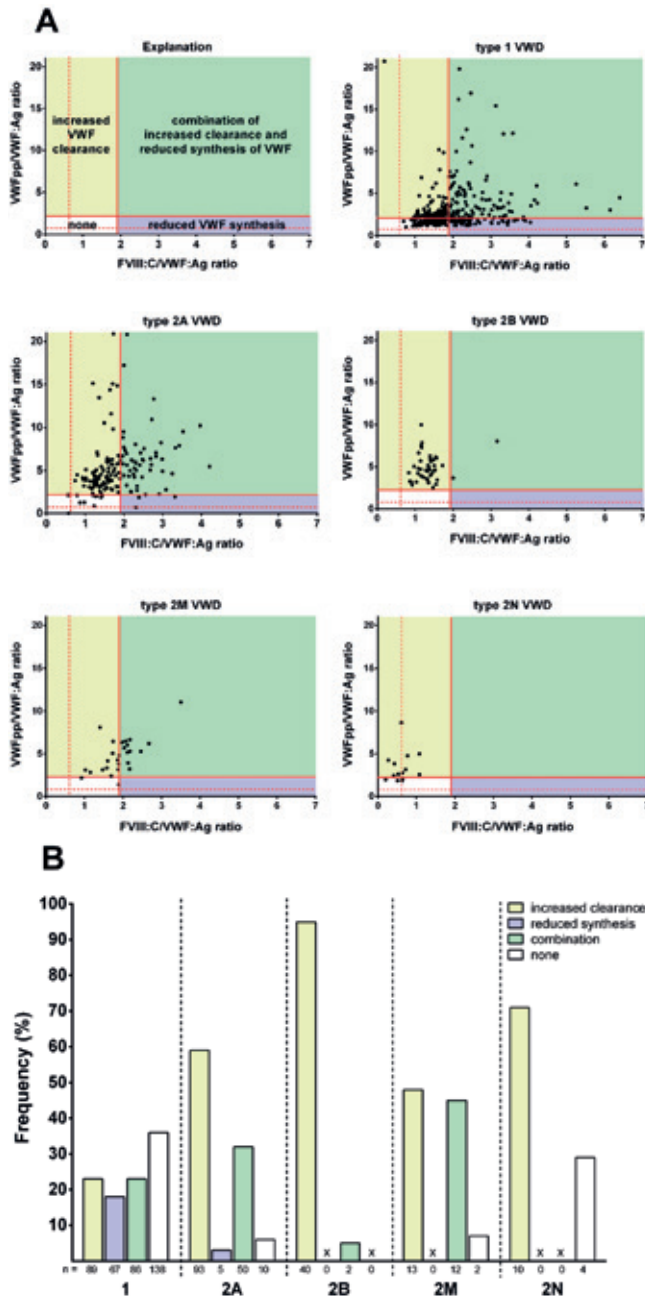


Figure 2: Pathophysiological mechanisms per type of VWD **A:** Scatter plots of FVIII:C/VWF:Ag (x-axis) versus VWFpp/VWF:Ag (y-axis) for type 1 VWD and type 2A, 2B, 2M and 2N VWD. The yellow area represents the patients with increased clearance, the purple area the patients with reduced synthesis, the green area the patients with a combination of both mechanisms, and the white area represents the absence of these pathophysiological mechanisms. For a better presentation of the graphics: one type 1 VWD patient with a very high VWFpp/Ag ratio (33.7), and two type 1 VWD patients with high FVIII:C/VWF:Ag ratios (8.4 and 9.7) were omitted. **B:** Proportion of VWD patients with increased clearance (yellow), reduced synthesis (purple), combination of increased clearance and reduced synthesis (green), and none of these mechanisms (white). X = not present.

Pathophysiological mechanisms in type 2 VWD variants

Based on increased VWFpp/VWF:Ag or FVIII:C/VWF:Ag ratios, the majority of type 2A VWD was characterized by an increased clearance of VWF (93/158, 59%), followed by a combination of increased clearance and reduced synthesis (50/158, 32%). In only five (of 158, 3%) patients with 2A VWD, reduced synthesis of VWF predominated. In ten (of 158, 6%) 2A patients, the VWD was characterized by neither increased clearance nor reduced synthesis. Ninety-five percent (40/42) of type 2B patients showed an increased VWFpp/VWF:Ag ratio with a normal FVIII:C/VWF:Ag ratio, suggesting that 2B VWD is completely characterized by an increased clearance of normally synthesized and secreted VWF. The pathophysiological mechanisms of type 2M VWD were an increased clearance in almost half of the patients (13/27, 48%) and a combination of increased clearance and reduced synthesis in the other half (12/27, 44%). None of the 2M patients had an increased FVIII:C/VWF:Ag ratio, indicating that 2M VWD is not characterized by reduced synthesis solely. Ten (of 14, 71%) 2N patients had an increased VWFpp/VWF:Ag ratio, indicating that apart from reduced FVIII binding the type 2N VWD is also characterized by increased clearance (Figure 2).

Type 3 VWD patients

Thirty-seven patients with VWF:Ag and VWF:Act <5 IU/dL were classified as type 3 VWD, according to the current ISTH guidelines [16]. VWFpp was undetectable in 59% of the (22/37) type 3 patients, which confirms the definition of type 3 VWD with complete absence of VWF. We therefore designated these patients: “true type 3 VWD patients”. In all other type 3 patients (15/37, 41%), VWFpp was detectable with a median of 72 U/dL [IQR 37-94] (table 3).

Table 3. Characteristics of type 3 VWD patients with and without VWFpp

Characteristics		Type 3 VWD patients		p-value
		without VWFpp n=22	With VWFpp n=15	
Age (years)		22 (2-60)	35 (4-65)	0.636
Male sex, n (%)		9 (41)	7 (47)	0.729
Blood group O, n (%)		8 (36)	12 (80)	0.009
VWFpp (U/dL)		0 [0-0]	72 [37-94]	<0.001
VWF:Ag (IU/dL)		0 [0-1]	4 [2-4]	<0.001
VWF:Act (IU/dL)		0 [0-0]	1 [0-3]	0.036
FVIII:C (IU/dL)		2 [1-3]	9 [8-13]	<0.001
Multimers	absent	19 (86)	3 (20)	<0.001
	abnormal	3 (14)	12 (80)	
Bleeding score		19.5 [11.3-23.8]*	14.0 [7.0-17.0]	0.025
Mutations identified [†]		10 out of 12 homozygous or compound heterozygous for null alleles	14 patients genotyped, 10 of whom had a mutation that is associated with increased clearance	

*2 missing, [†]11 missing. Levels and bleeding score are presented as median and 25-75% interquartile range [IQR]. Age is presented as median and range. Abbreviations: VWD = von Willebrand disease; VWFpp = von Willebrand factor propeptide; VWF:Ag = von Willebrand factor antigen; VWF:Act = von Willebrand factor activity; FVIII:C = factor VIII coagulation activity. VWFpp, VWF:Ag, VWF:Act and FVIII:C levels were measured centrally at time of inclusion in the study.

Compared with the “true” type 3 VWD patients with complete absence of both VWFpp and VWF:Ag, type 3 patients with detectable VWFpp had higher VWF:Ag levels (0 vs. 4 IU/dL, $p<0.001$), higher VWF:Act levels (0 vs. 1 IU/dL, $p=0.036$), and higher FVIII:C levels (2 vs. 9 IU/dL, $p<0.001$). The frequency of blood group O was significantly higher in the type 3 patients with detectable VWFpp than in the “true” type 3 patients (12/15 vs. 8/22, $p=0.009$).

In 12/22 “true” type 3 patients, the VWF gene was sequenced. Ten of whom were homozygous or compound heterozygous for a null allele (p.R2535X, p.N2546Y, p.Y1570X, p.S330KfsX4, p.D1333EfsX43, and deletion of exons 17-25). Of the 15 patients with detectable VWFpp, the VWF gene mutation was known in 14. Ten of whom had missense mutations in regions associated with rapid clearance of VWF (p.R1205H and p.S1285P) or mutations that are considered to rapidly clear VWF from the circulation (p.Y1584C and p.Y1146C)[12,18,30,31]. Additionally, three were compound heterozygous for a null allele and an unknown defect on the other allele.

Bleeding phenotype, VWFpp and pathophysiological mechanism

Bleeding score was significantly higher in the “true” type 3 patients with undetectable VWFpp than in those with detectable VWFpp level: 19.5 [11.3-23.8] vs. 14.0 [7.0-17.0], $p=0.025$ (table 3). In patients with type 1 VWD, the BS was not associated with the pathophysiological mechanisms of VWD, i.e. increased clearance, reduced synthesis, combination of increased clearance and reduced synthesis, or none of these mechanisms: median BS 10.0 vs. 9.0 vs. 8.5 vs. 9.0 respectively (Figure 3A). However, all type 2 patients with increased clearance, reduced synthesis or combination, had significantly higher BS than type 2 patients with none of these pathophysiologic mechanisms ($p=0.010$). Median BS in type 2 patients with increased clearance (13.0 [9.0-17.5], $p=0.001$) or a combination of increased clearance and reduced synthesis (13.0 [8.0-16.3], $p=0.007$) was significantly higher than in those with normal ratios: (7.5 [5.5-10.8]). Median BS in type 2 VWD characterized by reduced synthesis was 17.0 [8.0-26.0] vs. 7.5 [5.5-10.8] in those with normal ratios ($p=0.051$) (Figure 3B).

Discussion

In the present study from the WiN-cohort, we present as first that VWFpp improved the discrimination of true type 3 VWD patients with a total absence of both VWF:Ag and VWFpp, and VWD patients with extremely rapid VWF clearance leading to very low VWF plasma levels. Moreover, the ratios of VWF:Ag with VWFpp or FVIII:C differ between type 1 and 2 VWD. Type 2 VWD was mainly characterized by an increased clearance of VWF and type 1 VWD had a more heterogeneous pathophysiology. Finally, low VWFpp was associated with higher BS in type 3 and type 2 VWD.

VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios have been shown to identify type 1 VWD patients by giving insight into the pathophysiological mechanisms behind the low

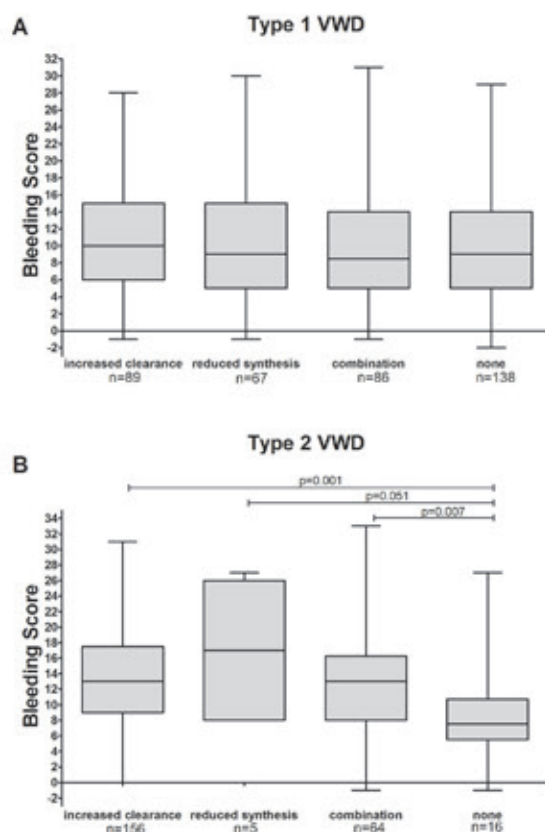


Figure 3: Bleeding score per pathophysiological mechanisms for type 1 and 2 VWD Bleeding score according to different pathophysiological mechanisms of VWD for (A) type 1 VWD and (B) type 2 VWD. Boxplots show median, 25-75% interquartile ranges and minimum and maximum score.

VWF levels, i.e. increased clearance and reduced synthesis of VWF [12,20,32]. We confirmed this in our study and showed that the pathophysiology of type 1 VWD is diverse, as our type 1 patients showed a mixture between VWF synthesis and clearance defects using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios. Almost two fifth of patients did not have increased ratios. Probably VWD in these patients results from other not yet identified mechanisms. This confirms the heterogeneity of type 1 VWD and the various causes of low VWF levels.

We hypothesized that as a result of our inclusion criteria (historically lowest VWF levels <30 U/dL), we selected more type 1 patients with accelerated clearance of VWF or reduced synthesis, and therefore patients with higher ratios. However, the VWFpp levels and ratios thereof in our type 1 patients are in agreement with a previous study in type 1 VWD patients [20]. This is remarkable as in the MCMDM-1VWD study also milder cases were included. Regression to the mean or the effect of aging on VWF and FVIII levels may have been observed [27].

We observed a higher VWFpp/VWF:Ag ratio in type 2 VWD patients compared with type 1 VWD patients. However, type 1 patients had a significantly higher FVIII:C/VWF:Ag ratio, revealing reduced synthesis as a pathophysiological mechanism of VWD in type 1, but rarely or not in type 2 VWD. This also suggests that these ratios may discriminate between type 2 and type 1 VWD, which has been stated before [33].

The main pathophysiological mechanism of type 2 VWD is increased VWF clearance, as the majority of type 2A, 2B and 2M patients showed an increased VWFpp/VWF:Ag ratio. In addition, a small proportion of type 2 patients showed a combination of reduced VWF survival and reduced synthesis, but this was only seen in 2A and 2M VWD. The effect of proteolysis of VWF on clearance has been argued [3,30,34,35]. We found no differences between 2A VWD with defects in multimerization and enhanced ADAMTS-13-mediated proteolysis, and 2M VWD with normal multimer patterns. Because of small sample size these findings should be interpreted with care. Our data indicate that 2B VWD is completely characterized by a normal synthesis and an increased clearance of VWF, which has been shown before in 18 type 2B VWD patients [36]. It has been suggested that the mutant VWF is cleared faster in these patients due to the spontaneous binding of VWF to platelets [17,19,37,38]. Our results also showed that the mutant type 2N VWF is cleared faster than normal VWF.

To the best of our knowledge, this is the first study to assess VWFpp in VWD patients with VWF:Ag and VWF:Act <5 IU/dL, the diagnostic criteria used in the current ISTH classification for type 3 VWD [16]. We showed that VWFpp is detectable in half of the type 3 VWD patients, indicating that VWF is actually produced but very rapidly cleared from the circulation in these patients. Our findings suggest that these type 3 VWD patients should actually reclassify as severe type 1 VWD patients. We therefore conclude that VWFpp can discriminate between “true” type 3 VWD with complete absence of both VWF:Ag and VWFpp, and severe type 1 VWD with extremely low VWF levels. A significant proportion of these severely affected type 1 VWD patients had blood group O, which may have contributed to the low VWF levels in these patients. Distinguishing between true type 3 VWD and severe type 1 VWD is of clinical importance as VWD patients with lack of VWF but detectable VWFpp have different clinical characteristics than those with a complete absence of both VWF and VWFpp.

Because VWF:Ag and FVIII:C levels are associated with bleeding phenotype in VWD [24,28,39], it may be expected that VWFpp also associates with bleeding phenotype in VWD patients: indirectly through its association with VWF:Ag or by binding to mature VWF in the circulation [40]. Interestingly, we found that the presence of VWFpp in patients with VWF:Ag levels <5 IU/dL correlates with a milder bleeding phenotype. We also observed that increased clearance, reduced synthesis and a combination thereof associates with higher bleeding scores in type 2 patients.

Mutation analysis of the VWF gene has been advised in type 2N VWD, type 2B VWD, and in type 3 VWD [41]. However, sequencing of the VWF gene is expensive and not

widely available. Looking at the molecular background of the type 3 VWD patients, we observed homozygosity and compound heterozygosity for null alleles in the true type 3 VWD patients with complete absence of VWF:Ag and VWFpp. In the type 3 VWD patients with detectable VWFpp, missense mutations in the D3 and D4 domain of the VWF gene were identified that are associated with accelerated clearance of VWF (p.R1205H; p.S1285P) [12,18,30]. Therefore assessing VWFpp may substitute for VWF gene analyses as it identifies carriers of two null alleles and thus “true” type 3 VWD. In addition, the MCMDM-1VWD study has recently shown that a high VWFpp/VWF:Ag ratio predicts the detection of VWF gene mutations in type 1 VWD patients [20]. Additionally, patients with mutations in regions of the VWF gene associated with reduced VWF survival, can be identified with the VWFpp/VWF:Ag ratio [12,18,30]. Based on our results and the current literature, we believe that measurement of VWFpp should be implemented as a standard diagnostic in VWD for patients with low VWF levels and may render mutation analysis of the VWF gene unnecessary.

VWFpp measurement is of clinical importance because its ratio with VWF:Ag predicts reduced VWF survival and may therefore predict desmopressin response. In patients with increased VWF clearance, VWF disappears rapidly after desmopressin administration. The desmopressin response in these patients is probably sufficient for hemostasis in case of minor bleeding, but inadequate in case of major bleeding or surgery because they lack a sustained response [13,18,42]. The FVIII:C/VWF:Ag ratio can also help in the classification of VWD patients, as those patients with an increased FVIII:C/VWF:Ag mainly classify as type 1 VWD. With the current novel insights into the pathophysiology and molecular biology of VWD the classification of VWD should be reviewed in the near future.

In conclusion, VWFpp discriminates between true type 3 VWD patients with complete lack of VWF and severe type 1 VWD patients with very low VWF levels. An increased FVIII:C/VWF:Ag ratio may help identify type 1 VWD patients. This study shows the clinical significance of the VWFpp assay; it is of added value for the classification of VWD, for the assessment of the bleeding phenotype, for genetic counseling, and it also has therapeutic consequences.

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FWG Leebeek and J Eikenboom designed research, analyzed and interpreted data, and wrote the manuscript. YV Sanders performed research, analyzed and interpreted data, and wrote the manuscript. D Groeneveld performed research, analyzed and interpreted data, and critically reviewed the manuscript. K Meijer, K Fijnvandraat, MH Cnossen, JG van der Bom, BAP Laros-van Gorkom, and EP Mauser-Bunschoten: designed research, analyzed and interpreted data, and critically reviewed the manuscript. M Coppens and J de Meris: analyzed and interpreted data, and critically reviewed the manuscript. All authors gave their consent to the final version of the manuscript.

Disclosures of Conflicts of Interest

FWG Leebeek received research support from CSL Behring for performing the WiN-study and has served on advisory boards of CSL Behring and Baxter. J Eikenboom received research support from CSL Behring and he has been a teacher on educational activities of Roche. EP Mauser-Bunschoten received research support from CSL Behring, Bayer, Baxter, Novo Nordisk, Pfizer and Sanquin. JG van der Bom has received unrestricted research/educational funding for various projects from the following companies: Bayer Schering Pharma, Baxter, CSL Behring, Novo Nordisk, and Pfizer. In addition, she has been a consultant to Baxter and Pfizer, and she has been a teacher on educational activities of Bayer Schering Pharma. MH Cnossen has received unrestricted research/educational funding for various projects from the following companies: Bayer Schering Pharma, Baxter, Novo Nordisk and Pfizer. K Meijer received research support from Bayer and Baxter, served on an advisory board for

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Part 3

Aspects of VWF beyond hemostasis

CHAPTER

7

Dysregulated secretion of angiogenic mediators in patients with moderate and severe von Willebrand Disease

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Abstract

Objective: It has recently been shown that von Willebrand factor (VWF) can act as a negative regulator of angiogenesis. VWF is stored in endothelial cells in Weibel-Palade bodies (WPB) together with angiopoietin-2, osteoprotegerin and galectin-3. These proteins are known to mediate angiogenesis. We hypothesized that defects in VWF, potentially leading to impaired or loss of WPB formation, result in dysregulated release of angiogenic mediators in the circulation. **Approach and Results:** We measured plasma levels of VEGF, angiopoietin-1 and 2, osteoprotegerin and galectin-3 in 380 type 1, 239 type 2 and 36 type 3 von Willebrand Disease (VWD) patients and 100 healthy controls. VWD patients had higher angiopoietin-1 and lower angiopoietin-2 levels compared with controls. VEGF levels were also higher in VWD patients and dependent upon VWF levels in plasma. These levels were higher in type 3 compared to type 1 patients. Type 1 and 2 VWD patients had lower angiopoietin-2 levels compared with controls, while type 3 VWD patients had similar angiopoietin-2 levels as controls. Type 3 VWD patients had higher osteoprotegerin levels compared with controls. **Conclusions:** Patients with VWD have 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 are frequently observed in VWD patients.

Introduction

Von Willebrand factor (VWF) is mainly produced in endothelial cells, although a small fraction of the total VWF pool is synthesized in megakaryocytes [1-3]. Upon formation of the multimeric VWF structure, the protein is either constitutively secreted into the blood, or stored in cell-specific organelles. In endothelial cells VWF is stored in the Weibel-Palade bodies (WPB) [4]. VWF is essential for the formation of these WPBs, because absence of VWF leads to the loss of WPB formation [5]. Cells that normally do not express VWF form pseudo-WPB when transfected with VWF [6,7].

Although VWF is best known for its role in hemostasis [1,8], other functions have been identified in recent years, indicating that this protein is involved in multiple vascular processes (reviewed in [9]). Vascular malformations in von Willebrand disease (VWD) patients, most frequently angiodysplasia in the gastrointestinal tract, have repeatedly been reported [10,11]. These malformations can be responsible for severe, intractable bleeding which is often not responsive to VWF replacement therapy and thus represent a significant unmet clinical challenge [12]. The pathological mechanism underlying vascular malformations in VWD is still largely unexplained. Recently it has been shown that VWF can act as a negative regulator of angiogenesis *in vitro* [13], by inhibition of endothelial tube formation, migration and proliferation,

in a Vascular Endothelial Growth Factor Receptor (VEGFR)-2 dependent process. Increased angiogenesis induced by VWF knockdown in endothelial cells was associated with increased angiopoietin-2 (Ang-2) release into the medium. Blood Outgrowth Endothelial Cells (BOECs) of type 1 and 2 VWD patients confirmed that low VWF levels lead to increased angiogenesis *in vitro* [13].

The angiogenic mediators Ang-2, osteoprotegerin (OPG), galectin-3 (Gal-3) are synthesized in endothelial cells and stored in the WPB together with VWF [14-16]. Angiopoietin-1 (Ang-1) and Ang-2 work together with Vascular Endothelial Growth Factor-A (VEGF) to mediate angiogenesis. Ang-2 is an antagonist of Ang-1 and both angiopoietins bind to the TIE-2 receptor on endothelial cells [17]. Increased levels of Ang-2 results in destabilized vessels, while increased VEGF levels can lead to vascular malformations in humans [18]. OPG, a soluble decoy receptor for both receptor activator of nuclear factor-kappaB ligand (RANKL) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL), is a positive regulator of microvessel formation, promoting both endothelial cell sprouting and proliferation [19]. Gal-3 modulates VEGF-mediated angiogenesis by binding to integrin $\alpha V\beta 3$ on endothelial cells, subsequently activating the signalling pathways that promote the growth of new vessels [20]. Since VWF is essential for the formation of WPB, other WPB components such as Ang-2, OPG and Gal-3 are dependent on VWF for their storage and secretion. We hypothesize that reduced levels or defects in VWF, which can lead to impaired WPB formation or complete loss of WPB, result in dysregulated release of other components of the WPB. The aim of this study was therefore to investigate plasma levels of angiogenic mediators in patients with various types of VWD in order to obtain more insight in the development of vascular malformations frequently observed in these patients.

Materials and Methods

Patients and controls

Stored plasma samples from the nationwide cross-sectional study among VWD patients in the Netherlands, the 'Willebrand in the Netherlands' (WiN) study [21-23] were used for this study. Patients diagnosed with type 1, 2 and 3 VWD who fulfilled the following inclusion criteria were initially included: (i) hemorrhagic symptoms or a family history of VWD; and (ii) historically lowest levels of VWF ≤ 30 U/dL (VWF:Ag and/or VWF:RCO) and/or FVIII:C ≤ 40 U/dL. Exclusion criteria for the current study were: patients who (i) were pregnant at time of inclusion, (ii) had received blood products, (iii) had received DDAVP infusion 72 hours prior to inclusion. Healthy individuals, who participated in the ATTAC study [24], for which VWF:Ag and blood group were known, were included to obtain reference values, and matched for age, sex and blood group. A total of 100 controls were included in this study. The Medical Ethical Committees at all participating centers approved this study and written informed consent was obtained from all study participants.

Laboratory Measurements

Details regarding the blood sampling procedure, questionnaires and laboratory measurements of VWF:Ag and VWF:Act have been described in more detail by De Wee *et al.* [22], Sanders *et al.* [23] and De Bruijne *et al.* [24] VWFpp was measured as previously described [25]. At inclusion, peripheral venous blood was collected in tubes containing 3.2% (0.105 M) trisodium citrate. Subsequently, patient samples were centrifuged twice at 2,200 x g for 10 minutes at room temperature, control samples were centrifuged at 2000 x g for 10 minutes and plasma was additionally centrifuged at 20.000 x g for 10 minutes. Citrated platelet-poor plasma was aliquoted and stored at -80°C. Angiopoietin-1 levels were measured using the Human Angiopoietin-1 DuoSet ELISA kit (DY923, R&D Systems Europe, Abingdon, Oxon, UK). Angiopoietin-2 levels were measured using the Human Angiopoietin-2 DuoSet ELISA kit (DY623), galectin-3 levels with the Human Galectin-3 DuoSet ELISA kit (DY1154), osteoprotegerin with the Human Osteoprotegerin/TNFRSF11B DuoSet ELISA kit (DY805), and VEGF levels with the Human VEGF DuoSet ELISA kit (DY293B, all R&D systems).

Statistical Analysis

Because data were not normally distributed, plasma levels are presented as median and 25-75% IQR. The Kruskal-Wallis test was used to test differences between types 1, 2A, 2B, 2M, 2N, 3 VWD and the control group. The Mann-Whitney U test was then used to detect differences between the groups. Correlations between different parameters were determined by Spearman correlation coefficient. A spearman r_s of <0.20 was regarded as very weak, 0.20-0.39 as weak, 0.40-0.59 as moderate and ≥ 0.6 as a strong correlation. Statistical analyses were performed with SPSS for Windows, version 22.0 (SPSS Inc, Chicago, USA). A p-value <0.05 was considered statistically significant.

Results

A total of 804 moderate and severe VWD patients participated in the WiN-study. From 681 patients plasma samples were obtained, 26 patients were excluded because of pregnancy ($n=7$), recent use of desmopressin or replacement therapy ($n=17$) or insufficient plasma sample ($n=2$). Therefore a total of 655 VWD patients were included in this study; 380 patients were classified as type 1 VWD, 239 as type 2 VWD and 36 as type 3 VWD according to the 2006 classification [26]. Fifteen patients who were initially diagnosed with type 3 VWD, had VWFpp levels >5 U/dL, and as a consequence these patients were included in the type 1 VWD group for the current analysis. Patient characteristics are given in Table 1.

Angiogenic mediators according to age, VWF:Ag levels and VWD

Both in VWD patients and controls OPG and VWF:Ag levels correlated with age. In VWD patients VEGF, OPG and Gal-3 levels correlated significantly with VWF:Ag levels ($r = -0.129$, $r = 0.089$, $r = 0.083$) (Table 2). Considering all VWD patients together, patients had higher Ang-1 (median [IQR], 0.91 [0.51-1.56] ng/ml) and VEGF levels

Table 1. Baseline characteristics

Characteristics	VWD Patients (n=655)	Controls (n = 100)
Age (years), median (range)	44 (1-83)	43 (18-56)
Male sex, n (%)	250 (38)	38 (38)
VWD type, n (%)		
1	395 (60)	
2	239 (36)	
2A	156	
2B	43	
2M	26	
2N	14	
3	21 (3)	
Blood group O, n (%)*	396 (61)	56 (56)
VWF:Ag (IU/dL), median [IQR]	29 [18-45]	91 [77-125]
VWF:CB (IU/dL), median [IQR]	22 [7-51]	108 [88-139]
VWF:Act (IU/dL), median [IQR]	23 [8-52]	
FVIII:C (IU/dL), median [IQR]	51 [32-73]	
Bleeding score, median [IQR]†	11 [6-16]	
Bleeding in last year, n (%) ‡		
Yes	180 (31)	
GI bleeding	9 (5)	
No	420 (69)	

*6 missing, †32 missing, ‡ 46 missing. VWF:Ag, von Willebrand factor: Antigen; VWF:CB, von Willebrand factor: Collagen Binding activity; VWF:Act, von Willebrand factor: Activity; FVIII:C, factor VIII coagulation activity; GI, gastrointestinal.

Table 2. Correlations between parameters

Relation	Group	Spearman r	P value
VEGF and VWF:Ag	Patients	-0.129	0.001
OPG and VWF:Ag	Patients	0.089	0.023
Gal-3 and VWF:Ag	Patients	0.083	0.033
VEGF and Age	Patients	-0.28	<0.001
OPG and Age	Patients	0.382	<0.001
Gal-3 and Age	Patients	0.26	<0.001
OPG and Age	Controls	0.294	0.003
VWF:Ag and Age	Patients	0.276	<0.001
VWF:Ag and Age	Controls	0.203	0.043

(median 5 [0-17] pg/ml) than healthy controls (0.14 [0.03-0.34] ng/ml, $p=0.0002$ and 0 [0-10] pg/ml, $p<0.0001$). Ang-2 levels were lower in VWD patients (0.12 [0-0.26] ng/ml) than in controls (0.20 [0.12-0.33] ng/ml, $p<0.0001$), while OPG and Gal-3 levels did not differ between patients and controls (Table 3).

Median Ang-1 levels were higher in type 1 (0.89 [0.51-1.54] ng/ml, $p<0.0001$), type 2 (0.95 [0.5-1.66] ng/ml, $p<0.0001$) and type 3 (1.32 [0.79-1.97] ng/ml, $p<0.0001$) VWD patients than in healthy controls (0.14 [0.03-0.34] ng/ml) (Table 3). In contrast, median levels of Ang-2 were lower in type 1 (0.1 [0-0.24] ng/ml, $p<0.0001$) and type 2 VWD patients (0.14 [0-0.29] ng/ml, $p=0.003$) than in healthy controls (0.20 [0.12-0.33] ng/ml). Type 3 VWD patients had comparable Ang-2 levels (0.16 [0.04-0.29] ng/ml, $p=0.17$) as controls (Table 3). Median VEGF levels were higher in type 1, 2 and 3 VWD patients compared with healthy individuals (4 [0-15] pg/ml, $p<0.001$ for type 1 patients, 4 [0-21] pg/ml, $p<0.001$ for type 2 patients and 16 [7-52] pg/ml, $p<0.0001$ for type 3 patients vs 0 [0-10] pg/ml for controls) (Table 3).

Type 3 VWD patients tended to have higher Ang-1 levels than type 1 VWD patients (median 1.32 [0.79-1.97] ng/ml vs 0.89 [0.51-1.54] ng/ml, $p=0.07$) (Table 3). Ang-2 levels were lower in type 1 VWD patients compared with type 2 VWD patients (0.1 [0-0.24] ng/ml vs 0.14 [0-0.29] ng/ml, $p=0.04$) (Table 3). In addition, type 3 VWD patients had the highest VEGF levels compared with both type 1 (16 pg/ml vs 4 pg/ml, $p=0.0014$) and type 2 VWD patients (16 pg/ml vs 4 pg/ml, $p=0.006$) (Table 3). OPG levels did not differ between type 1, 2 or 3 VWD patients and controls, although there was a trend towards higher levels in the type 3 VWD (1.28 [0.98-1.86] ng/ml) compared with type 1 (1.1 [0.85-1.44] ng/ml, $p=0.08$), type 2 VWD (1.1 [0.84-1.56] ng/ml, $p=0.15$) patients

Table 3. Angiogenic parameters in VWD patients and controls.

Angiogenic mediator	Controls (n=100)	All Patients (n=655)	Type 1 (n=395)	Type 2 (n=239)	Type 3 (n=21)
Ang-1	0.14	0.91	0.89	0.95	1.32
[ng/ml]	(0.03-0.34)	(0.51-1.56) ^a	(0.51-1.54) ^a	(0.50-1.66) ^a	(0.79-1.97) ^a
Ang-2	0.20	0.12	0.10	0.14	0.16
[ng/ml]	(0.12-0.33)	(0-0.26) ^a	(0-0.24) ^{a,1}	(0-0.29) ^c	(0.04-0.29)
VEGF	0	5	4	4	16
[pg/ml]	(0-10)	(0-17) ^b	(0-15) ^{a,2}	(0-21) ^{b,2}	(7-52) ^a
OPG	1.04	1.1	1.10	1.10	1.28
[ng/ml]	(0.77-1.29)	(0.85-1.48)	(0.85-1.44)	(0.84-1.56)	(0.89-1.86)
Gal-3	2.12	2.0	2.06	1.95	1.68
[ng/ml]	(1.6-2.76)	(1.28-2.77)	(1.36-2.78)	(1.2-2.78)	(0.93-2.70)

Variables are expressed as medians (IQR). ^a $p<0.0001$, ^b $p<0.001$, ^c $p<0.01$ compared with controls.

¹ $p<0.05$ compared with type 2. ² $p<0.01$ compared with type 3.

and controls (1.0 [0.77-1.29] ng/ml, $p=0.02$) (Table 3). Also Gal-3 levels did not differ between type 1, 2 or 3 VWD patients and healthy controls, although the type 3 VWD patients had slightly lower plasma levels (1.68 [0.93-2.7] ng/ml) compared with controls (2.12 [1.6-2.76] ng/ml, $p=0.07$) and type 1 and 2 VWD patients (2.06 [1.36-2.78] ng/ml, $p=0.12$ and 1.95 [1.2-2.78] ng/ml, $p=0.26$ respectively) (Table 3).

Angiogenic markers according to subtypes of type 2 VWD

Since type 2 VWD patients have different qualitative defects in their VWF, there might be diverse consequences on the various WPB components in the different VWD type 2 subtypes. Type 2 VWD patients were subdivided based on the ISTH subtype classification [26]. Our study had 156 type 2A, 43 type 2B, 26 type 2M and 14 type 2N patients. As shown in Table 4, compared with controls type 2A, 2B, 2M and 2N VWD patients had higher Ang-1 levels ($p<0.0001$). The median Ang-1 levels are higher in the type 2A (1.04 [0.61-1.78] ng/ml, $p=0.0012$) and 2M VWD (1.11 [0.65-1.69] ng/ml, $p=0.02$) compared with type 2B VWD (0.70 [0.27-1.1] ng/ml). Ang-1 levels were slightly lower in the type 2N VWD group (0.74 [0.35-1.41] ng/ml), but this difference did not reach statistical significance (Table 4). Ang-2 levels were only significantly lower in type 2A (0.14 [0.02-0.30] ng/ml, $p=0.005$) patients compared with controls (0.2 [0.12-0.33] ng/ml) (Table 4). Although there was no difference in Ang-2 levels within the type 2 VWD group, the type 2B VWD patients (0.21 [0.03-0.34] ng/ml) had slightly higher levels compared with the other type 2 VWD patients (Table 4). VEGF levels were higher in type 2A (4 [0-18] pg/ml, $p=0.0048$), 2M (9 [0-28] pg/ml, $p=0.0038$) and 2N (7 [1.5-58] pg/ml, $p=0.016$) patients compared with controls (0 [0-10] pg/ml), but not in type 2B patients (3 [0-26] pg/ml, $p=0.054$). VEGF, OPG and Gal-3 levels did not differ between the different type 2 VWD patients (Table 4).

Table 4. Angiogenic parameters in type 2 VWD patients and controls.

Angiogenic mediator	Controls (n=100)	Type 2 (n=239)	Type 2A (n=156)	Type 2B (n=43)	Type 2M (n=26)	Type 2N (n=14)
Ang-1	0.14	0.95	1.04	0.70	1.11	0.75
[ng/ml]	(0.03-0.34)	(0.50-1.66) ^a	(0.61-1.78) ^{a,1}	(0.27-1.10) ^a	(0.65-1.69) ^{a,2}	(0.35-1.41) ^a
Ang-2	0.20	0.14	0.14	0.21	0.02	0.11
[ng/ml]	(0.12-0.33)	(0-0.29) ^c	(0.02-0.30) ^c	(0.03-0.34)	(0-0.32) ^d	(0.02-0.40)
VEGF	0	4	4	3	9	7
[pg/ml]	(0-10)	(0-21) ^b	(0-18) ^c	(0-26)	(0-28) ^c	(2-58)
OPG	1.04	1.10	1.12	1.09	0.95	1.43
[ng/ml]	(0.77-1.29)	(0.84-1.56)	(0.84-1.56)	(0.84-1.59)	(0.61-1.19)	(0.94-1.75)
Gal-3	2.12	1.95	1.98	1.67	2.4	1.48
[ng/ml]	(1.6-2.76)	(1.2-2.78)	(1.27-2.78)	(0.99-2.39)	(1.68-3.23)	(0.99-3.43)

Variables are expressed as medians (IQR). ^a $p<0.0001$, ^b $p<0.001$, ^c $p<0.01$, ^d $p<0.05$ compared with controls.
¹ $p<0.01$, ² $p<0.05$ compared with type 2B.

VEGF levels are dependent upon VWF levels in plasma

In order to study the association between VWF:Ag levels and the angiogenic markers, we divided the VWD patients into 4 groups based on their VWF:Ag level; <10 (n=79), 10-20 (n=115), 21-30 (n=154), and >30 IU/dL (n=306). As shown in Figure 1A, patients with VWF:Ag levels below 10 IU/dL had higher VEGF levels (14 pg/ml, IQR [4-42]) compared with patients with VWF:Ag levels between 10-20 IU/dL (5 [0-18] pg/ml, $p=0.0006$), patients with VWF:Ag levels between 21-30 IU/dL (3 [0-16] pg/ml, $p<0.0001$) and patients with VWF:Ag levels >30 IU/dL (4 [0-14] pg/ml, $p<0.0001$). If only patients with type 1 and 2 with VWF:Ag levels <10 IU/dL were considered, also these patients had the highest VEGF levels (Figure 1B,C). For the type 2 patients this was still observed after exclusion of type 2B patients (data not shown). Ang-1, Ang-2, OPG and Gal-3 levels did not differ between the subgroups based on VWF:Ag levels (data not shown).

Increased VWF clearance and impaired VWF synthesis

The ratio between VWF propeptide (VWFpp) and VWF:Ag (VWFpp/VWF:Ag) can be used to assess VWF clearance, while the ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag (FVIII:C/VWF:Ag) can be used to assess VWF synthesis [27]. A high VWFpp/VWF:Ag ratio indicates increased clearance of VWF and a high FVIII:C/VWF:Ag ratio decreased synthesis of VWF. Since Ang-2, OPG and Gal-3 are stored together with VWF inside the WPB, we investigated if increased VWF clearance and/or impaired VWF synthesis influences the plasma levels of the other WPB components. Within the type 1 VWD group we divided the patients into normal synthesis (n=219) or clearance (n=200) (based on the upper limit of normal range of VWFpp/VWF:Ag

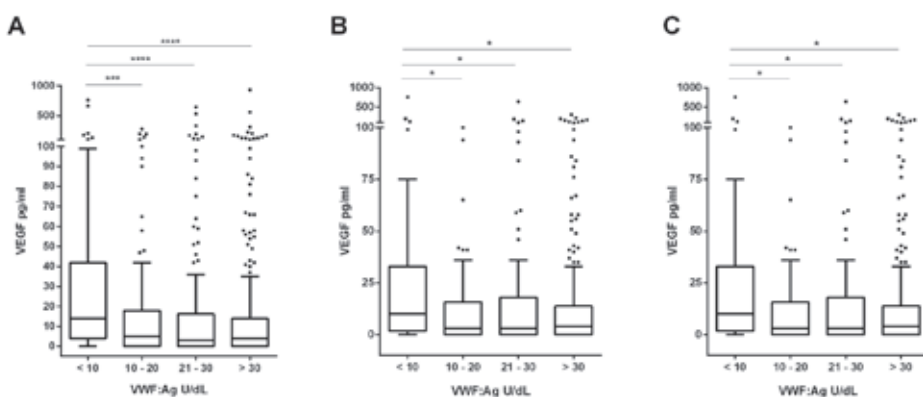


Figure 1. VEGF plasma levels in relation to VWF:Ag levels. All patients were divided into four groups based on their VWF:Ag level; < 10 IU/dL, 10 - 20 IU/dL, 21 - 30 IU/dL and > 30 IU/dL. Panel A shows VEGF plasma levels of all patients (< 10 IU/dL n=79, 10 - 20 IU/dL n=115, 21 - 30 IU/dL n=154, > 30 IU/dL n=306). In panel B only patients with type 1 VWD are plotted (n=34, n=50, n=79, n=232), in panel C only patients with type 2 VWD are plotted (n=24, n=65, n=75, n=74). Box plots show median values and interquartile ranges. Whiskers show Tukey hinges; black circles are outliers. Asterisks denote significant deviations, * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$, **** $P\leq 0.0001$.

and FVIII:C/VWF:Ag ratios as described by Eikenboom *et al.* [27]) or moderately and severely impaired synthesis (n=121 and 55) or increased clearance (n=137 and 58) (Figure 2). Median Ang-2 levels were higher in patients with very fast VWF clearance (arbitrary defined as a VWFpp/VWF:Ag ratio above 5) (0.16 [0.06-0.42] ng/ml), compared with patients with normal (0.09 [0-0.22] ng/ml, p=0.0021) or moderately increased clearance (0.09 [0-0.24] ng/ml, p=0.018) (Figure 2A). OPG and Gal-3 levels were similar in patients with normal and increased clearance of VWF (Figure 2B-C). Although not significant, Ang-2 levels were lower in patients with normal VWF synthesis (ratio of FVIII:C/VWF:Ag $\leq$ 1.9) compared with impaired VWF synthesis (ratio above 1.9) (Figure 2D). OPG levels were similar between patients with normal and impaired VWF synthesis (Figure 2E). Patients with normal VWF synthesis had significantly higher Gal-3 levels (2.15 [1.56-2.86] ng/ml) compared with patients with moderately impaired synthesis (1.91 [1.28-2.67] ng/ml, p=0.045) and severely impaired synthesis (1.74 [1.09-2.77] ng/ml, p=0.028) (Figure 2F).

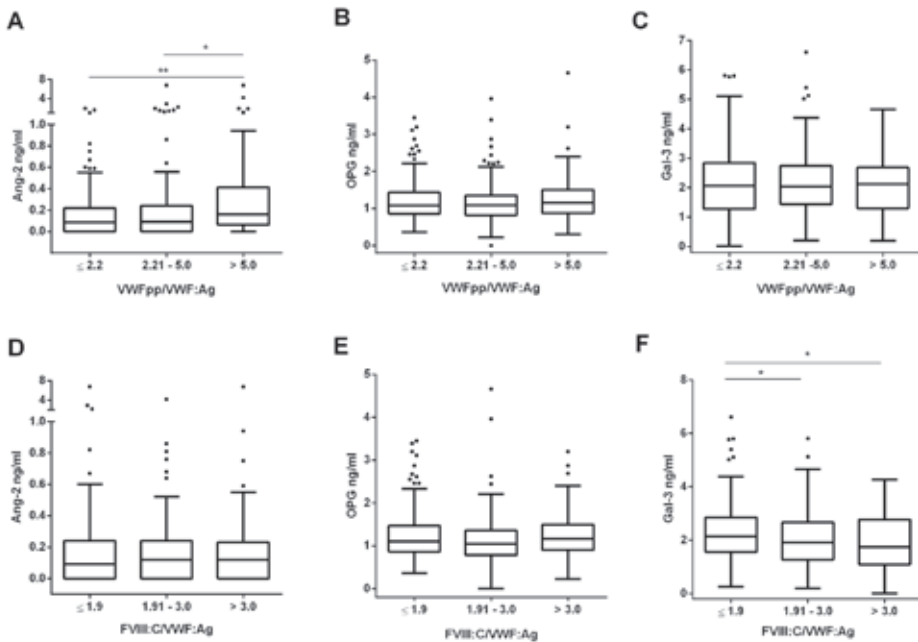


Figure 2. Plasma levels of WPB components Ang-2, OPG and Gal3 in relation to VWF synthesis and clearance. VWFpp/VWF:Ag was used as a parameter for VWF clearance rate (A-C). Within the type 1 VWD group (n=395), patients were categorized in three groups: VWFpp/VWF:Ag ratio of $\leq$ 2.2 (n=200), 2.21 – 5 (n=137) and $>$ 5 (n=58). Panel A shows levels of Ang-2, panel B shows OPG levels and panel C shows Gal-2 levels. The FVIII:C/VWF:Ag ratio was used as a parameter for VWF synthesis (D-F). Type 1 VWD patients were categorized into three groups based on their FVIII:C/VWF:Ag ratio: $<$ 1.9 (n=219), 1.9 – 3 (n=121) and $>$ 3 (n=55). Panel D shows levels of Ang-2, panel E shows OPG levels and panel F shows Gal-2 levels. The cutoffs are based on the upper limit of normal range as described by Eikenboom *et al.* [27]. Box plots show median values and interquartile ranges. Whiskers show Tukey hinges; black circles are outliers. Asterisks denote significant deviations, * P $\leq$ 0.05, ** P $\leq$ 0.01.

Angiogenic mediators and lack of Height Molecular Weight (HMW) Multimers

Angiodysplasia is mostly reported in type 2A, 2B [28-30] and type 3 VWD [10] patients, i.e. in patients lacking HMW multimers. Patients were divided into normal VWF multimer pattern (total n=339, type 1=300, type 2M =25, type 2N=14) or lacking the HMW multimers (total n=111, type 2A=76, type 2B=35) based on multimer analysis. We observed higher Ang-2 levels in the subgroups of VWD patients lacking the HMW multimers (0.14 [0.02-0.29] ng/ml) compared with those with normal multimeric pattern (0.09 [0-0.24] ng/ml, $p=0.02$) (Figure 3B). Although not significant, also Ang-1 levels are increased in patient lacking the HMW multimers (0.89 [0.51-1.47] ng/ml vs 0.99 [0.60-2.15] ng/ml, $p=0.09$), while Gal-3 levels were lower in these patients (2.0 [1.3-2.8] ng/ml vs 1.73 [1.2-2.58], $p=0.0733$) (Figure 3A, E). VEGF and OPG levels were comparable between the two patient groups (Figure 3C-D).

Angiogenic mediators and gastrointestinal bleeding

To investigate whether dysregulation of angiogenic mediators could contribute to vascular malformations leading to gastrointestinal bleedings, we analysed patients who experienced gastrointestinal bleedings in the last year ($n=9$) versus patients with

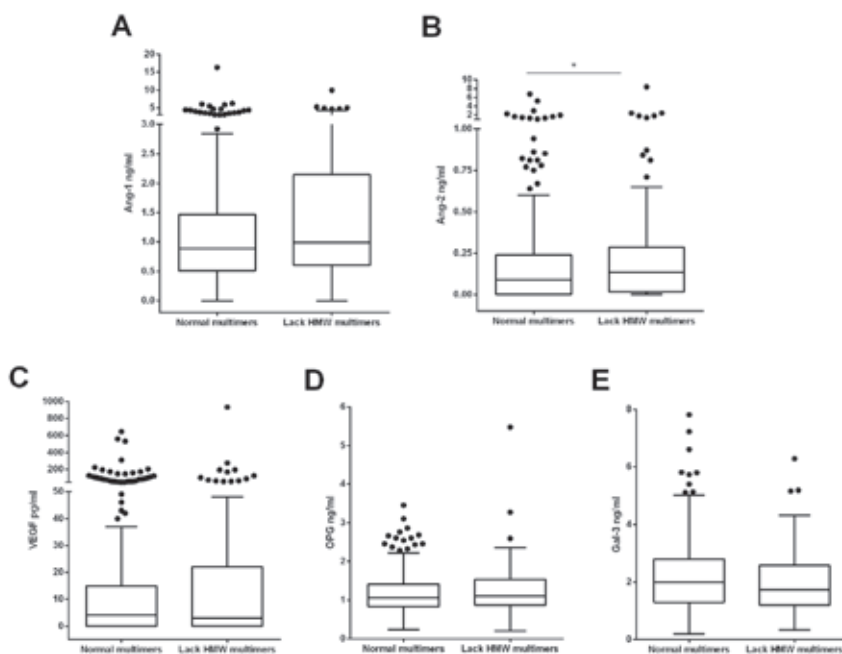


Figure 3. Plasma levels of angiogenic mediators in relation to VWF multimer pattern. Patients with normal VWF multimer pattern (Total n=339; type 1 = 300, type 2M = 25, type 2N = 14) and patients who lack the high molecular weight (HMW) multimers (Total n=111; type 2A = 76, type 2B = 35) are plotted for their plasma levels of (A) Ang-1, (B) Ang-2, (C) VEGF, (D) OPG and (E) Gal-3. Box plots show median values and interquartile ranges. Whiskers show Tukey hinges; black circles are outliers. Asterisks denote significant deviations, * $P \leq 0.05$.

bleedings elsewhere (n=180) and patients without bleedings in the year before inclusion (n=420). We observed higher Ang-2 levels in patients who bled (0.15 [0.01-0.29] ng/ml) compared with patients who did not experience bleedings (0.09 [0-0.25] ng/ml, p=0.03). Due to a lack of power, considering the small group of VWD patients with gastrointestinal bleedings we could not detect other differences between those with and without GI bleeding.

Discussion

VWF is a negative regulator of (*in vitro*) angiogenesis [13] and is stored in WPBs in endothelial cells. Besides VWF also other angiogenic mediators are stored within the WPBs. In this study we investigated plasma levels of angiogenic mediators in patients with VWD in order to obtain more insight in the development of vascular malformations frequently observed in patients with VWD. We observed that several of the measured angiogenic mediators are significantly different in VWD patients compared to healthy controls. Also within the VWD patient groups variations in these levels were observed.

We found higher Ang-1 and VEGF plasma in patients with VWD, with the highest levels in type 3 patients, which is in line with a previously published study [31]. VEGF is the best characterized pro-angiogenic endothelial growth factor [32,33]. At high concentrations however, VEGF induces an irregular vasculature with fragile and frequently hemorrhagic vessels, reminiscent of angiodysplasia [34] and increased VEGF levels have been reported in patients presenting with angiodysplasia and in patients with hereditary hemorrhagic telangiectasia [35,36]. Higher VEGF levels in VWD patients could therefore contribute to vascular malformations frequently seen in these patients [12]. However, care should be taken interpreting the increased VEGF and Ang-1 plasma levels. Since platelets store a large amount of Ang-1 and VEGF, circulating levels of these proteins may be biased by platelet count, *in vivo* platelet activation and unintentional platelet activation during blood collection [37]. Centrifugation steps were slightly different between patients and controls and we cannot exclude that this might have led to differences in *ex vivo* platelet activation. Since the first step of centrifugation is similar between patients and controls, and most platelets are discarded after this step, potential *ex vivo* activation is presumably comparable between patients and controls. Nonetheless, plasma collection was identical for all VWD patients, and thus within the VWD group this is not an issue. Platelet counts were not documented in this study, but most VWD patients have normal platelet numbers [36]. It has been shown that megakaryocytopoiesis is modified by the enhanced VWF-GPIb α interactions in patients with type 2B VWD [38], and VEGF and Ang-1 levels could be influenced by the abnormal megakaryocytopoiesis. However, when type 2B patients were excluded from the type 2 groups for analysis, differences were still observed. VEGF is also produced by endothelial cells and the higher levels

of VEGF in VWD patients could be a consequence of perturbed intracellular and extracellular pathways leading to increased VEGF expression in the absence of VWF [12,39]. This may also explain why patients with the lowest VWF:Ag levels (type 3 VWD patients and patients with a VWF:Ag <10 IU/dL) have the highest VEGF levels.

We speculated that defects in VWF, potentially leading to impaired or absent WPB formation, enhanced the release of other components of the WPB. In accordance with this hypothesis we found higher Ang-2 and OPG levels in type 3 VWD patients compared with type 1 and 2 VWD patients. However, the opposite was observed for Gal-3 levels; Gal-3 levels were the lowest in type 3 VWD patients. In VWF deficient mice Gal-3 levels were also reduced compared with wild-type mice [14], confirming the results observed in our type 3 VWD patients. In VWF deficient mice the reduced Gal-3 levels were corrected following hydrodynamic gene transfer of VWF, indicating that VWF contributes to the regulation of Gal-3 plasma levels. In the circulation Gal-3 is bound to VWF [14,40] and in the absence of VWF, Gal-3 co-precipitated with FVIII [40]. In type 3 VWD patients FVIII levels are very low (FVIII:C 1 IU/dL, IQR 1-3 in our group), because the half-life of free FVIII is very short compared with VWF-bound FVIII. In the absence of VWF, levels of circulating Gal-3 could be lower because complex formation with VWF, which may promote the circulatory lifespan of Gal-3, cannot occur or complex formation with free FVIII in the absence of VWF, promotes faster clearance.

The decreased Ang-2 levels observed in VWD patients compared with controls is contradictory to previous published results obtained from cultured endothelial cells from VWD patients [12,41]. The difference could be explained by lack of a clearance mechanism in the culture experiments. Since Ang-2 levels were measured in supernatants of cultured cells, clearance of Ang-2, which could play a role *in vivo*, is not affected in those experiments. Furthermore, Ang-2 levels were only measured in VWF siRNA-treated human umbilical vein endothelial cells, and levels in BOECs from VWD patients were unfortunately not reported. Both Ang-1 and Ang-2 bind to the TIE-2 (Tyrosine kinase with Immunoglobulin-like and EGF-like domains-2) receptor on endothelial cells [17,42] and activation of TIE-2 by Ang-1 binding induces intracellular signalling pathways leading to down-regulation of Ang-2 production in endothelial cells [42]. This interaction however, does not occur in cultured endothelial cells because Ang-1 is absent in culture experiments.

The pathological mechanism underlying angiodysplasia in VWD is still largely unexplained. We observed higher Ang-2 levels in patients who had bleedings compared with patients who did not experience bleedings, but due to the small group of patients (n=9) with gastrointestinal bleedings we could not detect further significant differences. Higher Ang-2 levels, combined with lower Ang-1 levels could contribute to the formation of fragile vessels seen in angiodysplasia, but more patients with a recent history of gastrointestinal bleeding should be analyzed to validate this hypothesis.

With regard to the WPB components Gal-3, Ang-2 and OPG, 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, information is scarce on this issue, and it would be interesting to examine 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, such as galectin-1, connective tissue growth factor and insulin-like growth factor binding protein-7, it is very likely that VWF plays an exceedingly complex role in regulating angiogenesis at different levels. Characterisation of the molecular pathways through which VWF regulates angiogenesis can provide novel therapeutic targets for the treatment of angiodysplastic gastrointestinal bleeding, since prophylaxis is less successful at reducing gastrointestinal blood loss than it is for reducing blood loss at different sites in the body [43].

In conclusion, VWD patients have several abnormalities in the levels of angiogenic mediators in plasma compared to controls. Impaired formation or loss of WPB in VWD patients leads to both increased and decreased plasma levels of angiogenic mediators compared with healthy individuals. The dysregulation between these angiogenic mediators could contribute to the vascular malformation frequently observed in these patients.

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CHAPTER

8

Angiogenic characteristics of blood outgrowth endothelial cells from patients with von Willebrand disease

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Abstract

Endothelial von Willebrand factor (VWF) has been shown to negatively regulate angiogenesis. Blood Outgrowth Endothelial Cells (BOECs) isolated from von Willebrand disease (VWD) patients showed enhanced *in vitro* angiogenesis compared with healthy control BOECs. Characterisation of the angiogenic response of BOECs from VWD patients is limited and differences between the different types of VWD have not been investigated in detail. The aim of this study was to further explore the potential pathogenic role of VWF mutations in regulating angiogenesis. We isolated BOECs from 4 healthy individuals, 10 patients with VWD and 1 heterozygous carrier of a type 2N mutation and measured cell migration and tube formation. Migration velocity and total tube formation was similar between VWD patients and controls in general. BOECs from the type 3 VWD patient and one type 2B patient showed increased migratory velocity and tube formation. Directionality was impaired in 8 out of 10 VWD BOECs and the ability to form tubes was limited to early passage numbers, but not for BOECs from healthy controls. In conclusion, BOECs from VWD patients did not have enhanced *in vitro* angiogenesis compared with healthy controls, but individual patients showed both increased and decreased angiogenic potential. Almost all VWD patients showed impaired directional migration. BOECs can be a useful tool for *ex vivo* assessment of endothelial cell function in patients with different types of VWD. But limitations, such as early loss of angiogenic capacity, should be taken into account when studying BOECs from VWD patients.

Introduction

Von Willebrand factor (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 [1]. A decreased concentration or abnormal function of VWF is responsible for von Willebrand disease (VWD), the most common inherited bleeding disorder [2]. A reduced concentration of structurally normal VWF is classified as type 1 VWD. Qualitatively abnormal variants of VWF are classified as type 2 VWD (with subgroups 2A, 2B, 2M and 2N), while a complete deficiency of the VWF protein is classified as type 3 VWD [3]. Von Willebrand factor (VWF) is mainly produced in endothelial cells, while a small contribution comes from synthesis in megakaryocytes [4,5]. Synthesized VWF is either constitutively secreted into the blood, or stored in cell-specific organelles. VWF is stored in the alpha-granules in platelets, while in endothelial cells it is stored in the Weibel-Palade bodies (WPB) [6].

Until recently, there was limited access to endothelial cells (EC) from VWD patients and this has been a barrier to better understand the cellular pathophysiology of VWD. Studies on cellular effects of VWF mutants have been carried out mainly in non-EC lines such as HEK293 or COS cells. However, those cell lines have their limitations

as they do not entirely mimic the endothelial cell milieu. While VWF storage and secretion can be assessed with VWF transfections in cell types such as HEK293 [7-9], these cells do not have the typical endothelial characteristics, such as expression of endothelial cell markers and the capacity of forming capillary-like structures in matrigel. Blood Outgrowth Endothelial Cells (BOECs) provide a useful system to study the storage and secretion of VWF in patients with VWD. BOECs can be derived from human peripheral blood mononuclear cells [10]. In culture, these BOECs have the typical endothelial cobblestone morphology, express endothelial cell markers such as CD34, VWF, vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion molecule (PECAM)[11], and are capable of forming capillary-like structures on Matrigel [12]. BOECs from several type 1 and 2 VWD patients have been successfully isolated and used to study the storage and secretion of VWF in VWD disease [13,14].

In recent years it has become clear that VWF has a versatile function beyond hemostasis. Endothelial VWF has been implicated in pathophysiological processes, such as tumor metastasis, cell proliferation and inflammatory processes [15]. Starke *et al* [16], demonstrated that endothelial VWF is also a negative regulator of angiogenesis. VWF knockdown in HUVECs led to increased *in vitro* angiogenesis. BOECs isolated from type 1 and 2 VWD patients showed increased migration and tube formation compared with BOECs isolated from healthy individuals [16]. Whether this also holds for type 3 VWD BOECs is unknown, since successful isolation has not been reported yet. Characterisation of the angiogenic characteristics of BOECs from VWD patients is still very limited and differences between the different types of VWD have not been investigated in detail. The aim of this study was to further explore the potential pathogenic role of VWF mutations in regulating angiogenesis. To this end, we isolated BOECs from type 1, 2 and 3 VWD patients and characterized their angiogenic capacity.

Methods

Patients and Controls

BOECs were successfully isolated from the following individuals: 1) Four type 1 VWD patients heterozygous for the mutations p.Arg1205His, p.Arg1205His/p.Arg924Gln, p.Tyr1584Cys, p.Cys2693Tyr. 2) Four type 2B VWD patients heterozygous for the mutations p.Arg1308Cys (two patients), p.Val1316Met and p.Pro1337Leu. 3) One VWD patient with an unidentified mutation leading to a type 2A phenotype. 4) An asymptomatic heterozygous carrier of the type 2N mutation p.Arg854Gln. 5) One type 3 VWD patient compound heterozygous for the p.Arg2535* mutation and a second unknown mutation. 6) Four healthy individuals. Plasma VWF:Ag levels were measured as previously described [17]. Angiopoietin-2 plasma levels were measured using the Human Angiopoietin-2 DuoSet ELISA kit (DY623, R&D Systems Europe, Abingdon, Oxon, UK). The study protocol was approved by the Leiden University Medical Center ethics review board. Written informed consent was obtained from all participating subjects before blood collection in accordance with the Declaration of Helsinki.

BOECs isolation, characterisation and culture

BOECs were isolated and cultured as previously described [14]. Briefly, 80ml of peripheral blood from each participant was drawn in 10ml citrated tubes (S-Monovette, Sarstedt, Nümbrecht, Germany). Citrated blood was diluted 1:1 with phosphate buffered saline (PBS) and buffy coat mononuclear cells were isolated by gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare, Diegem, Belgium) at 800 x *g* for 30 minutes, washed twice with PBS. Cell pellets were resuspended in EGM-2 medium (Lonza, Breda, The Netherlands) supplemented with 20% fetal calf serum (Gibco, Life Technologies, Grand Island, NY, USA) and a cocktail of growth factors (EGM-2 bullet kit, Lonza, Breda, The Netherlands). The cells were seeded onto 6-well tissue culture plates pre-coated with rat tail collagen type 1 (BD, Biosciences, Bedford, Massachusetts, USA). Culture medium was refreshed daily until the first colony appeared. Medium was then refreshed every 2 days. Cells were used for experiments at passages 2 to 8 unless stated otherwise.

FACS analysis

BOECs were washed once with PBS containing 1% bovine serum albumin and 0.01% sodium azide and were incubated with primary antibody or isotype control for 30 minutes at room temperature in the dark. Primary mouse monoclonal antibodies (all purchased from BD Biosciences) against human CD14-APC, human CD45-PE/cy5, human CD31 (PECAM), human KDR-PE and human CD144 (VE-Cadherin)-APC were used. A secondary antibody conjugated to Alexa Fluor 488 was used to label CD31. In separate tubes, cells were incubated with isotype controls. Samples were fixed in 1% paraformaldehyde and analyzed by FACS (LSRII, BD Biosciences). Data were analyzed using FACSDiVa software (BD Biosciences).

Confocal immunofluorescence microscopy

BOECs were grown on collagen coated coverslips. Confluent cells were fixed, permeabilized and stained essentially as previously described [8]. Polyclonal sheep antihuman antibody VWF-FITC (ab8822, Abcam, Cambridge, UK), monoclonal antibody CD62P (Clone AC1.2, BD Biosciences), polyclonal rabbit antihuman β -catenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal mouse antihuman NOS type III antibody (BD Biosciences), and polyclonal rabbit antihuman protein disulfide isomerase (PDI) antibody A66 (obtained from prof. I. Braakman, Department of Chemistry, Utrecht University, The Netherlands) were used to visualize VWF, P-selectin, β -catenin, eNOS and PDI respectively. Alexa 488 and Alexa 594-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Samples were analysed by Leica SL confocal laser scanning microscopy with a 40x and 63x oil objective (Leica Microsystems).

In vitro Matrigel Assay

Matrigel assays were performed according to the manufacturer's instructions. Growth factor reduced Matrigel (BD Biosciences) was thawed overnight on ice. For-

ty-eight-well tissue culture plates were coated with 150 μ l/well matrigel for 30 min at 37°C. BOECs from both VWD patients and from healthy donors were cultured for 4 hours in EGM-2 medium. Cells were subsequently seeded onto matrigel (BD Biosciences) coated wells at 50,000 cells per well in a 48-well plate in EGM-2 medium. Twenty-four hours after seeding images were taken by Leica DM IL LED inverted microscopy equipped with the Leica DFC295 digital camera (Leica Microsystems). Characterisation of the capillary-like structures was analyzed using the online software program WimTube (Wimasis Image Analysis, Ibidi GmbH, München, Germany) [18]. BOECs at different stages of cell passage were used to investigate the behaviour of the cells during prolonged culture. Three independent experiments in duplicate were performed for each BOEC.

Wound-healing Assay

BOECs were grown in complete EGM-2 medium supplemented with 20% fetal calf serum until a confluent monolayer was formed in a 24-well plate coated with collagen, washed twice with PBS and further migration experiments were performed in EGM-2 medium. Actinomycin D (2 ng/ml) (Sigma, St Louis, MO, USA) was added to the cells to inhibit proliferation in order to exclude bias related to proliferative effects. A thin 'wound' was introduced by scratching the bottom of the well with a pipette tip. Culture plate was placed in a Leica DMI6000 inverted microscope equipped with an environmental chamber for the control of temperature, CO₂ concentration, and humidity. Images were taken every hour using a Leica DF350FX CCD camera, until 24 hours after scratching. Images were analyzed using Fiji (ImageJ, National Institutes of Health, Bethesda, Maryland, USA). The cell covered area (μ m²) was measured for every image and subsequently the increase of cell-covered area per time interval was calculated. For the cell front migration velocity the total area was multiplied by the increase of cell-covered area, and this number was divided by the time interval. The result is the normalized cell front migration velocity in μ m/h. This ratio was converted to μ m/min. Three independent experiments in duplicate were performed for each BOEC. For individual cell migration, images were analysed using the Fiji manual tracking plug-in (ImageJ). Chemotaxis and Migration tool (Ibidi) was used to plot trajectories and calculate individual cell migration velocities. According to tracking instructions, around 25-30 individual cells in one well were tracked to calculate the individual cell migration velocity and average directionality. The average directionality is calculated by dividing the mean Euclidean distance (the shortest linear distance travelled from starting to end point of a tracked cell) of all tracked cells by the mean accumulated distance of all tracked cells. A value of 1 equals a straight-line migration from start to endpoint.

Statistics

Variables are expressed as means (with standard deviations, SD) or medians [with interquartile range, IQR]. Continuous data were tested for normality and analyzed by t-test, Mann-Whitney U test or ANOVA with a Dunnett post hoc test as appropriate.

P values of 0.05 or less were considered statistically significant. Statistical analysis was performed using GraphPad Prism, version 6.00 for Windows (GraphPad Software Inc, La Jolla, CA, USA).

Results

Isolation of BOECs from different VWD patients

We isolated circulating progenitor cells from patients with VWD and healthy controls and cultured them to expand BOECs. BOECs were successfully isolated from 10 VWD patients (4 with type 1, 5 with type 2 and 1 with type 3), 1 carrier of type 2N VWD and 4 healthy controls (Table 1). All patients had reduced plasma VWF:Ag levels compared with healthy controls. The type 3 VWD patient had VWF:Ag levels below the detection limit of the assay. Plasma levels of angiopoietin-2, an angiogenic protein that is stored within Weibel-Palade bodies together with VWF, were significantly lower in the VWD patients (mean $\pm$ SD, 0.85 ± 0.53 ng/ml) compared with healthy donors (2.1 ± 1.4 ng/ml, $p=0.02$) (Table 1).

Characterisation of type 3 VWD BOEC

We successfully isolated BOECs from a type 3 VWD patient. The BOECs were derived from a type 3 VWD patient compound heterozygous for a nonsense mutation p.Arg2535* and an unknown mutation on the other VWF allele. Plasma VWF:Ag levels were below the detection limit of 5 U/dl, no VWF multimers could be visualized on multimer gel, and the inheritance pattern in the family was autosomal recessive confirming the type 3 VWD diagnosis. The BOECs colony showed the typical endothelial

Table 1. Characteristics of investigated subjects

Control /Patient nr	Amino acid change	Nucleotide change	VWD Type*	VWF:Ag U/dL	Ang-2 ng/ml
1**	n/a	n/a	n/a	80	1.188
2**	n/a	n/a	n/a	110	1.277
3**	n/a	n/a	n/a	91	4.085
4**	n/a	n/a	n/a	120	1.821
1	Unknown	Unknown	2A	62	0.840
2**	p.Arg854Gln	c.2561G>A	2N	73	1.053
3	p.Arg1205His	c.3864G>A	1	15	0.832
4	p.Arg1205His/p.Arg924Gln	c.3864G>A and c.2271G>A	1	11	0.412
5	p.Arg1308Cys	c.3922C>T	2B	21	0.588
6	p.Arg1308Cys	c.3922C>T	2B	26	0.485
7	p.Val1316Met	c.3946G>A	2B	36	0.525
8	p.Pro1337Leu	c.4010C>T	2B	47	2.017
9**	p.Tyr1584Cys	c.4751A>G	1	48	0.417
10***	p.Arg2535 stop codon/unknown	c. 7603C>T	3	<5	1.631
11**	p.Cys2693Tyr	c.8078G>A	1	46	0.525

n/a, not applicable; VWD, von Willebrand disease; VWF:Ag, von Willebrand factor antigen; U/dL, units per decilitre; Ang-2, angiopoietin-2. * VWD type according to the 2006 classification [3]. **BOECs from these controls, patients and heterozygous type 2N carrier have been described in detail by Wang et al.[14] ***BOEC from a compound heterozygous type 3 patient for whom the mutation on the second allele is unknown.

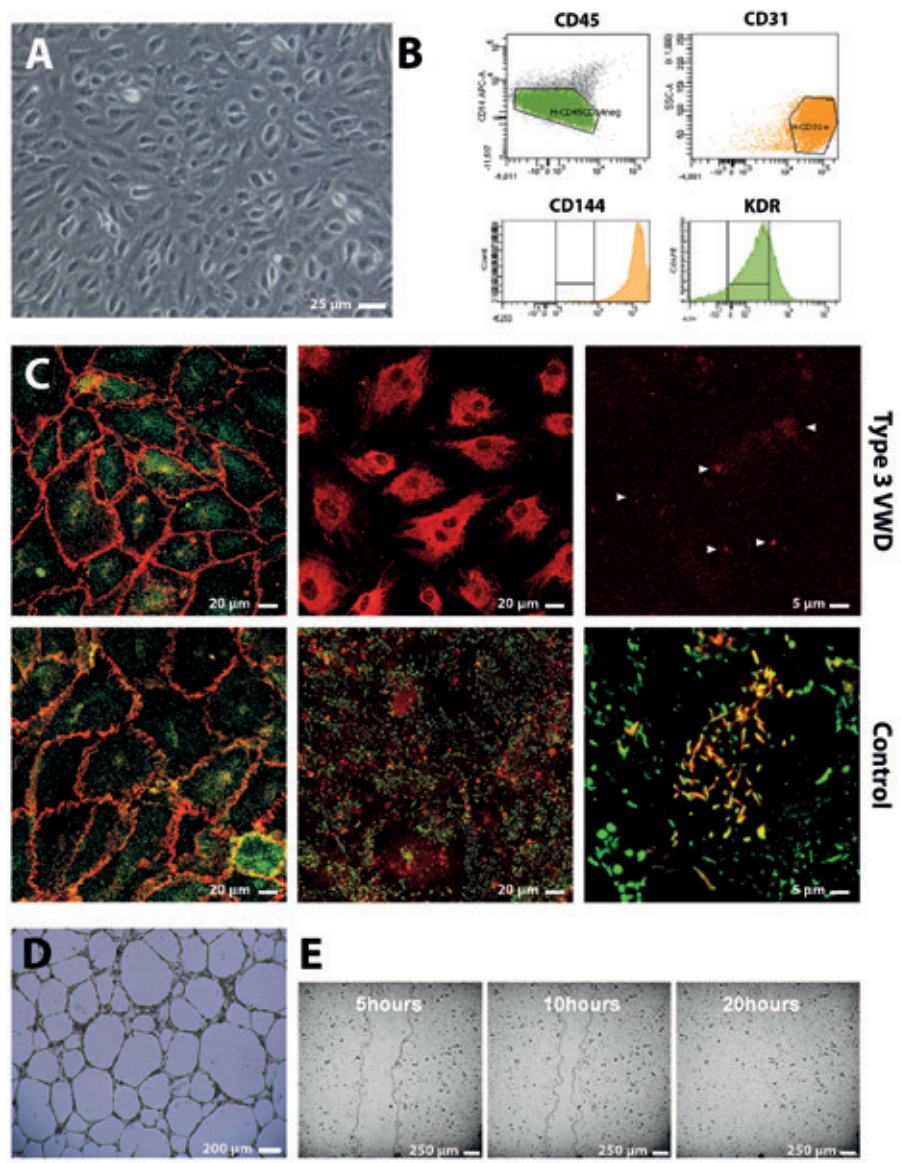


Figure 1. Phenotypic and functional analysis of a type 3 VWD BOEC. (A) Cobblestone-like morphology under bright field microscopy of BOECs isolated from a type 3 VWD patient compound heterozygous for the p.Arg2535* mutation and a second unknown mutation. (B) FACS analysis demonstrate that BOECs were negative for monocyte markers CD14 and CD45, but positive for endothelial cell markers CD31 (PECAM), KDR (VEGFR) and CD144 (VE-Cadherin). Isotype controls are shown as grey lines. (C) Fluorescence microscopy of type 3 VWD BOECs (upper images) and BOECs from a healthy control (lower images). Left panel: co-staining for eNos (endothelial nitric oxide synthase, green) and β -Catenin (red). Middle panels: co-staining for VWF (green) and PDI (red). Right panels: co-staining for VWF (green) and P-selectin (red). White arrows indicate diffuse P-selectin staining. Images were taken by Leica SL confocal laser scanning microscopy with a 40x or 63x/1.40 NA oil objective. (D) BOECs formed capillary-like structures in Matrigel. (E) BOECs were able to migrate and closed the open area in a wound healing assay within 20 hours.

cobblestone morphology (Figure 1A). Further characterisation by FACS analysis confirmed that these BOECs are endothelial cells as they expressed the classic endothelial cell markers VE-cadherin (CD144), KDR (VEGFR2) and PECAM (CD31)(Figure 1B). On the other hand, these cultured cells were negative for the monocyte markers CD45 and CD14 (Figure 1B). Confocal microscopy revealed that these cells also express β -catenin and to some extent endothelial nitric oxide synthase (eNOS)(Figure 1C, left image, top). In BOECs from healthy individuals we could demonstrate the elongated WPB with co-localization of VWF with P-selectin (Figure 1C, right image, bottom). VWF was not detectable in BOECs from this type 3 VWD patient, and staining for P-selectin was faint and rather diffuse with the presence of some small granules, which are most likely lysosomes. Therefore no apparent WPB were detected in the endothelial cells isolated from this patient (Figure 1C, middle and right images, top). BOECs from the type 3 VWD patient were functional endothelial cells as they were capable of forming capillary-like structures in Matrigel coated wells (Figure 1D), as well as migrate and close a wound in a wound-healing assay (Figure 1E).

Migration behaviour of BOECs from VWD patients

The median cell front migration velocity did not differ between BOECs of controls (median [IQR], 0.46 [0.39-0.56] $\mu\text{m}/\text{min}$) and BOECs of VWD patients overall (0.37 [0.27-0.48] $\mu\text{m}/\text{min}$)(Table 2 and Figure 2A). However, BOECs from patient with the p.Pro1337Leu mutation and the type 3 VWD patient had faster cell front migration

BOEC	Cell front migration velocity ($\mu\text{m}/\text{min}$)	Individual cell migration velocity ($\mu\text{m}/\text{min}$) [†]	Accumulated distance (μm)	Euclidean distance ^{††} (μm)	Directionality ^{†††}
Control 1	0.38 $\pm$ 0.10	0.46 $\pm$ 0.10	527 $\pm$ 109	398 $\pm$ 102	0.75
Control 2	0.51 $\pm$ 0.06	0.43 $\pm$ 0.07	431 $\pm$ 109	288 $\pm$ 122	0.66
Control 3	0.41 $\pm$ 0.08	0.46 $\pm$ 0.08	677 $\pm$ 161	496 $\pm$ 139	0.73
Control 4	0.58 $\pm$ 0.0009	0.51 $\pm$ 0.12	448 $\pm$ 110	323 $\pm$ 120	0.71
2A-1 (Unknown)	0.25 $\pm$ 0.03 ^c	0.40 $\pm$ 0.14	620 $\pm$ 228	374 $\pm$ 276	0.56
Carrier of p.Arg854Gln	0.41 $\pm$ 0.013	0.47 $\pm$ 0.08	802 $\pm$ 198 ^a	350 $\pm$ 155	0.45
p.Arg1205His	0.32 $\pm$ 0.08	0.40 $\pm$ 0.09	668 $\pm$ 134 ^c	351 $\pm$ 129	0.51
p.Arg1205His/p.Arg924Gln	0.35 $\pm$ 0.06	0.45 $\pm$ 0.11	559 $\pm$ 106	320 $\pm$ 108	0.58
p.Arg1308Cys-1	0.41 $\pm$ 0.002	0.45 $\pm$ 0.13	647 $\pm$ 185	291 $\pm$ 198	0.43
p.Arg1308Cys-2	0.37 $\pm$ 0.06	0.42 $\pm$ 0.11	567 $\pm$ 150	267 $\pm$ 126	0.47
p.Val1316Met	0.27 $\pm$ 0.002 ^c	0.46 $\pm$ 0.10	553 $\pm$ 168	343 $\pm$ 165	0.61
p.Pro1337Leu	1.29 $\pm$ 0.58 ^c	0.60 $\pm$ 0.13 ^a	352 $\pm$ 61 ^c	314 $\pm$ 65	0.81
p.Tyr1584Cys	0.48 $\pm$ 0.12	0.43 $\pm$ 0.11	710 $\pm$ 245 ^b	386 $\pm$ 211	0.55
p.Arg2535*/unknown1	0.74 $\pm$ 0.019 ^b	0.58 $\pm$ 0.11 ^a	568 $\pm$ 159	399 $\pm$ 147	0.70
p.Cys2693Tyr	0.23 $\pm$ 0.04 ^b	0.46 $\pm$ 0.17	780 $\pm$ 335 ^a	411 $\pm$ 176	0.55

All data indicate mean $\pm$ SD. [†] Around 25-30 individual cells were tracked for each BOEC. ^{††} The euclidean distance is the direct (linear) distance between starting point and end point of a cell track. ^{†††} The directionality is calculated by dividing the mean euclidean distance of the tracked cells by the mean accumulated distance. A value of 1 equals a straight-line migration from start to endpoint. ¹ BOEC from a compound heterozygous type 3 patient for whom the mutation on the second allele is unknown. ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$ compared with controls.

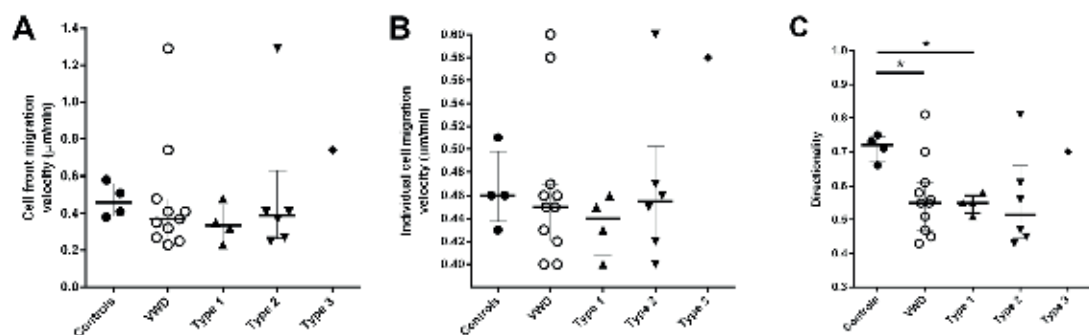


Figure 2. Migration behavior of BOECs from healthy individuals and VWD patients. BOECs were grown until they formed a confluent monolayer. A wound was introduced by scratching the bottom of the well with a pipette point. Images of migrating cells were taken every hour using a live-cell imaging microscope set-up for up to 24 hours after scratching. (A) Collective migration of the front cell layer for BOECs from healthy individuals and VWD patients. Around 25-30 individual cells were tracked using the manual tracking plug-in of the ImageJ software Fiji to measure individual cell migration velocity (panel B) and directionality (panel C). Directionality is calculated by dividing the Euclidean distance by the accumulated distance. Black bars indicate the medians with IQR. * $p < 0.05$.

velocity than healthy control BOECs (Table 2). On the other hand, BOECs from some patients (2A-1, p.Val1316Met, and p.Cys2693Tyr) had significantly lower cell front migration velocity compared with BOECs from healthy individuals (Table 2). We did not observe a difference in the cell front migration velocity between type 1 (0.34 [0.25-0.45] $\mu\text{m}/\text{min}$) and type 2 (0.39 [0.27-0.63] $\mu\text{m}/\text{min}$) VWD patients. Compared to the median cell front migration velocity of type 1 and 2 VWD patients, the BOECs from the type 3 VWD patient migrated faster (Figure 2A). The addition of actinomycin D, which inhibits proliferation without inducing cell death, in the wound-healing assay rules out that proliferative effects are responsible for the observed differences. Live-cell imaging of the cells showed that cells did not proliferate during the wound-healing assay.

Since cells can also migrate in other directions than the (open) wounded area, we also measured the velocity rate of individual cells by tracking cells during migration. Individual cell migration velocity did not differ between controls (0.46 [0.44-0.50] $\mu\text{m}/\text{min}$) and patients overall (0.45 [0.42-0.47] $\mu\text{m}/\text{min}$) (Table 2 and Figure 2B). However in accordance with the cell front migration velocity, individual cells from the patient with the p.Pro1337Leu mutation (mean $\pm$ SD, 0.60 ± 0.13 $\mu\text{m}/\text{min}$) and the type 3 VWD patient (0.58 ± 0.11 $\mu\text{m}/\text{min}$) also migrated significantly faster compared with individual cells from healthy individuals (0.47 ± 0.03 $\mu\text{m}/\text{min}$, $p < 0.001$). While BOECs from patients with the 2A, the p.Val1316Met and the p.Cys2693Tyr mutation had significantly slower cell front migration velocity, individual cells migrated with comparable rates as control BOECs (Table 2 and Figure 2B). The impaired migration velocity of the front layer was due to a loss of directionality towards the wounded area (Table 2). The directionality of BOECs from VWD patients overall (0.55 [0.47-0.61]) was impaired

compared with healthy controls (0.72 [0.67-0.75], $p=0.03$). Type 1 VWD patients (0.55 [0.52-0.57]) also had impaired directionality compared with controls (0.72 [0.67-0.75], $p=0.03$). Although the median directionality of BOECs from type 2 patients was even lower this did not reach statistical significance (0.52 [0.45-0.66], $p=0.11$) (Table 2 and Figure 2C). Individually, BOECs from VWD patients showed impaired directional migration towards the open wound area in most cases. Only BOECs from the patient with the p.Pro1337Leu mutation and type 3 VWD patient showed comparable directional migration compared with healthy controls (Table 2 and Figure 3).

Capacity to form capillary-like structures of BOECs from VWD patients

We investigated the angiogenic properties of BOECs from VWD patients in an *in vitro* tube formation assay on growth-factor reduced Matrigel. This assay measures the ability of endothelial cells to form structures which involves endothelial cell adhesion, migration and eventually tubule formation. Total tube length was similar between BOECs isolated from VWD patients overall (3.1 [2.3-3.6] cm) and BOECs isolated from healthy individuals (3.3 [3.0-3.3] cm) (Table 3 and Figure 4A). However BOECs from the patients with the p.Pro1337Leu and p.Tyr1584Cys mutations and type 3 VWD patient showed significantly increased total tube length compared with controls, while BOECs from patients with the p.Arg1205His, p.Arg1205His/p.Arg924Gln, p.Arg1308Cys-2, p.Cys2693Tyr mutation showed significantly decreased total tube length compared with BOECs from healthy donors (Table 3). This decrease in total tube length was accompanied by a decrease in branching points and loops and these BOECs also formed less tubes compared to control BOECs. On the other hand, BOECs that formed more tubes compared with control BOECs, also had a smaller average tube length than control BOECs (Table 3). The number of branching points, total tubes and the average length of a tube did not differ between BOECs from VWD patients overall and BOECs from healthy individuals (Figure 4B-D and Table 3). Although we could not detect statistically significant differences, type 1 VWD patients had lower total tube length, less branching points and formed less tubes than type 2 VWD patients and the type 3 VWD patient, while their average tube length was slightly higher. (Table 3, Figure 4A-C).

BOECs from VWD patients lose their angiogenic capacity earlier

BOECs from both healthy individuals and VWD patients gradually lost their capacity to form capillary-like tubules on Matrigel (Figure 5). However, while BOECs from healthy individuals were still capable of forming tubes at passage number 10 and began gradually losing their capacity around passage 15, BOECs from VWD patients lost their capacity to form capillary-like tubules much earlier on. The ability to form capillary-like tubules began to decrease at around passage 7 and 8 for BOECs isolated from VWD patients (Figure 5). Interestingly, BOECs isolated from the type 3 VWD patient with the p.Arg2535* mutation quickly lost their ability to form tubules on Matrigel (Figure 5, lower panel). While the capacity to form capillary-like tubules was declining for VWD BOECs, their migration velocity remained similar for up to passage number 8 (*data not shown*).

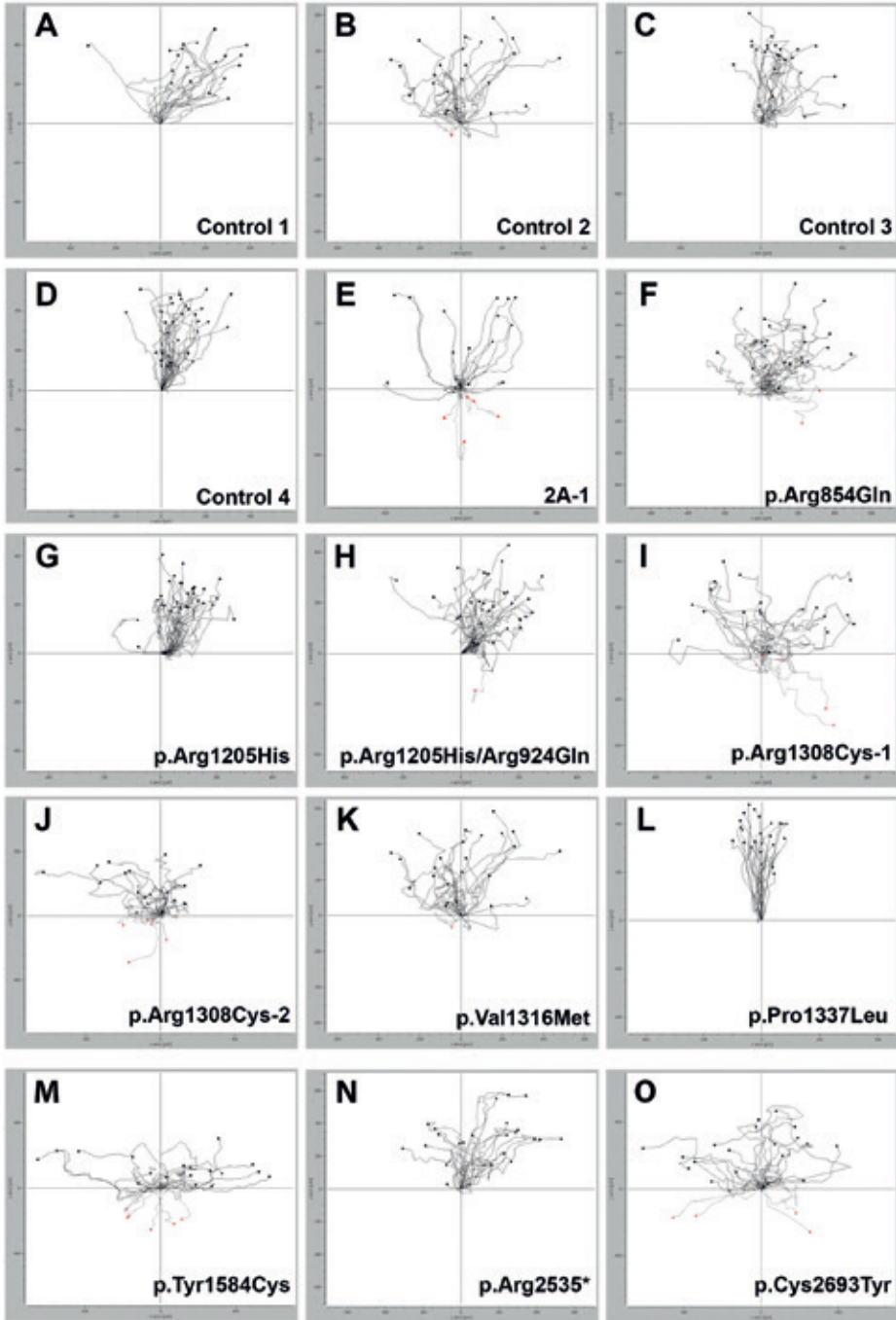


Figure 3. Trajectory plots of individual cell migration of BOECs. Around 25-30 individual cells were tracked using the manual tracking plug-in and recorded data was analyzed using the chemotaxis and migration tool from Ibidi. Trajectory plots of BOECs show individual cells migrating into a wounded area. The starting point of each cell trajectory is plotted at the center of the graph and the wounded area is upwards of the horizontal line which goes through zero on the y-axis. Upwards movement of cells indicates direction towards the wound, downwards movement of cells indicates direction away from the wound. Black dots indicate cells that are moving upwards to the wounded area, red dots indicate cells that are moving downwards away from the wounded area.

Table 3. Characterisation of capillary-like tubules of BOECs from controls and VWD patients								
BOEC	Covered Area (%) [†]	Total Tube length (cm)	Total branching Points (n)	Total Loops (n)	Total Tubes (n)	Mean Tube length (μm)	Mean Loop area (cm)	Mean Loop Perimeter (μm)
Control 1	21 ± 4	2.91 ± 0.34	89 ± 19	27 ± 9	170 ± 36	174 ± 23	8.46 ± 2.99	1427 ± 413
Control 2	26 ± 8	3.31 ± 0.23	113 ± 15	41 ± 12	208 ± 40	163 ± 23	4.86 ± 0.74	986 ± 142
Control 3	29 ± 5	3.26 ± 0.39	106 ± 23	41 ± 13	185 ± 38	180 ± 18	5.78 ± 1.50	1068 ± 164
Control 4	22 ± 4	3.31 ± 0.20	111 ± 15	36 ± 9	217 ± 23	153 ± 7	5.76 ± 0.80	1240 ± 136
2A-1 (Unknown)	29 ± 3	3.43 ± 0.41	126 ± 25	38 ± 10	245 ± 52 ^d	142 ± 15 ^d	5.93 ± 1.27	1204 ± 140
Carrier of p.Arg854Gln	33 ± 1 ^d	3.38 ± 0.20	120 ± 13	42 ± 5	216 ± 25	158 ± 16	4.59 ± 0.16	954 ± 54
p.Arg1205His	12 ± 3 ^b	1.40 ± 0.36 ^a	38 ± 13 ^a	10 ± 7 ^b	73 ± 17 ^a	193 ± 19 ^d	6.42 ± 4.62	1028 ± 226
p.Arg1205His/ p.Arg924Gln	12 ± 0 ^c	2.49 ± 0.01 ^c	73 ± 0 ^d	19 ± 1 ^d	153 ± 4	163 ± 5	8.88 ± 1.68	1545 ± 183
p.Arg1308Cys-1	23 ± 2	3.12 ± 0.20	105 ± 9	27 ± 8	203 ± 15	154 ± 5	5.69 ± 1.46	1219 ± 254
p.Arg1308Cys-2	13 ± 2 ^c	2.31 ± 0.20 ^a	63 ± 6 ^c	9 ± 2 ^b	150 ± 9	153 ± 4	6.28 ± 2.57	1199 ± 444
p.Val1316Met	30 ± 0	3.03 ± 0.04	53 ± 11	15 ± 1	100 ± 21	181 ± 6	4.77 ± 0.76	871 ± 65
p.Pro1337Leu	30 ± 6	4.61 ± 0.46 ^a	218 ± 35 ^a	82 ± 23 ^a	399 ± 76 ^a	117 ± 13 ^a	2.63 ± 0.51 ^d	797 ± 73 ^d
p.Tyr1584Cys	29 ± 4	4.05 ± 0.09 ^a	157 ± 2 ^a	48 ± 3	327 ± 17 ^a	128 ± 8 ^b	4.89 ± 0.50	1223 ± 105
p.Arg2535*/ unknown ¹	28 ± 5	3.63 ± 0.11 ^d	134 ± 7 ^d	53 ± 2 ^d	232 ± 12	157 ± 5	4.10 ± 0.32	908 ± 75
p.Cys2693Tyr	16 ± 2 ^c	2.20 ± 0.26 ^a	60 ± 9 ^b	16 ± 2 ^c	120 ± 17 ^c	185 ± 11	10.65 ± 3.08 ^c	1528 ± 344 ^d

[†] All data indicate mean ± SD. ¹ BOEC from a compound heterozygous type 3 patient for whom the mutation on the second allele is unknown. ^a p<0.0001, ^b p<0.001, ^c p<0.01, ^d p<0.05 compared with controls.

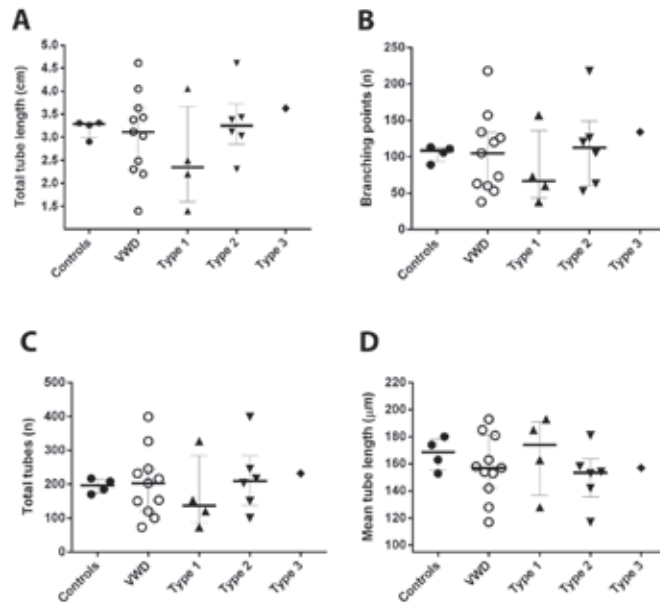


Figure 4. Characterization of capillary network formation of BOECs from controls and VWD patients. BOECs from healthy donors and VWD patients were seeded onto Matrigel coated wells and images were taken after 24 hours. Total length of all tubes (panel A), the number of branching points (Panel B), the number of tubes formed (Panel C) and the average length of the tubes (Panel D) was characterized with the WimTube online Software from Ibidi. Individual patient and control BOECs are plotted. Data represent the mean value of three independent experiments done in duplicate. The black bars indicate the medians with IQR.

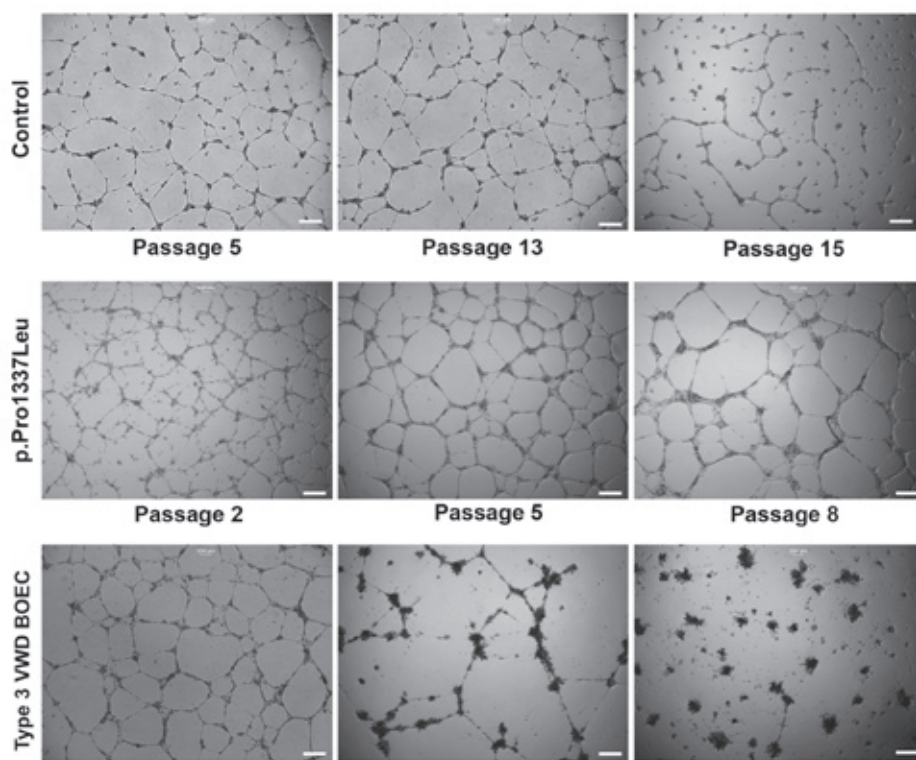


Figure 5. Capillary formation of BOECs from control and VWD patients at different passage numbers. BOECs from healthy control and VWD patients were seeded on Matrigel coated wells and images were taken after 24hours. Upper pictures are representative results of tube formation in BOECs from healthy control at passage number 5, 13 and 15. Middle pictures are capillary formation of BOECs from a patient with the p.Pro-1337Leu mutation at passage numbers 2, 5 and 8. Lower pictures are from BOECs derived from the type 3 VWD patient compound heterozygous for the p.Arg2535* mutation at passage numbers 2, 4 and 5. White scale bar indicates 200 μ m.

Discussion

Characterisation of the angiogenic capacity of BOECs from VWD patients is still limited and potential differences in angiogenic capacity between the different types of VWD have not been investigated in detail. In this study we aimed to further explore the potential pathogenic role of VWF mutations in regulating angiogenesis by studying BOECs. We isolated BOECs from healthy individuals, a carrier and patients with type 1, 2 and 3 VWD and characterized their angiogenic capacity by cell migration and Matrigel assays. Taken all patients together, we did not observe a difference in *in vitro* angiogenesis between BOECs from VWD patients and BOECs from healthy donors with regards to migration velocities or tube formation. Several BOECs showed decreased angiogenic potential (less tube formation and slower migration velocity) compared with control BOECs. However, BOECs isolated from the patient with the

Table 4. Summary of angiogenic characteristics of BOECs harbouring VWF mutations.

BOEC	Cell front velocity [†]	Individual cell velocity	Directionality	Tube formation
2A-1 (Unknown)	↓↓	=	↓	=
Carrier of p.Arg854Gln	=	=	↓	=
p.Arg1205His	=	=	↓	↓↓
p.Arg1205His/p.Arg924Gln	=	=	↓	↓
p.Arg1308Cys-1	=	=	↓	=
p.Arg1308Cys-2	=	=	↓	↓
p.Val1316Met	↓↓	=	↓	=
p.Pro1337Leu	↑↑	↑	=	↑↑
p.Tyr1584Cys	=	=	↓	↑
p.Arg2535* / unknown [‡]	↑	↑	=	↑
p.Cys2693Tyr	↓↓	=	↓	↑

[†] Based on Tables 2 and 3, = is comparable, ↓ is mild decrease, ↓↓ is severely decrease, ↑ is mild increase and ↑↑ is substantially increase compared with healthy control BOECs. [‡] BOEC from a compound heterozygous type 3 patient for whom the mutation on the second allele is unknown.

p.Pro1337Leu mutation and the type 3 VWD patient had significantly increased migration velocity and increased tube formation compared with control BOECs (for a summary see Table 4). Although not statistically significant, type 2 VWD BOECs and the type3 VWD BOEC had higher angiogenic capacity than BOECs isolated from type 1 VWD patients. The directionality of BOECs from VWD patients overall was significantly impaired compared with healthy controls. Only 2 VWD patients had normal directional migration. Interestingly, the potential to induce tube formation was declining in BOECs from VWD patients over subsequent cell passages, and especially the type 3 VWD BOEC clone lost its ability to form capillary-like structures *in vitro* at an early passage number.

Knockdown of VWF with siRNA in HUVECS led to a loss of directional migration in the study of Starke *et al.* [16] BOECs from our type 3 VWD patient, which did not express any detectable VWF, did not have impaired directional migration. However, we do observe loss of directionality in most of the other BOECs from our VWD patients compared with BOECs from healthy individuals. Only 2 out of 11 patients had normal directionality. Starke *et al.* [16] unfortunately did not report on the directionality of the BOECs they isolated from their VWD patients, so we cannot compare results between BOECs. In our wound-healing assay BOECs from VWD patients did not migrate faster than BOECs from healthy controls. This is in contrast to the reported data from Starke *et al.* [16], which showed increased migration compared with healthy controls. However, they also showed that BOECs from VWD patients had increased proliferation. We added actinomycin D to the medium in our wound-healing assay to inhibit proliferation of the BOECs, without inducing apoptosis of the cells. The increased proliferative and migratory effects seen by Starke *et al.*, suggests that the results observed in their study were most likely related to alterations of the proliferative rather than migratory phenotype. Furthermore, BOECs from their VWD patients showed increased total tube length compared to BOECs from healthy donors. We did not observe an increased

total tube formation in BOECs from VWD patients in general, although some patients had significantly increased tube formation. This discrepancy between their results and our data could reflect individual differences between the chosen healthy donors and/or the included VWD patients. The variation in their assays in both their control and VWD group is large, and while we do see a large variation between our VWD patients, the variation among our healthy controls is small.

While the VWF production in BOECs is stable for at least 10 passages in culture[14], the ability of BOECs to form tubular structures on Matrigel was declining earlier in patients with VWD. This reduced angiogenic response is most likely not the result of potentially progressive decrease in VWF production, since the reduced angiogenic response was already noticeable around passage 7-8. Furthermore, BOECs from the type 3 VWD patient were capable of forming numerous tubes on Matrigel at early passages when VWF was already absent. The reduced capacity of forming capillary-like structures might be attributable to decreased expression of other angiogenic proteins like VE-Cadherin, VEGF, Galectin-3 or $\alpha v\beta 3$ [19-22]. The tube formation assay covers several processes involved in angiogenesis such as adhesion, migration, protease activity and tube formation. The migratory ability of the BOECs did not decline as fast as the tube formation, and this suggests that the impaired tube formation is attributable to other processes beyond the migratory response.

Although we could not observe a significant difference between type 1 and 2 VWD patients, our data suggests that BOECs from type 1 VWD patients have lower angiogenic capacity than BOECs from type 2 and 3 VWD patients. The angiogenic capacity varied significantly between VWD patients, although variation within the same patient in independently performed assays was small. Variability may be overcome by isolating BOECs from a larger number of patients and controls. This might be a solution when investigating differences between the different VWD types in general. However, for investigating effects of specific VWF mutations this will not be feasible, as the number of patients carrying a specific mutation is generally limited.

We succeeded in isolating BOECs from a type 3 VWD patient, and BOECs obtained from this patient showed enhanced angiogenic capacity. Interestingly, we could not detect cellular presence of VWF, and P-selectin staining was faint and diffuse, indicating lack of WPB formation. This is in line with previous data obtained from BOECs isolated from type 3 VWD dogs [23]. Our study included only one type 3 VWD patient, and BOECs from more type 3 VWD patients should be isolated to substantiate the observed increased angiogenic capacity. Unfortunately, several attempts to establish BOECs from another type 3 VWD patient were unsuccessful. To date success in isolating BOECs from patients with type 3 VWD is limited, but published data shows that there is variation in the cellular VWF presence in BOECs isolated from the several type 3 VWD patients [24,25]. While BOECs from our patient showed a complete absence of VWF, presenting a true VWF “null” endothelial cell, others have showed diffuse

staining of VWF in type 3 VWD BOECs inside and outside the endoplasmic reticulum [24,25]. The presence of diffuse VWF in some type 3 VWD BOECs may influence the angiogenic capacity of those BOECs and it would be interesting to study angiogenesis in BOECs from type 3 VWD patients with different VWF genotypes and phenotypes.

In conclusion, we did not observe an increased angiogenic potential in BOECs from VWD patients compared with healthy controls, but individual patients showed both increased and decreased angiogenic potential. BOECs from VWD patients provide an easily accessible model to investigate the pathogenic role of VWF in regulating angiogenesis in VWD. However, the loss in angiogenic phenotype at later passages should be taken into account when performing angiogenesis assays with BOECs from VWD patients.

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Authorship Contributions

D.J. Groeneveld designed the study, performed the research and data analysis and wrote the manuscript; T. van Bekkum, R.J. Dirven and J.W. Wang performed part of the research and reviewed the manuscript; J. Voorberg discussed and interpreted the data and reviewed the manuscript; P.H. Reitsma discussed and analysed the data, and reviewed the manuscript; J. Eikenboom designed the study, interpreted the data, and contributed to writing of the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

The authors have no conflicts of interest to disclose.

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CHAPTER

9

Summary & Perspectives

Introduction

Almost 90 years ago, the first case of what is now known as von Willebrand disease (VWD), was published in a Finish 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 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 $\alpha_2\beta_1$ 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

via www.vwf.group.shef.ac.uk). 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 *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 **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 HMW 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. Therapeutic inhibitors of VWF-mediated platelet adhesion and activation may inhibit the progression of inflammatory diseases like atherosclerosis, hepatosteatosi and ischemic stroke. With regard to the non-hemostatic functions of VWF, many opportunities also remain to be explored. The combination of data obtained from both clinical and fundamental 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.

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CHAPTER

10

Nederlandse Samenvatting

Introductie

Bijna 90 jaar geleden werd de eerste casus van wat nu bekend staat als ziekte van von Willebrand (VWD) gepubliceerd in een Fins wetenschappelijk tijdschrift [1,2]. Prof. dr. Erik von Willebrand beschreef in dit tijdschrift zijn eerste casus van een erfelijke bloedingsziekte. Ongeveer 30 jaar later concludeerden Nilsson en collega's dat de verminderde stolselvorming ontstond door het ontbreken van een plasma factor; de von Willebrand factor (VWF) [3,4]. VWF is het best bekend voor wat betreft zijn drie klassieke hemostatische functies: (i) als dragereiwit voor stollingsfactor VIII, (ii) binding aan blootgesteld collageen bij vasculaire schade en (iii) als mediator bij het aantrekken van bloedplaatjes naar plekken van vasculaire schade [5]. De afgelopen jaren is duidelijk geworden dat VWF een veelzijdig eiwit is dat behalve bij hemostase, ook betrokken is bij verscheidene pathofysiologische processen, zoals tumormetastase, angiogenese, cel proliferatie en ontstekingsprocessen [6]. Dus, afgezien van de duidelijke rol in hemostase, is recent duidelijk geworden dat VWF een veel complexer en veelzijdiger eiwit is dan aanvankelijk gedacht werd. Verschillende aspecten - van synthese, secretie en klaring tot functies in de circulatie - van dit eiwit zijn bestudeerd en de resultaten zijn in dit proefschrift opgeschreven.

Deel 1: Synthese en secretie aspecten van VWF

VWD is de meest voorkomende erfelijke bloedingsziekte en wordt gekenmerkt door verlaagde plasma VWF niveaus of functioneel abnormale VWF [7,8]. VWD is onderverdeeld in drie groepen op basis van het type defect. Type 1 VWD wordt gekenmerkt door een gedeeltelijk kwantitatieve afname van VWF, terwijl type 3 VWD gekenmerkt wordt met een (bijna) volledige afwezigheid van VWF. Type 2 VWD omvat alle kwalitatieve disfuncties van VWF en is onderverdeeld in 4 subtypen: 2A, 2B, 2M en 2N. Lage plasmaspiegels bij patiënten met VWD zijn het gevolg van mutaties in het VWF gen die leiden tot verminderde synthese, verminderde secretie, verhoogde klaring of een combinatie daarvan. In ongeveer 30 tot 40% van de indexpatiënten wordt geen (oorzakelijke) mutatie gevonden [9-11], wat suggereert dat andere factoren buiten het VWF gen de plasmaspiegels kunnen bepalen. In **hoofdstuk 2** geven we een overzicht van de factoren die VWF plasmaspiegels kunnen beïnvloeden. Factoren zoals ABO bloedgroep [12], bloedplaatjes $\alpha_2\beta_1$ polymorfisme [13], leeftijd [12,14], en hormonale veranderingen [15,16] hebben grote invloed op VWF plasmaspiegels en / of bloedingsfenotype van de patiënten. Dit illustreert dat VWD een complexe multifactoriële ziekte is waar genetische- en omgevingsfactoren bijdragen aan het variabele fenotype van de ziekte. Dus ondanks het groeiende inzicht in de pathofysiologie van VWD, is de diagnose vaak nog moeilijk vanwege de vele factoren die van invloed zijn op VWF plasmaspiegels.

Het VWF molecuul bevat een groot aantal cysteïneresiduen die ofwel intramoleculaire of intermoleculaire disulfidebruggen vormen. Intramoleculaire disulfidebruggen zijn belangrijk voor de VWF monomeer structuur, terwijl intermoleculaire disulfidebrug-

gen essentieel zijn voor de vorming van de hoogmoleculaire multimeren (HMW) [17]. Cysteïne mutaties in VWF zijn vaak geïdentificeerd in patiënten met VWD (VWF databank, te raadplegen via www.vwf.group.shef.ac.uk). Het merendeel van de cysteïne mutaties leidt tot een verlies van intramoleculaire disulfidebruggen en zijn geassocieerd met lage VWF plasmaspiegels. Opvallend is dat de 5 geïdentificeerde cysteïne mutaties die betrokken zijn bij intermoleculaire disulfidebruggen (p.Cys1099Tyr, p.Cys2771Ser/Tyr and p.Cys2773Arg/Ser) zijn geassocieerd met normale VWF plasmaspiegels. Deze verschillende effecten van de twee soorten cysteïne mutaties deed ons veronderstellen dat een verminderde intramoleculaire of intermoleculaire disulfidebrugvorming in VWF verschillende effecten op de biogenese van Weibel-Palade bodies (WPB), de endotheel specifieke opslagorganellen voor VWF, kan hebben. Door het analyseren van de vorming van pseudo-WPB door expressie van VWF varianten met verstoorde intramoleculaire (p.Cys1130Phe en p.Cys2671Tyr) of intermoleculaire (p.Cys2773Ser) disulfidebruggen in **hoofdstuk 3**, hebben we geconcludeerd dat natuurlijke mutaties van cysteïnen, betrokken bij de vorming van interchain disulfidebruggen de opslag in WPB en secretie van VWF niet beïnvloeden, terwijl mutaties van cysteïnen die de intramoleculaire disulfidebruggen verstoren tot verminderde opslag en secretie VWF leiden door retentie in het endoplasmatisch reticulum.

Het is bekend dat sommige patiënten met VWD type 1 een verminderde respons op DDAVP behandeling laten zien [18]. Interessant is dat de VWF mutaties die in deze patiënten zijn geïdentificeerd allen binnen de A1-A3 domeinen van VWF liggen. Aangezien de DDAVP geïnduceerde toename van plasma VWF door exocytose van WPB komt, kan de vorming van WPB bij deze patiënten zijn aangetast door de mutaties. Expressie studies met varianten in de A-domeinen van VWF zijn beperkt. Daarom hebben we in **hoofdstuk 4** de biosynthese van zes VWF mutaties in de VWF A1-A3 domeinen gekarakteriseerd, die zijn gevonden in families die gediagnosticeerd zijn met VWD. We vonden dat alle mutanten, behalve de p.Arg1583Trp, een verminderde vorming van pseudo-WPB vertoonden. Bovendien vertoonden 2 mutanten (p.Leu1307Pro en p.Val1822Gly) ook een verminderd aantal pseudo-WPB en verhoogde retentie in het endoplasmatisch reticulum. Gereguleerde secretie van VWF na stimulatie van getransfecteerde HEK293 cellen reproduceert de *in vivo* situatie. Dit geeft aan dat het systeem met HEK293 cellen die VWF varianten tot expressie brengen die zijn gevonden in patiënten met VWD, goed kan worden gebruikt om defecten in gereguleerde secretie te bestuderen. De effecten die we zagen van de mutanten p.Ser1285Pro, p.Leu1307Pro en p.Tyr1584Cys op pseudo-WPB vorming en gereguleerde VWF secretie met HEK293 cellen werden bevestigd in de endotheelcellen (Blood Outgrowth Endothelial Cells) van de overeenkomstige patiënten [19].

Deel 2: Aspecten van VWF klaring

Zoals vermeld in **hoofdstuk 2**, zijn de ABO bloedgroepen één van de belangrijkste determinanten van VWF plasmaspiegels. VWF is een van de weinige plasmaeiwitten die de ABO bloedgroepsuikers bevat [20]. Deze ABH antigenen zijn toegevoegd aan de

N-gekoppelde oligosacharide ketens van VWF. Individuen met bloedgroep O hebben ongeveer 25% lagere VWF plasma niveaus in vergelijking met non-O individuen [21]. Personen met het Bombay bloedgroep fenotype, die geen ABH antigenen hebben, hebben zelfs lagere plasmaspiegels dan mensen met bloedgroep O [22]. Het exacte mechanisme achter de relatie tussen ABO bloedgroep en VWF niveau is onbekend, hoewel effecten op de klaring worden vermoed [23]. Daarom hebben we in **hoofdstuk 5** onderzocht of klaring van VWF afhankelijk is van de ABH antigenen aanwezig op het VWF molecuul. Door het zuiveren van humaan VWF van bloedgroep A, B en O donor- en, waren we in staat om de klaring van VWF met de verschillende bloedgroepen in VWF deficiënte muizen te bestuderen. We zagen echter geen verschil in klaring tussen A, B of O humaan plasma-gezuiverd VWF. In ons humane model, hebben we drie type 3 VWD patiënten een VWF concentraat (Haemate-P®) intraveneus toegediend. Haemate-P® bevat VWF van donoren met alle bloedgroepen, wat ons in staat stelde om A-VWF, B-VWF en O-VWF gelijktijdig te bestuderen in één individu. Snellere klaring van O-VWF aanwezig in de Type 3 VWD patiënten na infusie zou moeten leiden tot een relatieve toename van de belading van VWF met A- en B antigenen in de tijd. Opvallend genoeg vonden wij dat de belading van VWF met A en B antigenen vermindert tijdens de klaring van VWF in 2 van de 3 patiënten en in de derde patiënt bleef de belading gelijk. De afname van A en B antigeen belading van VWF werd bevestigd door Western blot. Op basis van de resultaten van ons muis - en menselijke model zijn we tot de conclusie gekomen dat er geen direct effect is op de klaring van VWF van ABH bloedgroepantigenen op VWF. We hebben aangetoond dat in een directe vergelijking binnen één individu O-VWF niet sneller geklaard wordt in vergelijking met A- of B-VWF. Preliminair resultaten over de farmacokinetische gegevens van infusies van Haemate-P® bij bloedgroep O en non-O type 3 VWD patiënten, lijken onze nieuwe hypothese te onderbouwen dat het de bloedgroep O status van het individu zelf is waardoor het VWF molecuul sneller klaart, maar dat dit onafhankelijk is van de ABO bloedgroep antigenen op VWF.

Snellere klaring van VWF is een van de mechanismen die leiden tot lagere VWF plasmaspiegels en vervolgens bloedingsfenotypes. VWF en VWF propeptide (VWFpp) worden uitgescheiden in equimolaire hoeveelheden in de circulatie, maar de halfwaardetijden verschillen. De ratio tussen deze twee parameters kan daarom gebruikt worden om onderscheid te maken tussen normale en versnelde klaring van VWF. Terwijl VWFpp en VWF differentieel worden geklaard, vormt stollingseiwit FVIII daarentegen een complex met VWF en dus zijn hun halfwaardetijden gerelateerd. De ratio tussen FVIII stollingsactiviteit (FVIII:C) en VWF:Ag (FVIII:C/VWF:Ag) kan gebruikt worden voor het identificeren van defecten in VWF synthese. De FVIII:C/VWF:Ag is verhoogd wanneer VWF synthese verminderd is, maar de verhouding blijft 1 wanneer VWF sneller wordt geklaard. Tegengestelde resultaten worden verkregen voor VWFpp/VWF:Ag, die met verminderde synthese ongewijzigd blijft, maar zal toenemen met verminderde halfwaardetijd van VWF [24]. Met behulp van de VWFpp/VWF:Ag en FVIII:C/VWF:Ag verhoudingen, hebben we in **hoofdstuk 6** onder-

zocht wat de pathofysiologische mechanismen zijn bij type 1, 2 en 3 VWD patiënten in de Willebrand in Nederland (WiN) cohort [25]. We vonden dat in type 1 VWD zowel verminderde aanmaak (verhoogde FVII:C/VWF:Ag) als versnelde klaring (verhoogde VWFpp/VWF:Ag) bijdragen aan het pathofysiologische fenotype bij deze patiënten. Dit is in lijn met eerder gepubliceerde data uit een grote Europese cohort van type 1 patiënten [24]. Interessant is dat in de type 2B patiënten verhoogde klaring frequent werd waargenomen. Dit is waarschijnlijk te wijten aan het feit dat type 2B VWF spontaan bindt aan bloedplaatjes in de circulatie. Het VWF-bloedplaatjes complex wordt geklaard uit de circulatie, wat leidt tot lagere VWF:Ag levels maar normale VWFpp niveaus. Een andere, onverwachte, bevinding was het meetbare niveau van VWFpp in een grote groep van type 3 VWD patiënten, terwijl hun VWF:Ag onder het criterium van 5 IU/dL lag. De meetbare hoeveelheid VWFpp geeft aan dat deze patiënten wel degelijk VWF synthetiseren en uitscheiden, maar dat het VWF waarschijnlijk zo snel geklaard wordt dat het VWF:Ag niveau zeer laag blijft. Inderdaad vonden wij bij de meeste van deze patiënten mutaties die bekend zijn met versnelde klaring zoals de p.Arg1205His en p.Ser1285Pro mutaties [26]. Voor de klinische behandeling zal dit echter niet van zoveel belang zijn, aangezien patiënten allen VWF concentraat zullen krijgen indien nodig, maar voor genetische counseling kan het waardevol zijn en het VWFpp kan als diagnostisch hulpmiddel het onderscheid maken tussen 'echte' type 3 VWD patiënten en type 1 VWD patiënten bij wie het VWF heel snel geklaard wordt.

Deel 3: Aspecten van VWF naast hemostase

Naast dat het een belangrijke rol in de hemostase speelt, lijkt VWF ook een negatieve regulator van (*in vitro*) angiogenese [27], de vorming van nieuwe bloedvaten uit reeds bestaande vaten. VWF is opgeslagen in WPBs in endotheelcellen. Maar naast VWF worden ook andere eiwitten opgeslagen in de WPB. Van meerdere van deze eiwitten is bekend dat ze betrokken zijn bij angiogenese, zoals angiopoietine-2 (Ang-2), galectin-3 (Gal-3) en osteoprotegerin (OPG). Omdat VWF essentieel is voor de vorming van WPBs, zijn deze factoren afhankelijk van VWF voor hun opslag in de WPB en de secretie in de circulatie. We speculeerden dan ook in **hoofdstuk 7** dat verstoorde WPB formatie en/of exocytose door VWF mutaties, zou leiden tot een ontregelde secretie van andere angiogene mediators. Met behulp van het WiN cohort (zie **hoofdstuk 6**) zijn 655 plasma monsters van type 1, 2 en 3 VWD patiënten geanalyseerd op hun angiopoietin-1 (Ang-1), Ang-2, Gal-3, OPG en vasculair endothelial growth factor (VEGF) niveaus. Vergeleken met controles, hebben patiënten hogere Ang-1 en VEGF concentraties. Type 1 en 2 patiënten hadden lagere Ang-2 niveaus, terwijl type 3 VWD patiënten hogere OPG concentraties hadden in vergelijking met controles. VEGF niveaus waren sterk afhankelijk van VWF plasmaspiegels, en waren het hoogst bij patiënten met type 3 VWD. Patiënten met VWD hebben dus diverse veranderingen in angiogene markers in het plasma. Mutaties in VWF, die leiden tot een verstoorde vorming van WPB, leiden echter niet consequent tot ontregelde secretie van andere WPB componenten. De variaties in angiogene mediators kunnen bijdragen aan de ontwikkeling van vasculaire misvormingen, zoals angiodysplasie die kunnen optreden in VWD.

Uit recent onderzoek is gebleken dat endotheel VWF *in vitro* angiogenese reguleert [27]. BOECs geïsoleerd uit bloed van type 1 en 2 VWD patiënten vertoonden verhoogde angiogenese in vergelijking met BOECs geïsoleerd van gezonde individuen. Of dit ook geldt voor type 3 VWD BOECs is onbekend, omdat succesvolle isolatie nog nauwelijks is gepubliceerd. Karakterisering van de angiogene capaciteit van BOECs van VWD patiënten is nog zeer beperkt en verschillen tussen de verschillende typen VWD zijn nog niet in detail onderzocht. Daarom hebben we in **hoofdstuk 8** de angiogene capaciteit van BOECs van patiënten met type 1 (n=4), type 2 (n=6) en type 3 (n=1) VWD onderzocht om verder te onderzoeken of BOECs als endotheelcel model te gebruiken zijn om zo de pathogene rol van VWF mutaties in het reguleren van angiogenese te bestuderen. De migratie snelheid en de totale tube formatie was vergelijkbaar tussen alle VWD patiënten en controles. Echter, BOECs van de type 3 VWD patiënt en één type 2B VWD patiënt vertoonden meer angiogene capaciteit dan controle BOECs. De directionaliteit van de migratie was aangetast in 9 van de 11 VWD patiënten. Tevens zagen we dat de angiogene respons van BOECs van VWD patiënten beperkt was tot vroege passagenummers. Hoewel BOECs van VWD patiënten een nuttig instrument kunnen zijn voor *ex vivo* bepaling van endotheliale cel functie bij patiënten met verschillende typen VWD, dient er ook rekening gehouden te worden met beperkingen (zoals vroeg verlies van angiogene capaciteit) bij het bestuderen van BOECs VWD patiënten.

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Acknowledgement

You know how it is.

You pick up a dissertation, flip through the pages right to the acknowledgements, and find that, once again, the author has thanked numerous people, except you.

Not this time.

Because we haven't yet met/have only a glancing acquaintance/are just crazy about each other/haven't seen each other in much too long/

are in some way related/worked together/will never meet, but will,

I trust, despite that, always think fondly of each other.

This one's for you.

With you know what, and you probably know why.

~ Adapted from Anansi Boys by Neil Gaiman, 2005 ~

Curriculum Vitae

Dafna J. Groeneveld werd geboren op 9 November 1982 te Steenwijk en behaalde in 2001 haar HAVO diploma aan de Thorbecke Scholengemeenschap (Zwolle). In datzelfde jaar begon zij aan de opleiding “Biologie en medisch laboratoriumonderzoek” aan het Saxion Hogeschool IJsselland (Deventer) met als specialisatie “klinische chemie”. Vanaf 2003 heeft ze drie stages gelopen. Haar eerste stage werd uitgevoerd op het laboratorium voor klinische chemie van het Meander Medisch Center (Amersfoort). Haar tweede stage volbracht zij bij de afdeling experimentele klinische chemie van het Academisch Medisch Centrum (Amsterdam) waar zij onder leiding van prof. dr. A. Sturk en dr. M. Peters onderzoek deed naar de betrouwbaarheid van de bloedingstijd en de Platelet Function Analyzer voor het detecteren van bloedingsneigingen. Haar afstudeerstage van 9 maanden volbracht zij onder leiding van prof. dr. T. Lisman bij de afdeling Trombose en Hemostase van het Universitair Medisch Centrum Utrecht waar zij werkte aan het identificeren van de bindingsplaats voor von Willebrand factor in collageen. Na het behalen van haar bachelor diploma in juli 2005 heeft zij een jaar als research analist gewerkt bij de afdeling Vasculaire Geneeskunde van het UMC Utrecht, waar ze de rol van endotheel- en gladde spier voorlopercellen in patiënten met eindstadium nierfalen onderzocht. In september 2006 begon zij aan de opleiding Forensic Science aan de Universiteit van Amsterdam. Haar master thesis “No enemy is worse than bad advice: a framework for validating forensic advice” was het resultaat van 6 maanden onderzoek bij de Forensische Samenwerking in de Opsporing (FSO, locatie Midden Nederland), een samenwerkingsverband tussen de politie en het Nederlands Forensisch Instituut. Na het behalen van haar masterdiploma, begon zij eind 2008 met haar promotieonderzoek bij de afdeling Trombose en Hemostase, onderdeel van het Eindhoven Laboratorium voor Experimentele Vasculaire Geneeskunde, in het Leids Universitair Medisch Centrum. Onder begeleiding van prof. dr. H.C.J. Eikenboom deed zij onderzoek naar verschillende aspecten van het stollingseiwit von Willebrand factor. De resultaten beschreven in dit proefschrift zijn op verschillende (inter)nationale congressen gepresenteerd en in 2012 ontving zij de NVTH award for scientific excellence in biochemical research van de Nederlandse Vereniging voor Trombose en Hemostase. Sinds november 2013 is zij werkzaam als post-doc bij de afdeling Chirurgisch Onderzoekslaboratorium van het Universitair Medisch Centrum Groningen. Onder begeleiding van prof. dr. R.J. Porte en prof. dr. T. Lisman werkt zij aan zowel klinische als fundamentele aspecten op het snijvlak van hematologie, hepatologie en chirurgie. Ze heeft als co-applicant zowel een beurs binnen gehaald van de Nederlandse Trombosestichting als de Grifols Antithrombin Research Award.

