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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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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 (causal) mutations were found, suggesting that other factors outside the VWF gene can determine VWF plasma levels. Factors such as ABO blood group, platelet α2β1 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. 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

<table>
<thead>
<tr>
<th>VWD Subtype</th>
<th>Description*</th>
</tr>
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<tbody>
<tr>
<td>Type 1</td>
<td>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.</td>
</tr>
<tr>
<td>Type 2A</td>
<td>Qualitative VWF defect resulting in a reduction of VWF-dependent platelet adhesion. Associated with absence of the largest multimers. Generally autosomal dominant.</td>
</tr>
<tr>
<td>Type 2B</td>
<td>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.</td>
</tr>
<tr>
<td>Type 2M</td>
<td>Qualitative VWF defect associated with specific defects in platelet/VWF interaction but with a normal range of multimers. Inheritance is autosomal dominant.</td>
</tr>
<tr>
<td>Type 2N</td>
<td>Qualitative VWF defect resulting from defective VWF binding to FVIII and consequently low levels of circulating FVIII. Inheritance is autosomal recessive.</td>
</tr>
<tr>
<td>Type 3</td>
<td>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.</td>
</tr>
</tbody>
</table>

*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 (MCMMDM-1VWD)” study, no mutation was found in 30% (45 out of 150) of the
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 then other factors can influence the test results.

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

<table>
<thead>
<tr>
<th>Candidate Mutation*</th>
<th>UK study (32 IC) n (%)</th>
<th>Canadian study (123 IC) n (%)</th>
<th>European study (150 IC) n (%)</th>
<th>Total (305 IC) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1584C</td>
<td>8 (25)</td>
<td>18 (14.6)</td>
<td>13 (8.7)</td>
<td>39 (12.8)</td>
</tr>
<tr>
<td>R1205H</td>
<td>4 (12.5)</td>
<td>3 (2.4)</td>
<td>10 (6.7)</td>
<td>17 (5.6)</td>
</tr>
<tr>
<td>R924Q</td>
<td>3 (9.4)</td>
<td>8 (6.5)</td>
<td>4 (2.7)</td>
<td>15 (4.9)</td>
</tr>
<tr>
<td>R854Q</td>
<td>3 (2.4)</td>
<td>3 (2.4)</td>
<td>5 (3.3)</td>
<td>8 (2.6)</td>
</tr>
<tr>
<td>R2464C</td>
<td>-</td>
<td>3 (2.4)</td>
<td>3 (2)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>P1266L</td>
<td>-</td>
<td>4 (3.3)</td>
<td>1 (0.7)</td>
<td>5 (1.6)</td>
</tr>
<tr>
<td>P2063S</td>
<td>-</td>
<td>3 (2.4)</td>
<td>1 (0.7)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>R1115C</td>
<td>-</td>
<td>-</td>
<td>4 (2.7)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>R1374C</td>
<td>-</td>
<td>-</td>
<td>4 (2.7)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>V1279I</td>
<td>-</td>
<td>4 (3.3)</td>
<td>-</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>C1130F</td>
<td>-</td>
<td>-</td>
<td>3 (2)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>C1130R</td>
<td>-</td>
<td>-</td>
<td>3 (2)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>M740I</td>
<td>-</td>
<td>-</td>
<td>3 (2)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>N1231T</td>
<td>-</td>
<td>3 (2.4)</td>
<td>-</td>
<td>3 (1)</td>
</tr>
<tr>
<td>R1325C</td>
<td>-</td>
<td>3 (2.4)</td>
<td>-</td>
<td>3 (1)</td>
</tr>
<tr>
<td>V1229G</td>
<td>-</td>
<td>3 (2.4)</td>
<td>-</td>
<td>3 (1)</td>
</tr>
<tr>
<td>C2304Y</td>
<td>-</td>
<td>-</td>
<td>2 (1.3)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>P1413L</td>
<td>-</td>
<td>1 (0.8)</td>
<td>1 (0.7)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>G1415D</td>
<td>-</td>
<td>-</td>
<td>2 (1.3)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>R1374H</td>
<td>-</td>
<td>-</td>
<td>2 (1.3)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>R1668S</td>
<td>-</td>
<td>2 (1.6)</td>
<td>-</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Y1146C</td>
<td>-</td>
<td>1 (0.8)</td>
<td>1 (0.7)</td>
<td>2 (0.7)</td>
</tr>
</tbody>
</table>

*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.
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

![Abnormal Multimer Pattern](image1.png)

![Normal Multimer Pattern](image2.png)

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](image)

**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-
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 to 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 α₂β₁ 807C allele, which is associated with low receptor density, was found significantly higher than in the normal population [56]. Low density of the integrin α₂β₁ 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 α₂β₁ 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 MCMDEM-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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