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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>
<thead>
<tr>
<th>VWD Subtype</th>
<th>Description*</th>
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<tbody>
<tr>
<td>Type 1</td>
<td>Partial quantitative deficiency of VWF.</td>
</tr>
<tr>
<td>Type 2A</td>
<td>Qualitative VWF defect resulting in a reduction of VWF-dependent platelet adhesion.</td>
</tr>
<tr>
<td>Type 2B</td>
<td>Qualitative VWF defect resulting in increased VWF-dependent platelet adhesion.</td>
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<tr>
<td>Type 2M</td>
<td>Qualitative VWF defect associated with specific defects in platelet/VWF interaction but with a normal range of multimers.</td>
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<tr>
<td>Type 2N</td>
<td>Qualitative VWF defect resulting from defective VWF binding to factor VIII (FVIII) and consequently low levels of circulating FVIII.</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.</td>
</tr>
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*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-

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**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 distinctegrin and metalloprotease with a thrombospondin type 1 motif member 13; MMP9, matrix metalloprotease 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 αvβ3 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 GPIbα 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 αvβ3. 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.
References

10. ISTH-SSC VWF Online Database. Available at: http://vwf.group.shef.ac.uk