Chapter 1

General introduction and outline of thesis

Von Willebrand disease

Von Willebrand disease (VWD) is the most common inherited bleeding diathesis in humans. It is caused by acquired or genetic defects in von Willebrand factor (VWF), a large multimeric plasma protein [1]. On the basis of the quantitative or functional defects in VWF, VWD is classified into three types: type 1 is characterized by low plasma VWF levels, type 2 is characterized by defective interaction of VWF with platelets or coagulation factor VIII (FVIII), and type 3 is characterized by a virtually complete deficiency of plasma VWF. Among all the index cases, type 1 VWD is the most common, while type 3 VWD is the most severe but also most rare subtype. Most mutations causing type 1 VWD are missense mutations (up to 75%), whereas only a minority of mutations causing type 3 VWD are missense mutations [1,2].

Von Willebrand factor

VWF is a multimeric hemostatic protein composed of a single subunit assembled into multimers that range in molecular mass from 500 to 20.000 kDa [3]. It plays pivotal roles in hemostasis by mediating adhesion and aggregation of platelets at sites of vascular injury, thereby promoting clot formation and tissue repair. VWF also carries FVIII and stabilizes it in the circulation (Figure 1A). VWF may contribute to the risk of thrombosis, may cause thrombotic thrombocytopenic purpura (TTP), or may lead to a bleeding tendency as seen in VWD. Its role in the hemostatic balance relies on the synthesis, storage, secretion and catabolism of VWF [1,3]. Given its roles in hemostasis and its interactions with other molecules (eg. collagen) and cells, VWF has also been implicated in other diseases such as stroke, coronary heart disease and diabetes [4]. This thesis focuses on the roles of VWF in VWD.

VWF is exclusively synthesized in vascular endothelial cells and in megakaryocytes [3,5]. The precursor protein of VWF is composed of a signal peptide (22 amino acids, aa) and of proVWF that has fourteen distinct domains in the order of D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (2791 aa). During its translocation to the endoplasmic reticulum (ER) the signal peptide is cleaved off, and the proVWF forms dimers in a "tail-to-tail" fashion through cysteines in its carboxy-terminal cysteine knot (CK) domain. proVWF dimers transit to the Golgi apparatus to assemble into multimers in a "head-to-head" fashion through the formation of intermolecular disulfide bonds between cysteine-residues in the D3 domain. Meanwhile, D1-D2 domains are cleaved off to form VWF propeptide (VWFpp, 741 aa), while the remaining domains form mature VWF (2050 aa). In the

trans Golgi network (TGN) VWFpp promotes mature VWF to assemble into high molecular weight (HMW) multimers. The HMW multimers subsequently aggregate into tubular structures which are packaged into α-granules in megakaryocytes, and into Weibel-Palade bodies (WPB) in endothelial cells (Figure 1B) [3,5,6].

Weibel-Palade body

WPB are endothelial cell-specific organelles that were discovered using transmission electron microscopy (TEM) and first described by Ewald Weibel and George Palade as follows: "a hitherto unknown rod-shaped cytoplasmic component which consists of a bundle of fine tubules, enveloped by a tightly fitted membrane, was regularly found in endothelial cells of small arteries in various organs in rat and

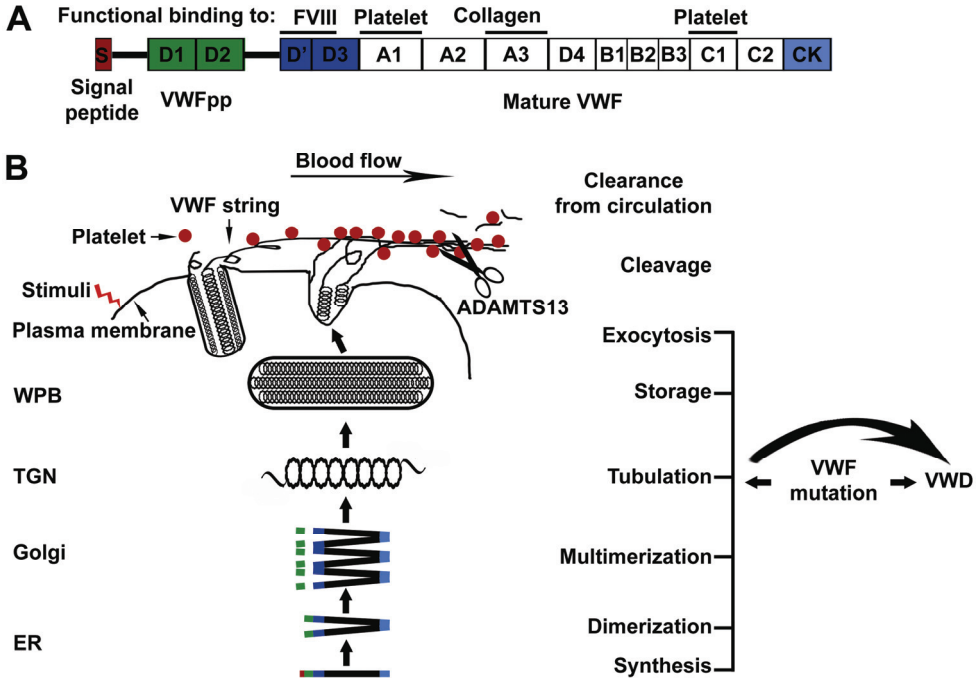

Figure 1. Schematic representation of the structure and metabolism of VWF. (A) The domain structure and functional binding sites of VWF. The signal peptide and VWFpp are cleaved upon translocation of proVWF into the ER and in the Golgi apparatus, respectively. (B) Synthesis, trafficking, proteolysis and clearance of VWF. The structure of precursor VWF protein is indicated by different colors: red for signal peptide; green for VWFpp; dark blue for D'D3 domains; black for A1-C2 domains; and light blue for CK domain.

man." [7]. The presence of WPB has been demonstrated in endothelial cells of almost all types of blood vessels from humans and other vertebrates. These organelles are enclosed by a membrane and are typically high µm in length) with regular internal striations under TEM [5].
WPB serve as an intracellular storage pool of VWF and of several cytokines including interleukin 8, tissue plasminogen activator (t-PA), P-selectin and angiopoietin 2 [8]. FVIII has also been found in WPB of the microvascular endothelial cells of the lung [9]. When the endothelium is exposed to certain stimuli such as DDAVP - a clinically applied agonist for the release of VWF to plasma - WPB undergo exocytosis and release their contents to the circulation or present them on the cell surface [6]. Upon exocytosis of WPB, VWF forms string-like structures on the cell surface that recruit platelets from the flowing blood and thereby promote clot formation and tissue repair. VWF can become cleaved by the protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) into smaller multimers and is cleared from the circulation in liver and spleen [3,10]. Interestingly, the existence of WPB solely depends on expression of its main component VWF. If there is no expression of VWF, endothelial cells do not make WPB. Vice versa, expression of VWF in other cell types such as in the human embryo kidney cell line HEK293 cells leads to de novo formation of elongated granules containing VWF that resemble WPB. These organelles are named pseudo-WPB [6,8].

Outline of the thesis

All aspects of VWF synthesis, storage, secretion and metabolism, including protease cleavage by ADAMST13 and clearance from the circulation are discussed in this thesis, with a focus on storage and secretion in the context of VWD (Figure 1). Since the biogenesis of WPB is driven by VWF, we hypothesize that structural changes in VWF caused by mutations as encountered in VWD patients may alter VWF storage and secretion and consequently contribute to the bleeding tendency seen in VWD, in particular in quantitative forms of VWD deficiency. Based on this hypothesis, we studied the effects of VWD variants on the formation, structure and regulated secretion of WPB with various techniques in order to advance the knowledge of molecular and cellular mechanisms underlying VWD.
In **Chapter** 2, the role of VWF in WPB formation and the effects of VWF mutations on WPB formation and function are reviewed in the context of VWD.

In **Chapter** 3, we studied the intracellular storage and regulated secretion of four missense mutations located in the D3 domain (p.Cys1060Tyr and p.Cys1149Arg) and the CK domain (p.Cys2739Tyr and p.Cys2754Trp) of VWF. All four cysteine mutations were originally identified in type 1 (p.Cys1060Tyr and p.Cys1149Arg) or type 3 (p.Cys2739Tyr and p.Cys2754Trp) VWD. Both confocal immunofluorescence microscopy and transmission electron microscopy were applied in this study. Albeit that D'D3 and CK domains of VWF are important for the formation and function of WPB, the missense mutations found in a subset of type 1 VWD patients who showed a reduced response to DDAVP treatment are mostly located in the A1-A3 domains of VWF. We therefore in **Chapter** 4 studied the intracellular storage and secretion of six VWF variants with missense mutations located in the A1-A3 domains. Three of these mutations, p.Leu1307Pro, p.Arg1374His, and p.Val1822Gly, were identified in VWD patients with apparently impaired response to DDAVP. One (p.Tyr1584Cys) mutation was identified in VWD patients who showed a partly reduced to normal response to DDAVP; and the other two mutations (p.Ser1285Phe and p.Arg1583Trp) were identified in VWD patients whose response to DDAVP infusion is not known.

In **Chapter** 5, we compared the effects of VWD variants with impaired VWF intrachain (p.Cys1130Phe and p.Cys2671Tyr) or interchain (p.Cys2773Ser) disulfide bound formation on the biogenesis and exocytosis of WPB. We postulate that mutations of cysteines involved in the formation of interchain disulfide bonds do not affect the storage and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds will reduce VWF storage and secretion.

In **Chapter** 6, we described the isolation of blood outgrowth endothelial cells (BOECs) from the peripheral blood of four VWD patients and one heterozygous carrier of a recessive VWF mutation. All these subjects are heterozygous for VWF missense mutations. We characterized the endothelial phenotypes of BOECs and studied the storage and regulated secretion of VWF in the cultured endothelial cells.

In **Chapter** 7, we established VWF strings on the cell surface of non-endothelial HEK293 cells and examined whether HEK293 cells provide a novel model-system for investigation of normal and mutant VWF in terms of VWF string formation and function.

In **Chapter** 8, we studied the pathophysiologic mechanisms of VWF mutations p.Arg1205His and p.Arg924Gln both in BOECs derived from two VWD type Vicenza patients and in HEK293 cells.

All the studies presented in this thesis are summarized and discussed in **Chapter** 9.

References

1. Sadler JE, Budde U, Eikenboom JC, Favaloro EJ, Hill FG, Holmberg L, Ingerslev J, Lee CA, Lillicrap D, Mannucci PM, Mazurier C, Meyer D, Nichols WL, Nishino M, Peake IR, Rodeghiero F, Schneppenheim R, Ruggeri ZM, Srivastava A, Montgomery RR, Federici AB. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost 2006; 4:2103-2114.
2. Wang JW, Eikenboom J. Von Willebrand disease and Weibel-Palade bodies. Hamostaseologie 2010; 30:150-155.
3. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem 1998; 67:395-424.
4. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J Thromb Haemost 2006; 4:1186-1193.
5. Wagner DD. Cell biology of von Willebrand factor. Annu Rev Cell Biol 1990; 6:217-246.
6. Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J. Functional architecture of Weibel-Palade bodies. Blood 2011; 117:5033-5043.
7. Weibel ER, Palade GE. New cytoplasmic components in arterial endothelia. J Cell Biol 1964; 23:101-112.
8. Metcalf DJ, Nightingale TD, Zenner HL, Lui-Roberts WW, Cutler DF. Formation and function of Weibel-Palade bodies. J Cell Sci 2008; 121:19-27.
9. Jacquemin M, Neyrinck A, Hermanns MI, Lavend'homme R, Rega F, Saint-Remy JM, Peerlinck K, Van RD, Kirkpatrick CJ. FVIII production by human lung microvascular endothelial cells. Blood 2006; 108:515-517.
10. Denis CV, Christophe OD, Oortwijn BD, Lenting PJ. Clearance of von Willebrand factor. Thromb Haemost 2008; 99:271-278.