

## Changing insights in the diagnosis and classification of autosomal recessive and dominant von Willebrand diseases 1980-2015

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### Abstract

The European Clinical Laboratory and Molecular (ECLM) criteria define 10 distinct Willebrand diseases (VWD): recessive type 3, severe 1, 2C and 2N; dominant VWD type 1 secretion/clearance defect, 2A, 2B, 2E, 2M and 2D; and mild type 1 VWD (usually carriers of recessive VWD). Recessive severe 1 and 2C VWD are characterized by secretion and multimerization defects caused by mutations in the D1-D2 domain. Recessive 2N VWD is a mild hemophilia due to D'-FVIII-von Willebrand factor (VWF) binding site mutations. Dominant 2E VWD caused by heterozygous missense mutations in the D3 domain is featured by a secretion-clearance-multimerization VWF defect. Dominant VWD type 2M due to loss of function mutations in the A1 domain is characterized by decreased ristocetin-induced platelet aggregation and VWF:RCo, normal VWF multimers and VWF:CB, a poor response of VWF:RCo and good response of VWF:CB to desmopressin (DDAVP). Dominant VWD type 2A induced by heterozygous mutations in the A2 domain results in hypersensitivity of VWF for proteolysis by ADAMTS13 into VWF degradation

products, resulting in loss of large VWF multimers with triplet structure of each individual VWF band. Dominant VWD type 2B due to a gain of function mutation in the A1 domain is featured by spontaneous interaction between platelet glycoprotein Ib (GPIb) and mutated VWF A1 followed by increased proteolysis with loss of large VWF multimers and presence of each VWF band. A new category of dominant VWD type 1 secretion or clearance defect due to mutations in the D3 domain or D4-C1-C5 domains consists of two groups: Those with normal or smeary pattern of VWF multimers.

**Key words:** Von Willebrand disease; Von Willebrand factor; ADAMTS13; DDAVP; Von Willebrand factor assays; Von Willebrand gene mutations

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**Core tip:** The European Clinical Laboratory and Molecular criteria define at least 10 distinct phenotypes of von Willebrand diseases (VWD) that have significant therapeutic implications. High quality von Willebrand factor (VWF) multimeric analysis and responses to desmopressin of FVIII:C and VWF parameters are of critical diagnostic importance to document the contribution of VWF secretion, clearance, proteolysis and multimerization defects to real life phenotyping of each individual VWD patient.

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## INTRODUCTION

Von Willebrand factor (VWF) is biosynthesized exclusively in vascular endothelium and megakaryocytes. The precursor protein proVWF consists of a signal peptide (22 amino acids, aa), the propeptide (741 aa) and the mature VWF monomer (2050 aa) (Figure 1)<sup>[1,2]</sup>. The intracellular uncleaved VWF (2791 aa) has 14 distinct domains from left to right: D1, D2, D', D3, A1, A2, A3, D4, B1-3, C1, C2 and CK (Figure 1). The exons which encode each domain are shown in Figure 1 above the VWF domain. The areas of VWF involved in binding specific functional factors are shown in Figure 1 below the VWF domains<sup>[1,2]</sup>. During the translocation of proVWF to the endoplasmic reticulum the signal peptide is cleaved off, and the proVWF forms dimers in a tail-to-tail fashion through cysteines in its carboxyterminal cysteine knot (CK) domain (Figure 2)<sup>[3-5]</sup>. ProVWF dimers transit to the Golgi apparatus as multimers through disulphide bonds between cysteine residues in the D1-3 multimerization

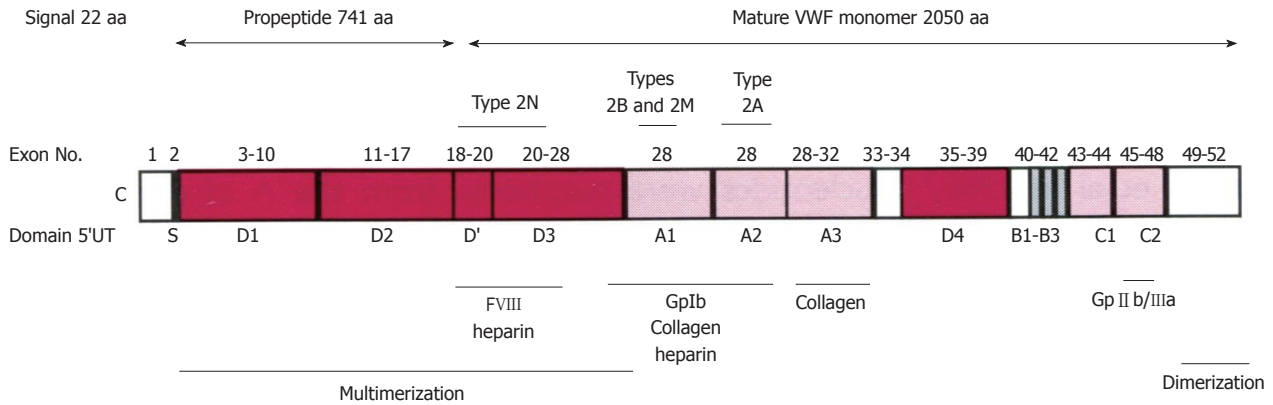
domain. Meanwhile, the D1-D2 domains are cleaved off to form the VWF propeptide (VWFpp, 741 aa), while the remaining domains from D' to CK form mature VWF (2050 aa, Figures 1 and 2). In the trans Golgi network, VWFpp promotes high molecular weight multimer formation in tubular structures, subsequently packaged in Weibel Palade bodies (WPB)<sup>[3-5]</sup>. When the endothelium is exposed to certain stimuli such as desmopressin (DDAVP), WPB undergo exocytosis and release their contents into the circulation or present them on the cell surface as string-like structures<sup>[3,4]</sup>. These high molecular weight VWF recruit platelets from the circulating blood to bind, upon which the ultralarge VWF are cleaved into the normal spectrum of high, intermediate and small strings (multimers) by the VWF cleavage protease ADAMTS13 at high shear stress in the endarterial circulation (Figure 2)<sup>[4,5]</sup>. At the time that VWF is secreted from WPB in the endothelial cell, the VWF propeptide (VWFpp = D1D2 domain) is cleaved off again at the furin cleavage site (Figure 3). Mutations in the D1 and D2 domains mean that the propeptide VWF cannot cleave off from the mature VWF, with the consequence of a VWF secretion and multimerization defect, explaining the loss of large VWF multimers in recessive severe type 1 and 2C disease (Figure 3).

## VWF-FVIII and VWF-platelet interactions

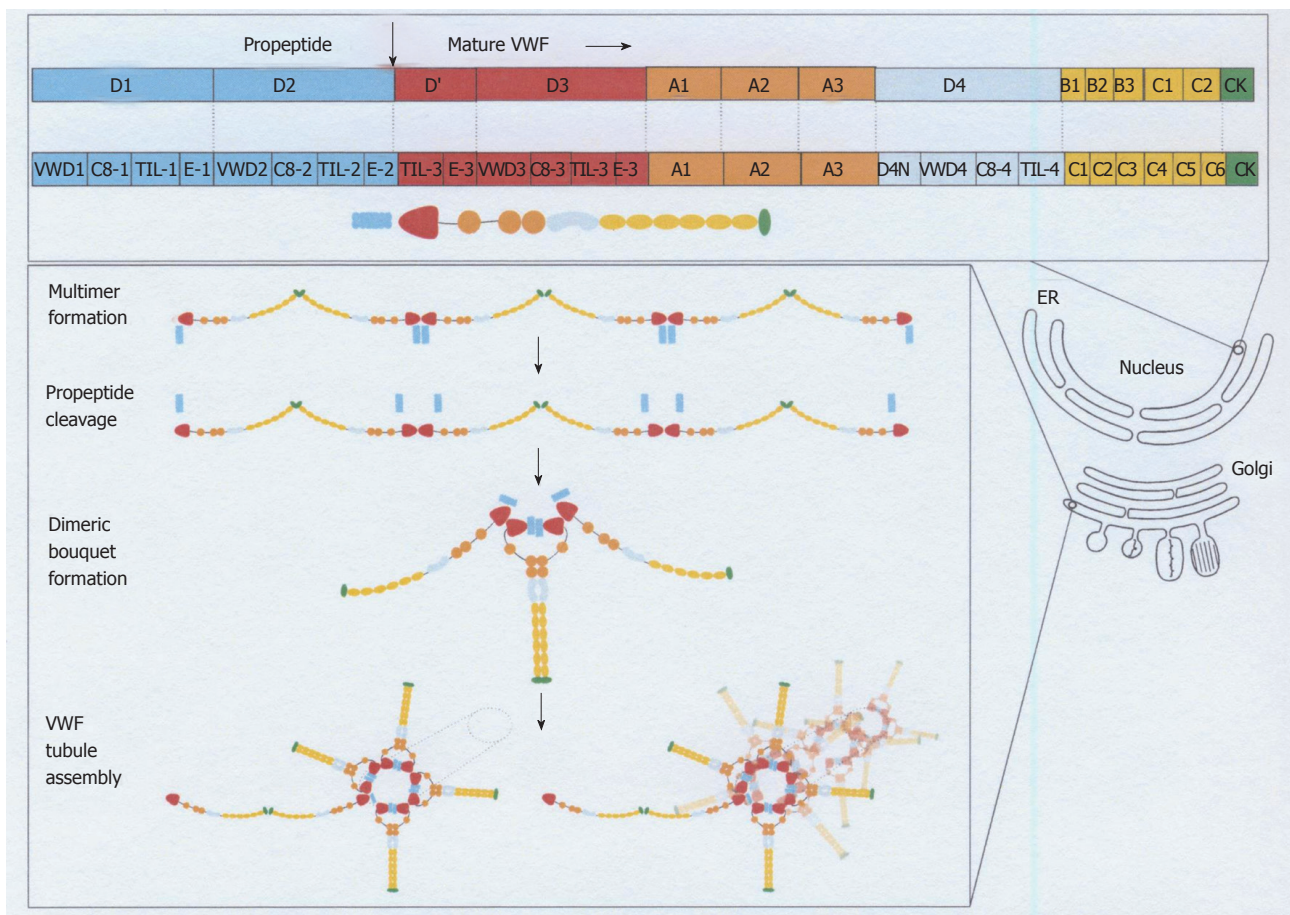
VWF circulates as a multimeric plasma glycoprotein with coagulation factor VIII (FVIII:C) bound to the D' domain of VWF<sup>[6]</sup>. FVIII is cleaved off from VWF by thrombin at sites of vascular injury. VWF circulates as large multimers as a function of the D3 multimerization and CK-terminal dimerization domains. Activated VWF and platelets mediate platelet adhesion to subendothelium and platelet aggregation at sites of vascular injury (Figure 4)<sup>[6]</sup>. At sites of vascular injury and high shear, activated platelets and activated VWF aggregate through binding of platelet GPIb to the VWF A1 domain. In the equilibrium state, with intact endothelial cells and no injured blood vessel, resting VWF circulates in globular form with resting platelets in the blood (Figure 4A). In this state, VWF is incapable of mediating platelet adhesion. After an injury of the endothelial cells, the activated and elongated VWF interacts with exposed collagen via VWF domains A1 and A3 and triggers the adhesion of activated platelets via VWF GPIb, collagen binding and GPIIb/IIIa domains (Figure 1). At low shear there is no binding between VWF domain A1 and platelet GPIb. At high shear rate the VWF globules elongate and make the VWF A1 domain accessible by the dissociation of domain A1 from A2 (Figure 4). Binding between GPIb of activated platelets to the GPIb receptor of VWF is immediately followed by cleavage of VWF in the A2 domain by ADAMTS13 (Figure 4).

## Von Willebrand disease type 1, 2 and 3

The introduction of ristocetin-based assays VWF:RCo and ristocetin induced platelet aggregation (RIPA), and the



**Figure 1 Structure and function relationship of the von Willebrand factor domains<sup>[1]</sup>.** The VWF is synthesized in endothelial cells as a large protein of 2813 amino acid (aa): signal prepeptide 22 aa, propeptide 741 aa, and the mature VWF monomer 2050 aa. D1-D2 pro-peptide is cleaved off at the furin cleavage site at time of secretion. VWF circulates bound to the FVIII at the D' FVIII binding domain. Below the figure are the areas of VWF involved in binding specific factors. VWF circulates as large multimers as a function of the D3 multimerization and CK dimerization domains. Source: Goodeve and Peake<sup>[1]</sup>. VWD: Von Willebrand disease; VWF: Von Willebrand factor.

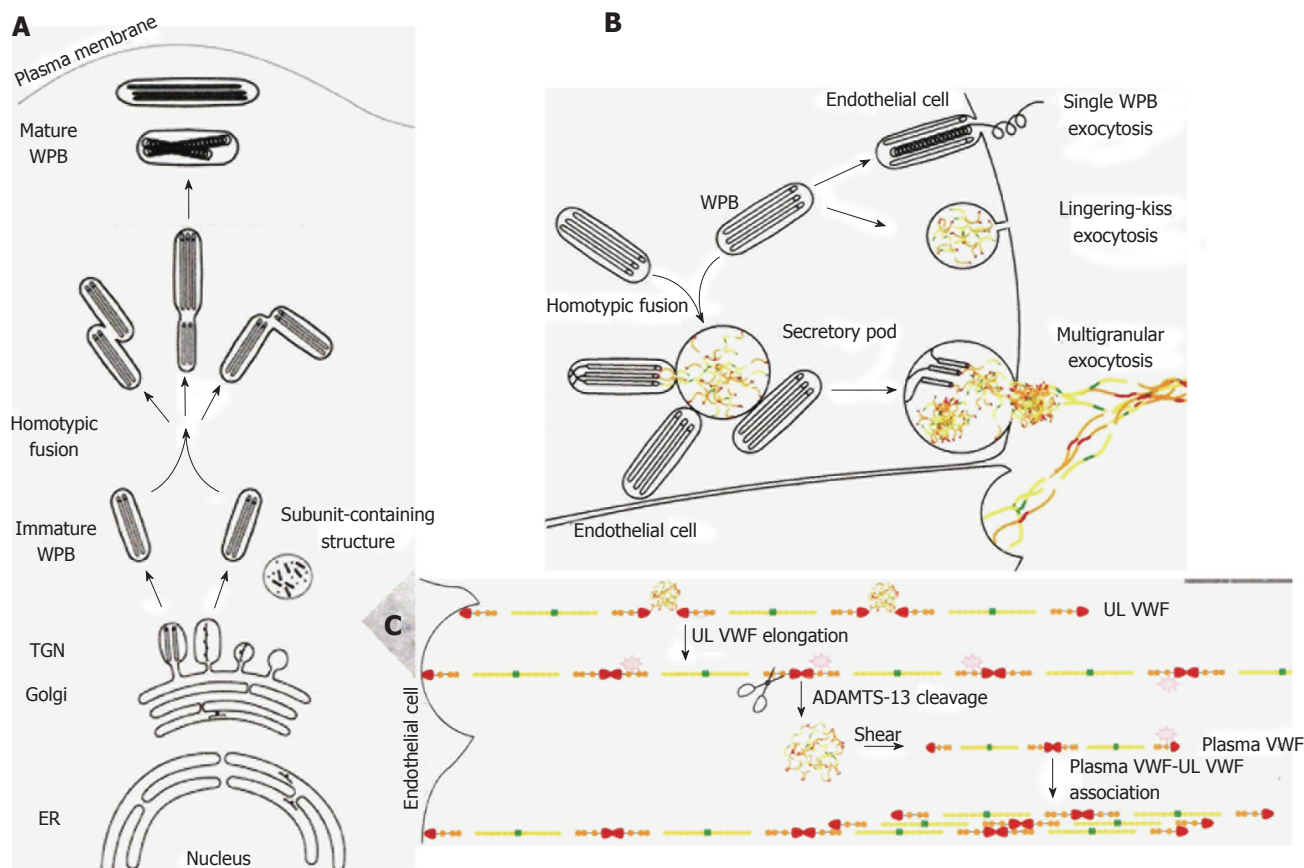


**Figure 2 Von Willebrand factor domain structure and assembly throughout the biosynthetic pathways in endothelial cells<sup>[3-5]</sup>.** The top panel shows the different domains of VWF as it is synthesized in the ER<sup>[4]</sup>. The arrow between the D2 domain and the D' domain indicates the furin cleavage site at 764 leading to the production of the VWF propeptide (VWFpp) D1-D2 (blue) and the mature VWD protein with the domains D', D3, A1, A2, D4, C1-6 and the cysteine knot (CK). The lower panel shows the assembly of VWF into multimers in the Golgi compartment, the cleavage VWFpp (blue), and the assembly of VWF into the dimeric bouquet at the trans-Golgi network (TNG). During the translocation of proVWF to the ER the signal peptide is cleaved off, and the proVWF forms dimers in a tail-to-tail fashion through cysteines in its carboxyterminal cysteine knot domain. ProVWF dimers transit to the Golgi apparatus to assemble into multimers in a "head-to-head" fashion through the formation of intermolecular disulphide bonds between cysteine residues in the D3 (multimerization) domain<sup>[4]</sup>. This is followed by the assembly of VWF in the Golgi network. ER: Endoplasmic reticulum; VWD: Von Willebrand disease; VWF: Von Willebrand factor. Source: Valentijn and Eikenboom 2013<sup>[4]</sup>.

VWF:RCO to FVIII:Ag (VWF:Ag) ratio, combined with VWF multimeric analysis in the 1970s were the first steps

in the classification of von Willebrand disease (VWD)<sup>[7,8]</sup>. In 1973, Firkin *et al*<sup>[7]</sup> discovered increased RIPA at low





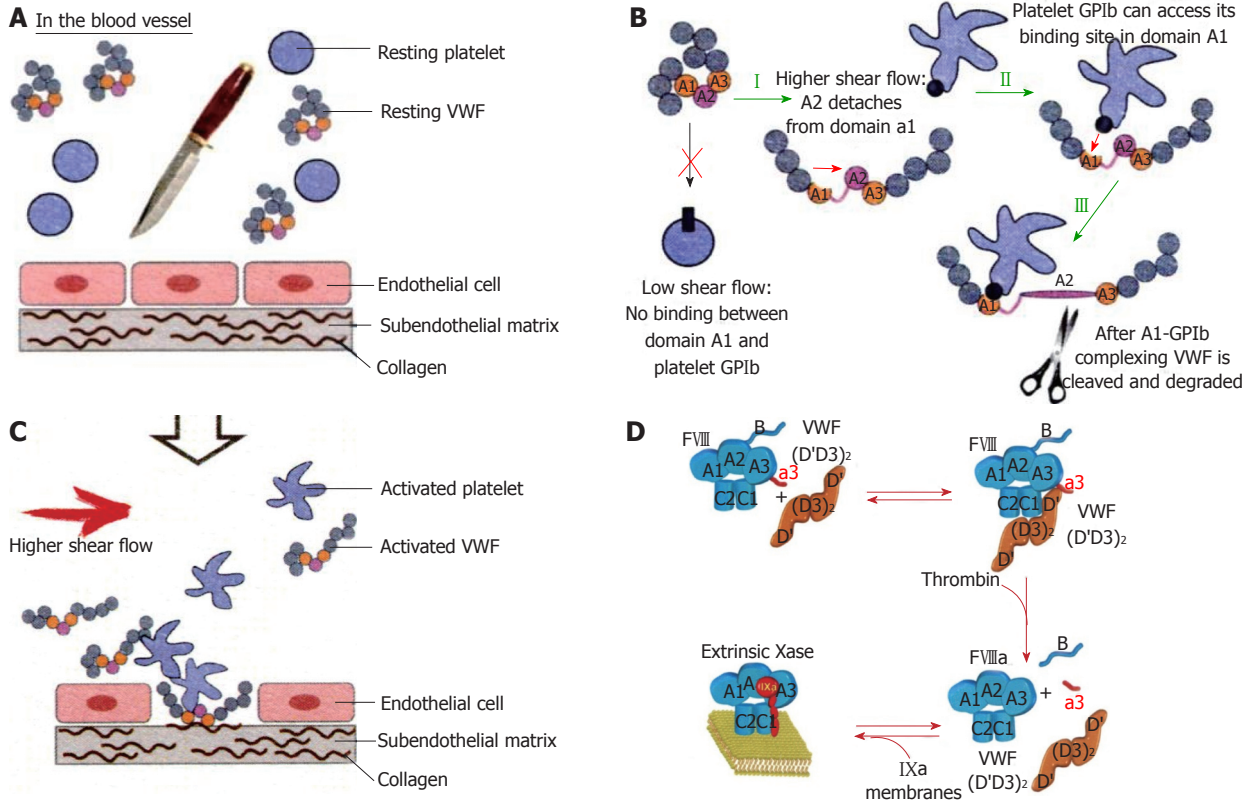
**Figure 3** A left biosynthesis pathway of Weibel-Palade body<sup>[4]</sup>. A, B: The different steps in WPB synthesis of von Willebrand factor (VWF) assembly at the level of endoplasmic reticulum (ER), at the trans-Golgi network (TGN) level (A), and VWF tubules are assembled and packed into budding vesicles prior to immature WPB formation. Homotypic fusion of WPB gives rise to the formation of WPB with different shapes. As WPB mature they become more electron-dense and reach the plasma membrane. B: Different modes of WPB exocytosis and VWF string formation on endothelial cells. In single WPB exocytosis mode, a single WPB fuses with the plasma membrane and ultra large VWF multimers (MM) are secreted. In lingering-kiss exocytosis mode (B), WPB round up and a small pore is formed with the plasma membrane, allowing the secretion of ultra large VWF MM. In multigranular exocytosis mode (B), WPB undergo homotypic fusion leading to the formation of a secretory pod that permits pooling of ultra large VWF MM prior to secretion<sup>[4]</sup>. After release, the ultra large vWF strings stick to the endothelial cell surface, attract platelets through platelet GpIb ligand and VWF GpIb receptor interaction, thereby activating the VWF cleavage site to be cleaved by ADAMTS13 at high shear stress in the endarterial circulation (C). WPB: Weibel-palade body. Source: Valentijn and Eikenboom 2013<sup>[4]</sup>.

ristocetin concentrations as a pathognomonic finding for VWD type IIB as a distinct bleeding diathesis. Ruggeri *et al.*<sup>[8]</sup> confirmed the association of heightened interaction between platelets and VWF in type IIB VWD. In contrast, RIPA was decreased or absent in type IIA VWD. The 1986 Zimmerman Classification of VWD<sup>[9]</sup> could distinguish five main variants of type 2 VWD: IIA, IIB, IIC, IIE and IID (Figure 5). Loss of large VWF multimers due to increased proteolysis into 176 kDa and 140 kDa degradation products is seen in VWD type IIA and IIB. In contrast, proteolytic VWF fragments (degradation products) are absent in VWD type IIC, IIE and IID as compared to VWF multimers in normal plasma<sup>[2,9,10]</sup>. Consequently, the loss of large VWF multimers in VWD 2C and 2E is not due to increased proteolysis, but caused by a multimerization defect due to mutations in the D1-D2 and D3 domains (Figures 6 and 7)<sup>[3,11,12]</sup>.

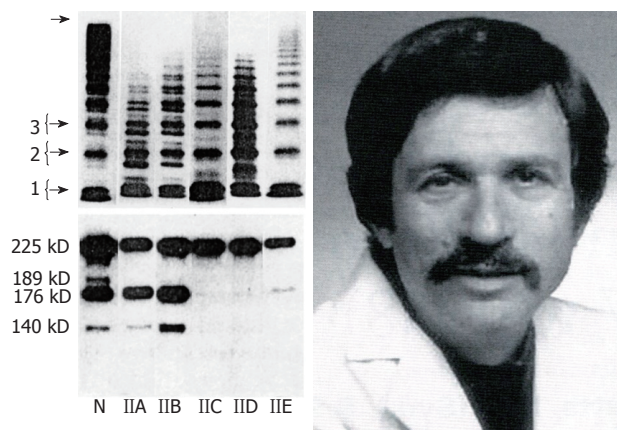
Three main categories of VWD can be distinguished: firstly, a category of recessive type 3, severe type 1 and 2C; secondly, a category of dominant type 1 and 2, and thirdly, large category of mild VWD with no

or low penetrance of bleeding manifestations<sup>[12-20]</sup>. Recessive VWD type 3, a hemophilia-like bleeding disorder with a complete absence of VWF and FVIII is caused by a homozygous or double heterozygous non-sense mutation in the VWF gene<sup>[21-23]</sup>. Recessive severe "type 1" VWD differs from "type 3" VWD by double heterozygosity for a non-sense/missense or two missense mutations with the presence of detectable VWF:Ag and FVIII:C levels between 0.09 and 0.40 U/mL<sup>[24-33]</sup>. Double null mutations in recessive type 3 VWD are distributed over all domains and exons of the VWF gene. Missense mutations causing recessive severe type 1 are mainly located in the exons 3 to 11 of the D1-D2 domains (e.g., D47H, S85P, Y87S, D141Y, D141N, C275S, W377C, I427N), and in exons 36 to 52 of the D4, B1-3, C1-2, CK domains (e.g., P2063S, C2174G, C2362F, N2546Y, C2671Y, C2754W and C2804Y)<sup>[24-33]</sup>.

The 2N mutations E787K, T791M and R816W cause a severe type 2N phenotype with less than 10% FVIII binding (FVIII:B) to VWF. Homozygous or double heterozygous R854Q mutations are the most frequent



**Figure 4** In the equilibrium state, with intact endothelial cells and no injured vessel, resting von Willebrand factor circulates as globules with resting platelets in blood (A). In this state, VWF is incapable of mediating platelet adhesion. After an injury of the endothelial cells, the activated VWF: Von Willebrand factor (VWF) interacts with exposed collagen via vWF domains A1 and A3 (orange parts) and triggers the adhesion of activated platelets via VWF domain A1 (B, C). At low shear there is no binding between VWF domain A1 and platelet GPIb. At high shear rate the VWF globules elongate and made the VWF A1 domain accessible by the dissociation of domain A1 from A2. High shear flow detaches the A2 domain from domain A1 (I). Binding between GPIb of activated platelet to the GPIb receptor of VWF (II), which is immediately followed by cleavage of VWF in the A2 domain by ADAMTS13 (III) (B). Courtesy of Dr Sandra Posch. Institute of Biophysics, Linz, Austria: sandra.posch@jku.at. FVIII:C is a heterodimer with a domain structure of A1-A2-B-A3-C1-C2 (upper left, Blue). FVIII:C circulates in complex with VWF through binding to the D'D3 domain, the FVIII binding site on VWF. Thrombin cleavage of FVIII liberates the a3 peptide and the B domain of FVIII (D), resulting in the dissociation of VWF from FVIII<sup>[6]</sup>.



**Figure 5** The 1986 Zimmerman Classification of Von Willebrand disease type IIA, IIB, IIC, IID and IIE<sup>[9]</sup> and Dr. Ted Zimmerman (1937-1988). SDS-agarose multimeric analysis of plasma VWF in normal plasma (N) and in VWD type IIA, IIB, IIC, IIE and IID. Left lower part: Immunoblots of VWF proteolytic degradation products show increased proteolysis in VWD type IIA and IIB, but not or even absent in VWD type IIC, IIE and IID (N = normal plasma). VWD: Von Willebrand disease; VWF: Von Willebrand factor.

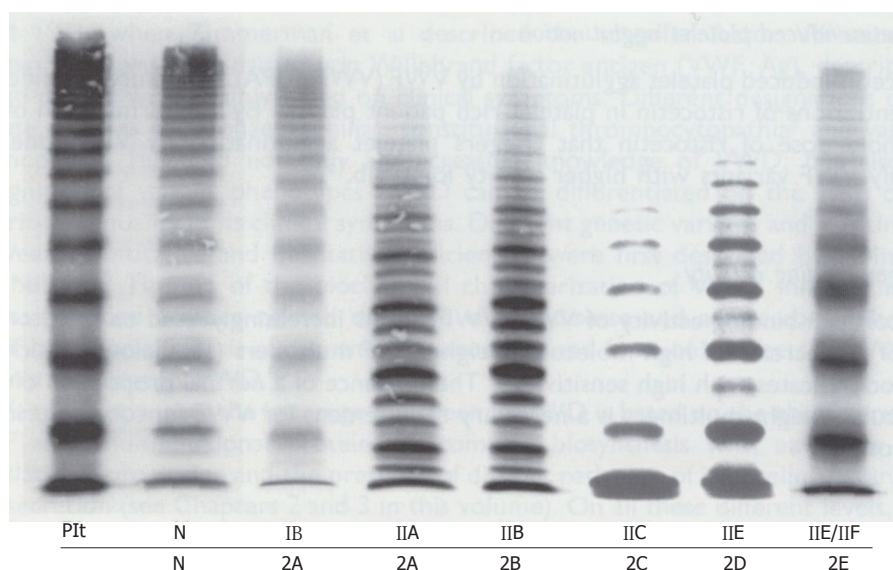
findings in type 2N and are associated with mild FVIII binding defects of around 25%<sup>[6,34,35]</sup>. A normal

multimer distribution is observed in non-cysteine mutated VWD 2N patients in whom bleeding episodes are similar to those in patients with mild/moderate hemophilia A, with bleedings occurring after trauma or surgery. Type 2N mutations that involve a cysteine (C788R/Y, Y795C, C804F and C858S/F) are associated with aberrant multimerization, poor secretion and reduced FVIII binding<sup>[34,35]</sup>. Three mutations (T791M, R816W and R845W) account for the majority of typical 2N cases with normal VWF multimers<sup>[33,34]</sup>. Patients with mild 2N VWD (e.g., homozygous R854W) can be treated for minor bleeds by DDAVP administration<sup>[18,35]</sup>. Obligate carriers of recessive type 3, recessive severe type 1 and recessive 2N VWD are heterozygous for a non-sense (null) or missense mutation, and are usually asymptomatic at VWF levels around 50 U/mL<sup>[16,32,33]</sup>.

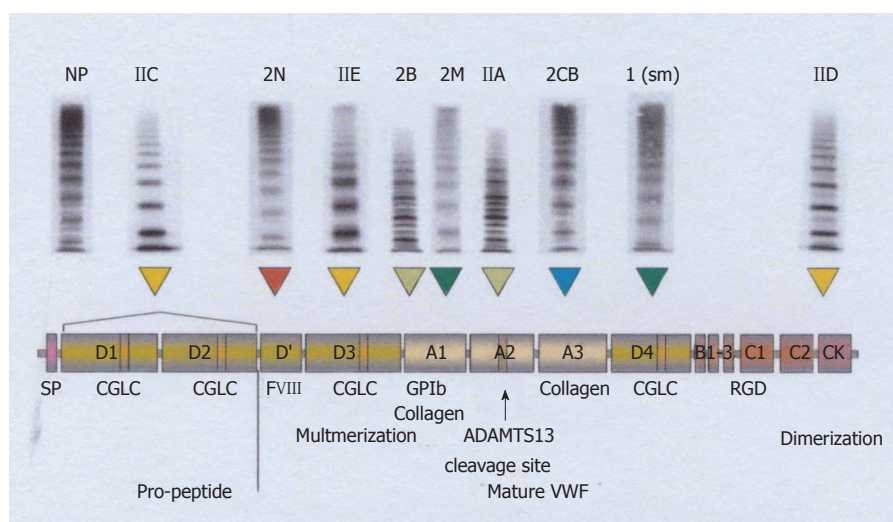
### Translation of VWD IIC, IIE, IIA, IIB and IID into 2C, 2E, 2M, 2A, 2B and 2D

The International Society on Thrombosis and Haemostasis (ISTH) classification of VWD is based on 5 relatively "insensitive" laboratory tests (FVIII:C, VWF:Ag, VWF:RCo, RIPA and VWF multimers in low resolution gels) (Table 1)<sup>[13-15]</sup>. The ISTH criteria cannot clearly





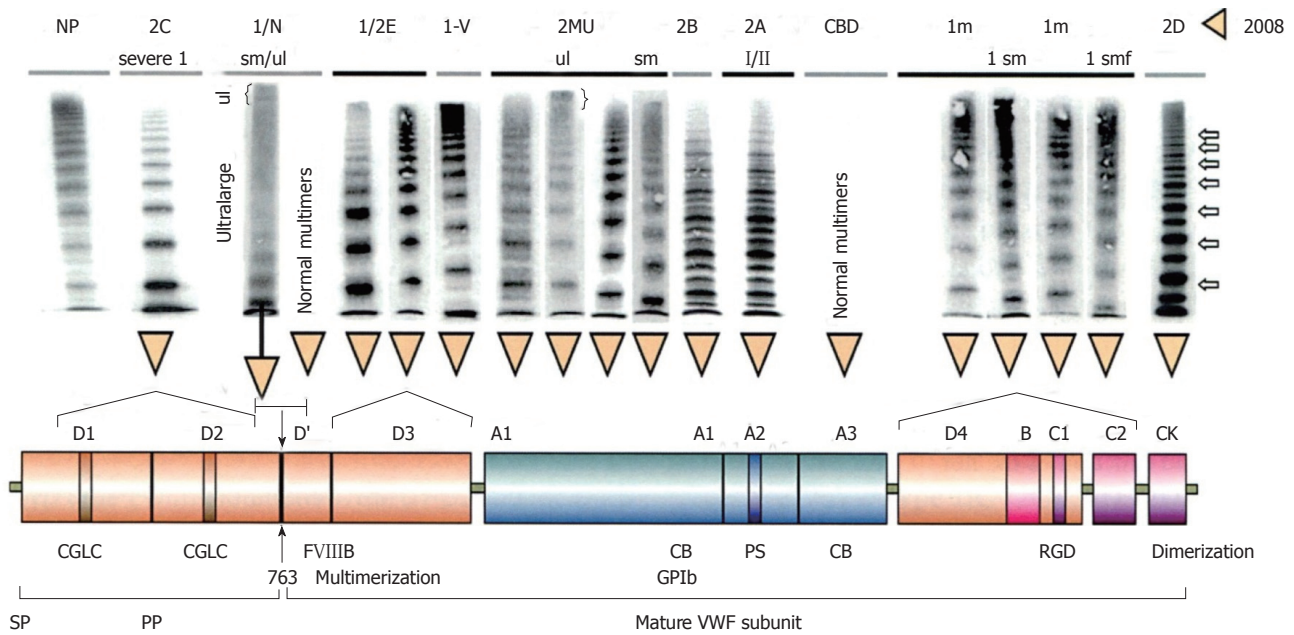
**Figure 6 Multimeric pattern.** VWF from plasma of patients with von Willebrand disease classified according to the ISTH criteria for VWD type IIA, IIB, IIC and IIE and the translation into the 2001 Hamburg criteria for 1B = 2M (not 2A), 2A, 2B, 2C, 2D and 2E anno 2001 compared to a normal control in high resolution gel concentration (1.5%) according to Schneppenheim *et al*<sup>[10]</sup>. Dominant 1B relatively lacking large VWF multimers (MM) = 2M (Michiels). Dominant IIA = 2A lack of large molecular weight MM and the outer sub-bands of the individual triplets are markedly pronounced indicating increased proteolysis as the cause of 2A. Dominant 2B cannot be distinguished from 2A by MM alone. Recessive IIC = 2C lack of large MM and absence of triplets. Low MM and especially the first band, which probably reflects protomer (dimer) and a tetramer, is markedly pronounced. IID = 2D intervening VWF band and an odd number of MM. 2E lack or relative decrease of large MM and absence of the outer sub-bands of the normal triplet structure. Triplets are lacking in 2C, 2D, 2E and 1B = 2M are lacking indicating the absence of proteolysis. VWD: Von Willebrand disease; VWF: Von Willebrand factor. Pit: Platelet VWF; N: Normal.



**Figure 7 Translation and integration of the 2006 International Society on Thrombosis and Haemostasis and the 2009 Hamburg classification.** VWD type 2 variants<sup>[20]</sup> (recessive IIC, recessive 2N, dominant IIE, 2B, 2M, IIA, 2CB 1 (sm) and IID related to clustered distribution of VWF gene mutations in the D1-D2 propeptide, D', D3, A1, A2, A3, D4 and CK domains respectively. For explanation see Figure 8: from left to right recessive 2N, recessive IIC → 2C, and dominant → IIE → 2E, 2B, 2M, IIA → 2A, 2 CB (collagen binding defect), 1 smeary pattern (sm) and IID → 2D (from ref.[20]). VWD: Von Willebrand disease; VWF: Von Willebrand factor.

distinguish the different variants of pronounced type 1, 2N, 2M and 2E VWD at VWF levels around and below 0.15 U/mL<sup>[15]</sup>. The ISTH mainly used a “lumping” instead of a “splitting” approach for the classification of type 2 VWD (Table 1). The ISTH criteria lumped several variants of VWD IIA, IIC, IID, IIE together into type 2A with loss of large VWF multimers<sup>[13-15]</sup>. The loss of large multimers in VWD 2 is due to various mechanisms: increased proteolysis in dominant 2A and 2B VWD, defective multimerization of VWF in recessive 2C

and dominant 2E, and defective dimerization of VWF (CK domain) in 2D VWD (Figures 7 and 8)<sup>[10-12,17-22]</sup>. Decreased RIPA due to loss of function in the interaction of platelet-GPIb-VWF is a typical feature of VWD 2M<sup>[18]</sup>. VWD 2M usually presents as pronounced type 1 VWD with normal VWF multimerization pattern<sup>[18,20]</sup>. VWD type 2M is frequently labeled by the ISTH classification as 2U, 2A-like or variant 2A with decreased RIPA and some loss of large VWF multimers<sup>[17,36]</sup>. VWD type Vicenza has “supranormal” VWF multimers and type



**Figure 8 Structure and function of normal von Willebrand factor protein<sup>[20]</sup>.** Mutations in the D1D2 domain prohibit the cleavage of VWFpp from mature VWF leading to a severe secretion and multimerization defects in recessive VWD 2C<sup>[16,31,33]</sup>. FVIII binding defects in the VWF D' domain either homozygous or double heterozygous causes recessive VWD 2N<sup>[34,35]</sup>. Dominant VWD type 2E due to heterozygous missense mutations in the D3 leads to a secretion clearance multimerization defect, VWD 2E<sup>[20,38,39]</sup>. Loss of function mutations in the VWF GPIb of the A1 domain induce dominant VWD 2M<sup>[18,36,37]</sup>. Dominant VWD 2A due to mutations in the A2 domain makes the mutant VWF hypersensitive to the VWF cleavage protease ADAMTS13 at the VWF cleavage site (1605-1606)<sup>[40-44]</sup>. Immediately after secretion the 2A mutated VWF is proteolysed with loss of large VWF multimers and typical triplet structure of each VWF band. Dominant VWD 2B due to gain of function mutation in the A1 domain accelerates the interaction of platelet-GPIb and VWF A1 followed by VWF proteolysis by ADAMTS 13 interaction<sup>[17,46]</sup>. This process starts immediately after secretion of the 2B mutated VWF and causes VWD 2B with loss of large multimers and typical triplet structure of each VWF band. A new category of VWD type 1 secretion defect (SD) is due to mutations in the D4,B1-3,C1-2<sup>[39,49]</sup> domains relabelled as the C1, C2, C3, C4, C5 and C6 domains of the VWF gene/protein<sup>[3-5]</sup>. Heterozygous mutations in the D4, C1-C6 domains result in VWD type 1 SD and have either normal multimers or abnormal multimers. Homozygous or double heterozygous mutations in the D4, C1-C6 domains are associated with severe VWD type 1<sup>[26-29]</sup>. Cysteine mutations in the CK dimerization domain, either heterozygous and homozygous or double heterozygous, are associated with VWD 2D<sup>[30]</sup>. VWD: Von Willebrand disease; VWF: Von Willebrand factor; CBD: Collagen binding defect.

**Table 1 Classification of von Willebrand disease according to International Society on Thrombosis and Haemostasis guidelines 1994-2007<sup>[13-15]</sup>**

1	Inherited VWD caused by genetic mutations at the VWF locus includes a broad spectrum of recessive and dominant variants of VWD
2	WD Type 1 is quantitative deficiency of VWF mainly based on a normal VWF:RCO/VWF:Ag ratio. Type 2 VWD is a qualitative deficiency of VWF as documented by a decreased VWF:RCO/VWF:Ag ratio. Type 3 refers to virtually complete deficiency of VWF
3	VWD Type 2 refers to qualitative variants with absence of high molecular weight VWF multimers and distinguishes 2A (IIA, IIB, IIE, and IID) 2B, 2M and 2N
4	VWD Type 2M or 2U is a distinct entity with decreased platelet dependent function (VWF:RCO) and presence of large VWF multimers
5	VWD Type 2A (IIA, IIB, IIE and IID) refers to qualitative variants with absence of HMW multimers, normal or decreased RIPA and decreased VWF: VWF:RCO/VWF:Ag ratio
6	VWD Type 2B is a qualitative variant with absence of HMW multimers, decreased VWF:RCO/VWF:Ag ratio and increased RIPA
7	VWD Type 2N is a mild hemophilia due to FVIII binding defect of VWF, presence of large VWF multimers, normal VWF:RCO/VWF:Ag ratio and decreased FVIII/VWF:Ag ratio

VWD: Von Willebrand disease; VWF: Von Willebrand factor; RIPA: Ristocetin-induced platelet aggregation.

1 phenotype due to increased clearance<sup>[18,32]</sup>. In type 1 phenotype the multimers are cleared too rapidly for ADAMTS13 mediated proteolysis to occur. In the ISTH classification, VWD 2N has normal VWF multimers, a typical type 1 VWD phenotype with low FVIII:C and decreased FVIII:C/VWF:Ag ratio<sup>[13-15]</sup>. Between 2001 and 2009 Schneppenheim *et al.*<sup>[10,11,20]</sup>, and Michiels *et al.*<sup>[17]</sup> translated and modified the ISTH classification of VWD type IIA, IIB, IIC and IIE (Table 1) into the European Clinical, Laboratory and Molecular (ECLM) criteria (Table 2) for VWD recessive 2C, recessive 2N, dominant 2E, 2M, 2A, 2B, 2CBD and 2D (Figures 6, 7 and 8)<sup>[32,33,37-39]</sup>.

The distinction of the dominant type 2 VWDs in the ECLM classification is based on typical VWF multimeric patterns for each type 2 VWD variant in high resolution gel concentration (1.5%)<sup>[10,11,20]</sup>.

Pronounced dominant type 1 VWD with VWF levels around and below 0.15 U/L using the ISTH criteria is seen in VWD type 1 secretion or clearance defects, and in VWD type 2E and 2M (Figure 8)<sup>[17,18,20]</sup>. Diagnostic differentiation of so-called severe type 1 VWD using the ISTH criteria remains a persistent problem in routine daily practice anno 2011 (Table 1). This can easily be overcome by the use and correct interpretation of

**Table 2 European Clinical, Laboratory and Molecular criteria of von Willebrand disease**

Mild type 1: VWF:Ag < 35%, normal VWF:CB/VWF:Ag and VWF:RCO/VWF:Ag ratio > 0.7
Type 1 with VWF:Ag above 35% with manifest bleeding can be included
Autosomal recessive VWD
Type 3 recessive with VWF:Ag and FVIII:C undetectable
Type 1 severe recessive VWD with VWF:Ag and VWF:RCo detectable < 5%, high FVIII:C/VWF:Ag ratio in particular after DDAVP
Type 2C recessive with increased FVIII:C/VWF:Ag ratio (secretion defect) and loss of large VWF multimers due to a multimerization defect caused by homozygous or double heterozygous mutations in the D1-D2 of the VWF gene (Figure 8)
Type 2N recessive with FVIII:C/VWF:Ag ratio < 0.5 due to FVIII-VWF binding defect caused by mutations in the D' FVIII-binding domain (Figure 8)
Type 2 autosomal dominant VWD 2A, 2B, 2E and 2M (Figure 8)
2A/2M: Decreased RIPA (Ristocetin Induced Platelet Aggregometry, 2B increased RIPA, decreased VWF:RCO/VWF:Ag ratio < 0.7
2A: Loss of large MM caused by increased VWF proteolysis due to mutations in the A2 domain of the VWF gene
2B: Increased RIPA (0.8 mg/mL) and thrombocytopenia with VWD type 2 due to gain of function mutation in the GpIb receptor in the A1 domain
2E: Type 1/2, loss of large multimers due to multimerization defect and increased clearance due to mutations in the D3 multimerization domain
2M: Decreased VWF:RCO/VWF:Ag ratio (< 0.6), normal VWF:CB/VWF:Ag ratio (> 0.7), decreased RIPA due to loss of function mutation in the A1 domain
2M-CBD: Collagen binding defect, VWF:RCO/VWF:Ag ratio > 0.7 and VWF:CB/VWF:Ag ratio < 0.7 due to mutation in the A3 domain

VWD: Von Willebrand disease; VWF: Von Willebrand factor; RIPA: Ristocetin induced platelet aggregation; MM: Multimers.

**Table 3 Desmopressin challenge test (0.3 µg/kg in 100 mL physiological saline intravenously over 30 min) proposed by the International Society on Thrombosis and Haemostasis**

Blood sample DDAVP	At 15 min	After DDAVP				After DDAVP 12 h
		1 h	2 h	4 h	6 h	
Ivy BT	+	-	+	-	-	+
PFA-100	+	+	+	+	+	+
RIPA	+	+	+	+	+	+
FVIII:C	+	+	+	+	+	+
VWF:Ag	+	+	+	+	+	+
VWF:RCO	+	+	+	+	+	+
VWF:CB	+	+	+	+	+	+
VWF:MM	+	+	+	+	+	+
VWF propeptide	+	+	+	+	+	+

This challenge test has been used at the Goodheart Institute, Rotterdam since 1992 to calculate the recovery and half life times of FVIII:C and VWF parameters for the diagnosis and characterization of VWD type 1, 2 and 3<sup>[18]</sup>. VWD: Von Willebrand disease; VWF: Von Willebrand factor; RIPA: Ristocetin induced platelet aggregation; DDAVP: Desmopressin; MM: Multimers.

VWF multimeric analysis and FVIII:C/VWF:Ag response curves to DDAVP<sup>[18-20]</sup>. VWF multimeric analysis using low and medium resolution gels clearly distinguishes VWD type 2A, 2B, 2E and 2M (Figure 8, middle part)<sup>[32]</sup>. The responses of FVIII and VWF parameters to intravenous DDAVP is an essential tool in the splitting approach of the ECLM classification; it allows to distinguish the various variants of dominant type 1 and 2, and elucidates the molecular differences between homozygous or compound heterozygous recessive type 3 and severe type 1 VWD<sup>[16,33]</sup>. The ECLM splitting approach uses sensitive and specific diagnostic tools with regard to structure and function defects of mutant VWF proteins (Table 2).

#### Characteristics of dominant type 1 VWD secretion defect, 2M and 2E

FVIII:C and VWF parameters in dominant VWD type 1 secretion defect are characterized by increased FVIII:

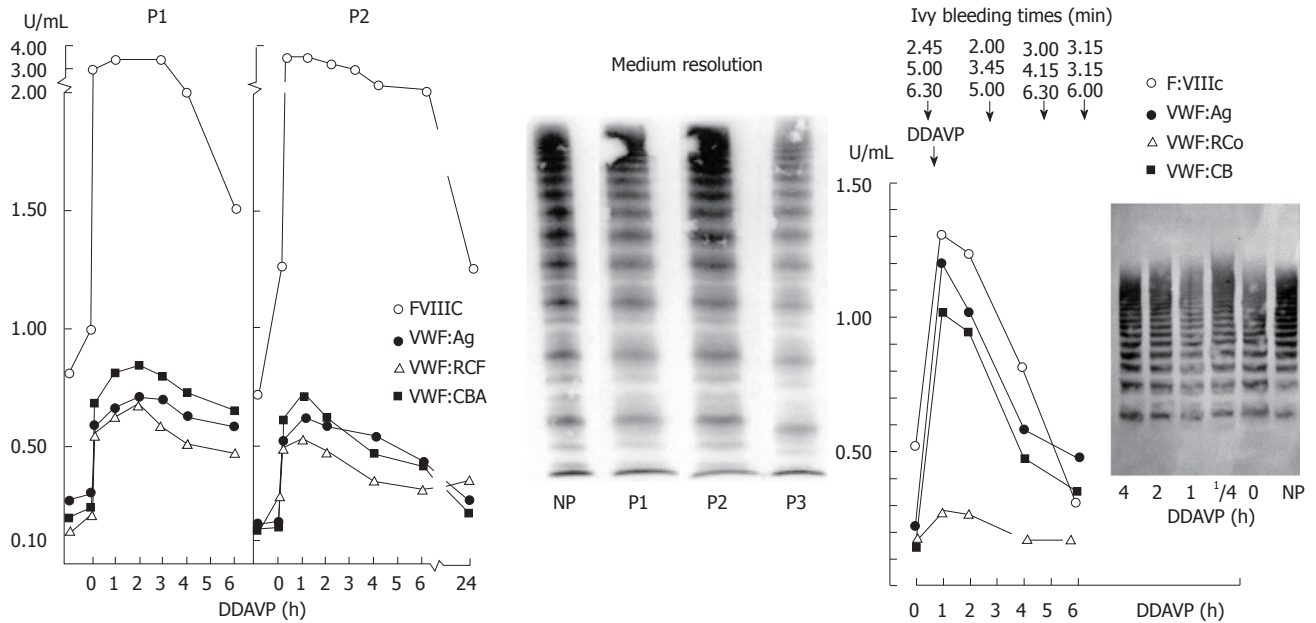
C/VWF:Ag ratio before and after DDAVP with restricted responses of the VWF parameters as compared to FVIII response to DDAVP (Figure 9)<sup>[18]</sup>. We studied three family index cases with pronounced autosomal dominant cases of VWD type 1, in whom the responses to DDAVP of all VWF parameters were very restrictive, whereas FVIII:C levels reached very high levels around 2.0 U/mL. This discrepancy of increased FVIII:C/VWF:Ag ratio and restricted responses to DDAVP of all VWF parameters is diagnostic for a pronounced VWD type 1 secretion defect<sup>[18,19]</sup> and is clearly different from VWD 2M (Figure 9)<sup>[18,20]</sup>. VWD 2M has normal VWF multimers before and after DDAVP (Figures 9 and 10) and the responses to DDAVP are poor for VWF:RCO, fairly good for VWF:CB, FVIII:C and VWF:Ag, followed by shortened half-life times of FVIII:C, VWF:Ag and VWF:CB, indicative for a clearance defect (Figures 9 and 10)<sup>[18,20,36,37]</sup>.

The response to DDAVP in a case of dominant VWD type 2E due to *W1120S* mutation in the A3 domain induced transient correction of PFA-100 closure time and restricted increase of VWF parameters from around 0.20-0.40 U/mL to around 1.0 U/mL (Figure 10). The VWD type 2E usually presents as laboratory phenotype 1 or 2, but the the multimeric pattern is characterized by loss of large multimers and the absence of the triplet structure of VWF bands due to mutations in the D3 multimerization domain (Figure 8)<sup>[38,39]</sup>.

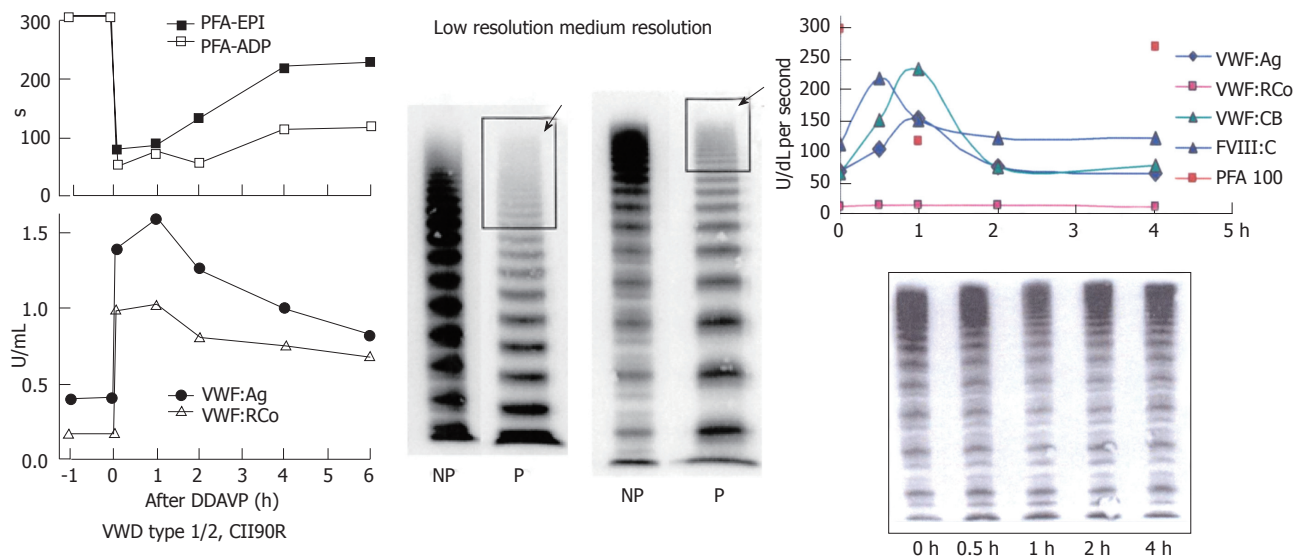
#### Dominant VWD type 2A Group I and II

The missense mutations *V1607D*, *S1506L*, *L1540P* and *R1568del* result in poor or no secretion of high molecular weight multimers due to intracellular proteolysis and impaired transport of VWF multimers between the endoplasmic reticulum and the Golgi complex (so-called VWD 2A Group 1 defect)<sup>[40-44]</sup>. Eight missense mutations in the A2 domain (*R1597W*, *G1505E*, *I1628T*, *L1503Q*, *M1528V*, *G1609R*, *I1628T*, *G1629E*, *G1631D* and *E1638K*) result in normal secretion of high molecular weight multimers, which are hypersensitive to ADAMTS13-induced proteolysis





**Figure 9 Restricted response of von Willebrand factor parameters to desmopressin.** Pronounced dominant VWD type 1 secretion defect (high FVIII:C/VWF:Ag ratio) with restricted response of VWF parameters to DDAVP as compared to completely normal responses of FVIII (high FVIII:C/VWF:Ag ratio) is indicative for VWD type 1 secretion defect (Left). Diagnostic differentiation of pronounced 1 VWD 1 secretion defect with normal VWF multimers (VWF MM according to Budde) and restricted decreased response to DDAVP of all VWF parameters in two members of one family (proband and her brother) vs pronounced case of dominant VWD 2M (Right) with normal VWF multimers before and after DDAVP<sup>[18]</sup>, a poor response of VWF:RCo to DDAVP and fairly good responses to DDAVP of FVIII:C, VWF:Ag and VWF:CB followed by shortened half life times of FVIII:C, VWF:Ag and VWF:CB indicative for rapid clearance defect. Dominant VWD type 2M (Michiels) is featured by loss of function mutation in the A1 domain, normal multimers, decreased to zero RIPA, low VWF:RCo activity, a secretion defect and rapid clearance<sup>[18]</sup>. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; NP: Normal plasma; P: Patient.

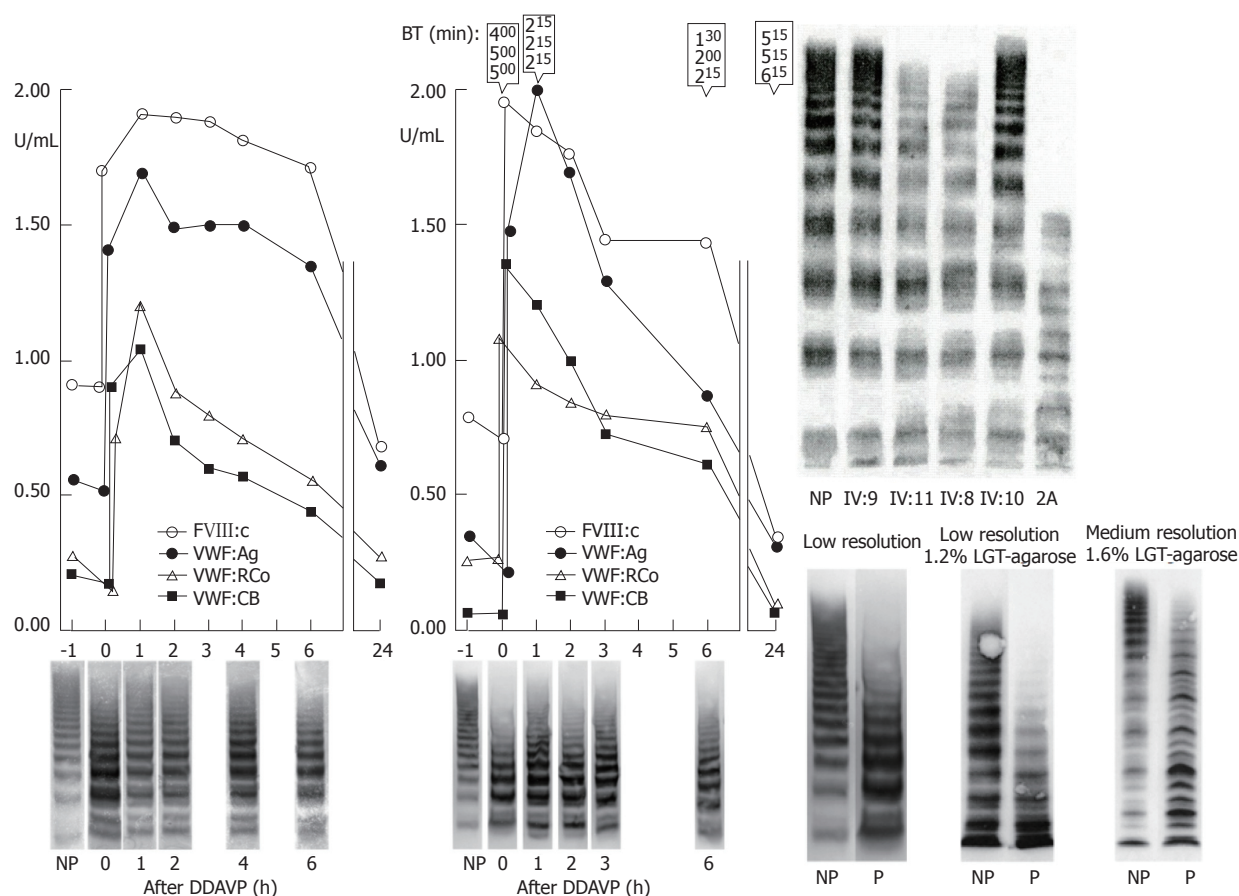


**Figure 10 Von Willebrand disease 2E (left) and von Willebrand disease 2M (right).** Left: Dominant VWD type 2E: multimerization defect with loss of large VWF multimers to W1120S mutation in the A3 domain. DDAVP induced transient correction of PFA-100 closure time and restricted increase of VWF parameters from around 0.20-0.40 U/mL to around 1.0 U/mL. In VWD type 2E, VWF multimeric pattern is characterized by a lack or relative decrease of large multimers and the absence of the outer sub-band of the normal triplet structure. Medium resolution gel according to Budde *et al.*<sup>[12]</sup>. Right: VWD 2M: Poor response of VWF:RCo to DDAVP, normal VWF multimers before and after DDAVP and good responses of FVIII, VWF:Ag and VWF:CB followed by shortened half-life time indicating rapid clearance defect of the FVIII-VWF complex on top of loss of VWF:RCo function in VWD 2M<sup>[20]</sup>. Medium resolution gel according to Budde *et al.*<sup>[12]</sup>. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; PFA-EPI: Platelet function analyzer, epinephrin; PFA-ADP: Platelet function analyser adenosine di-phosphate; NP: Normal plasma; P: Patient.

(so-called VWD 2A Group 2 defect)<sup>[40-43]</sup>. VWF of severe VWD 2A Group I is already proteolysed in endothelial cells before secretion, whereas VWF in mild to moderate VWD 2A Group II is secreted as large multimers, which

after secretion from endothelial cells are proteolysed due to hypersensitivity to ADAMTS13<sup>[40-44]</sup>.

Dominant VWD type 2A mutation V1499E in a large Dutch family is featured by normal RIPA, loss of large



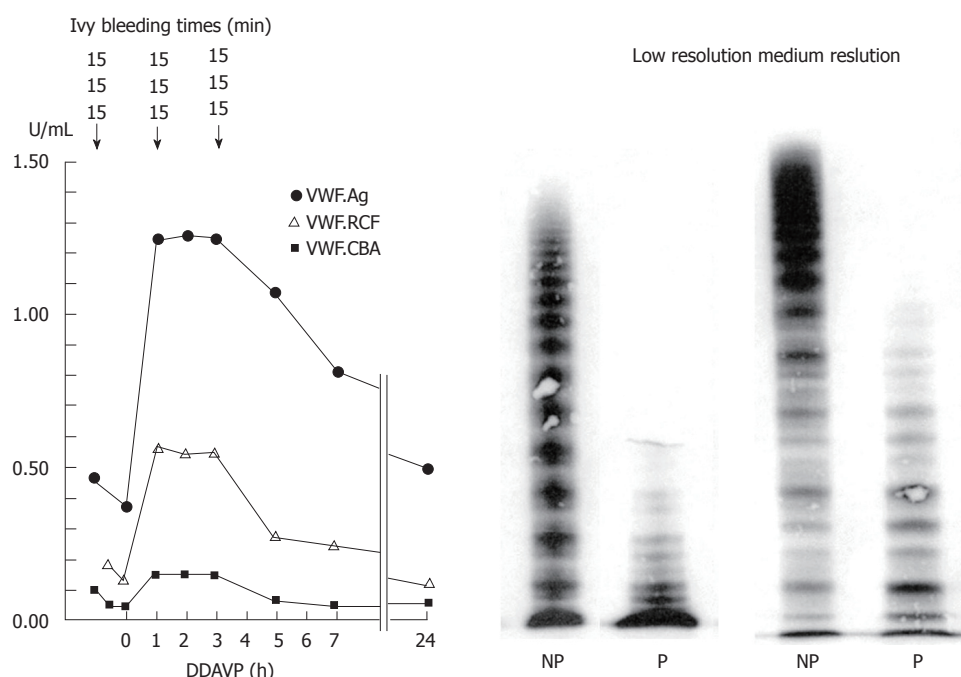
**Figure 11 Dominant von Willebrand disease type 2A mutation V1499E is featured by a normal ristocetin-induced platelet aggregation assay.** The loss of largest VWF multimers and increase of intermediate and small VWF multimers in low resolution gels (VWF multimeric pattern before DDAVP, lower left)<sup>[43,44]</sup>. The responses to DDAVP of FVIII:C and von Willebrand factor antigen (VWF:Ag) are normal. The responses to DDAVP of the functional VWF:RCo and VWF:CB are restricted to about 1 U/mL 1 h post-DDAVP with transient correction of Ivy bleeding times and transient reappearance of large VWF multimers in two cases of moderate dominant VWD type 2A (mutation V1499E). As compared to VWF:Ag and FVIII:C, the half life times of VWF:RCo and VWF:CB are shortened due to increased proteolysis of VWF multimers (Left). Lower right: Please note that the VWF multimers in low resolution gels in the Rotterdam laboratory and in the Hamburg Laboratory (Budde, middle lanes) clearly show the absence of large VWF multimers and no triplet of the individual VWF bands. The typical triplet structure of the individual VWF bands diagnostic for VWD type 2A was only seen in the medium resolution gels (right lanes) according to Budde. Upper right: The multimeric analysis of VWF from affected patients from the large Dutch family with dominant V1499E mutated VWD 2A in a third laboratory (Amsterdam)<sup>[43]</sup> show the loss of the largest VWF multimers as shown for 2 affected cases (IV:8 and IV:11) as compared to normal (NP) and 2 non-affected family members (IV:9 and IV:10). The loss of large multimers in V1499E mutated VWD patients was less pronounced as compared to a case of typical VWD 2A with the loss of large and some of the intermediate VWF multimers and a typical triplet structure of each VWF band in that laboratory<sup>[43]</sup>. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; NP: Normal plasma; P: Patient.

VWF multimers and increase of intermediate and small VWF multimers in low resolution gels (VWF multimeric pattern before DDAVP, Figure 11, lower left)<sup>[43,44]</sup>. The responses to DDAVP are normal for FVIII:C but restricted for the functional VWF:RCo and VWF:CB to about 1 U/mL 1 h post-DDAVP. Transient correction of Ivy bleeding times was associated with temporary reappearance of large VWF multimers indicating that the mutation V1499E belongs to VWD 2A Group II (Figure 11). The multimeric pattern of the V1499E mutant VWF was studied in three different laboratories. Low resolution gels in two laboratories clearly show the absence of large VWF multimers but no clear triplets of individual VWF bands (Figure 11). The triplet structure of the individual VWF bands diagnostic for VWD type 2A was only seen in the medium resolution gels (right lanes, Figure 11). Severe VWD 2A Group I is characterized

by pronounced triplet structure, absence of RIPA and prolonged Ivy bleeding times as shown in our case with the S1506L mutation in the A2 domain (Figure 12). The poor responses to DDAVP of the VWF parameters are completely in line with impaired assembling, transport and proteolysis of intracellular VWF multimers seen in severe VWD 2A Group I caused by mutations like S1506L (Figure 12).

### Dominant VWD type 2B

The key feature of VWD 2B is the loss of large VWF multimers (Figure 8, Table 2) due to increased proteolysis caused by increased interaction of platelets and mutated VWF in the A1 domain (increased RIPA)<sup>[18,20,45,46]</sup>. The process of increased VWF-GpIb-platelet interaction of mutant VWF in VWD 2B starts as soon as the mutant VWF enters the circulation (Figure 4).



**Figure 12** Absence of large and intermediate von Willebrand factor multimers in severe dominant von Willebrand disease type 2 A, with absence of ristocetin-induced platelet aggregation and strongly prolonged Ivy bleeding times in a case with the *S1506L* mutation<sup>[44]</sup>. The responses of VWF parameters to DDAVP are very poor with no correction of Ivy bleeding times (BT) and no re-appearance of large VWF multimers in this case with dominant severe VWD 2A Group I indicating impaired assembling, transport and proteolysis of intracellular VWF multimers caused by the mutation *S1506L* near to the VWF cleavage site in the A2 domain of the VWF gene. VWD: Von Willebrand disease; VWF: Von Willebrand factor; DDAVP: Desmopressin; NP: Normal plasma; P: Patient.

Clumps of mutant VWF-platelets are cleared from the circulation leading to thrombocytopenia upon DDAVP or stress. Federici *et al.*<sup>[46]</sup> evaluated the clinical and molecular predictors of thrombocytopenia and the risk of bleeding in 67 VWD 2B patients from 38 unrelated families. Thrombocytopenia was found in 30% at baseline and in 57% after stress conditions in only those with pronounced VWD 2B carrying the mutation<sup>[46]</sup>. Thrombocytopenia did not occur in 16 patients (24%) from 5 families with mild VWD 2B carrying the *P1266L* or *R1308L* mutation<sup>[46]</sup>. The *P1288L* and *R1308L* mutations are associated with a mild type 1 variant of VWD 2B with normal VWF:RCO/VWF:Ag ratios of 0.9 and 0.8 respectively, also seen in *P1266L*-mutated VWF in VWD Malmo and New York VWD phenotype 1B, who do have a mild bleeding illness with normal VWF:RCO/VWF:Ag ratios consistent with a laboratory VWD type 1B phenotype with increased RIPA<sup>[17,18]</sup>.

## MYSTIFICATIONS AROUND ISTH-DEFINED VWD TYPE 1

The European (EU) study on ISTH-defined type 1 VWD, named EU MCMDM-1VWD<sup>[44,45]</sup>, involved twelve partners in nine European countries, and aimed to recruit the whole spectrum of patients diagnosed by referring centres as having type 1 VWD, including the more severe and mildest cases, to try and represent the range of patients seen by other centers diagnosing type 1 VWD. The EU MCMDM-1VWD study recruited

148 evaluable families. The Canadian type 1 VWD study recruited 124 families from 13 Haemophilia Centres across Canada<sup>[47-49]</sup>. Analysis at both the recruitment centre and central laboratory of plasma samples was obtained on at least two occasions. The EU and Canadian VWD 1 multi-centre national/international studies have provided new insights into the molecular pathogenesis of type 1 VWD. In 2008, 117 different VWF mutations (80% missense, about 10% non-sense and about 10% splice site or transcription) were reported to be associated with type 1 VWD and were included in the ISTH VWF mutation database. When comparing the ECLM criteria in Table 2 with the ISTH criteria in Tables 1 and 3, there are several misclassifications of VWD in the European MCMDM-1VWD study. The European MCMDM-1VWD study did contain typical examples of recessive or heterozygous VWD type 2N (heterozygous *R816W*, *R854W* and *R854W/R924Q*, *R854W/null*) and typical cases of VWD 2M (*D1277-E78delinsl*, *R1315C*, *R1342CR1374C*, *R1374H*, *G1415D I1416N*)<sup>[44]</sup>. There were 3 cases with typical 2M VWD with abnormal multimers and 2 mutations (*R1315H/P1266L*, *R1315L/R934Q* and *R1374C/P2145S*) in which the 2M mutation has a dominant negative effect on the VWD type 1 mutation<sup>[47]</sup>. The mutations in exon 26, D3 domain, *R1130R/G/F*, *W1144G*, *Y1146C* and *C1190R* usually present with a laboratory phenotype VWD 1 but have abnormal VWF multimers with typical features of VWD 2E<sup>[39,49]</sup>. The majority of mild type 1 VWD cases in the Canadian study were in fact carriers of recessive severe type 1 VWD heterozygous for mutations mainly



located in the D1-D2 and D'domains (K762E, M771I, P812fs, Exon 21 skip, R924Q, R924W and C996E)<sup>[50,51]</sup>. A minority of ISTH-defined type 1 VWD patients in the Canadian study had missense mutations in the D3 (S1024fs, I1094T, in fact VWD 1/2E), A1 (F1280fs, R1379C, P1413L, Q1475X, in fact VWD 2M) or A2 domain (R1583W, and Y1584C)<sup>[52-54]</sup>. The combination of C1584/bloodgroup O is rather frequent and typically shows a good to normal response to DDAVP<sup>[53,54]</sup>. Carriers of recessive VWD type 3 or severe recessive type 1 VWD are asymptomatic or may manifest mild bleeding in particular when associated with blood group O<sup>[16,33]</sup>.

## CONCLUSION

The classification of VWD remains an important problem to this day. Several classifications have been proposed but none have proved to be ideal. The current ISTH classification is a lumping together of types based upon easily available but "insensitive" laboratory techniques, with especially type 2A as a collection of different pathophysiological entities. The ECLM criteria for VWD try to improve on this classification by including also the response to DDAVP, and have more regard to pathophysiology and the VWF domain structure.

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