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Review Article

Basic Insights into the Molecular Etiology and Pathology of Von Willebrand Factor (VWF) Behind the ISTH And ECLM Classifications of Von Willebrand Disease Using a Complete Set of VWF Parameters

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Abstract

The authors present a novel focus on the molecular basis of von Willebrand disease (VWD) phenotype classifications behind the International Society on Thrombosis and Haemostasis (ISTH) and the European Clinical Laboratory and Molecular (2019 ECLM). The ECLM molecular-based classification of VWD type 1, 2 and 3 in this study starts with the detection of the mutation defect located to the D1, D2, D", D3, A1, A2, A3, D4, C1 to 6 or the CK domains of the VWF gene and than phenotyping each individual VWD patient by a complete set of FVIII:C and von Willebrand factor (VWF) parameters. Recessive VWD type 3 is caused by homozygous or double heterozygous null/null mutations and present with pseudo-hemophilia A first decribed by Erik von Willebrand. Recessive severe VWD type 1 due to homozygous or double heterozygous missense mutation in the D1 domain is featured by persistence of proVWF as the cause of secretion/multimerization and FVIII binding defect mimicking VWD type 3 (pseudo-hemophilia A). Carriers of heterozygous/wild type mutations in the D1 and D2 domains have decreased values for VWFpp, VWFpp/Ag ratios (0.51 to 0.99) indicating a secretion defect. ISTH defined VWD patients type 1 or 2 due to a multimerization defect in the D3 domain typically have VWF:RCo/Ag and VWF:CB/Ag ratios around the cut off level of 0.70 are diagnosed as VWD type 1E and type 2E patients when the ECLM criteria are applied. Twenty two reported VWD type 1E and 2E patients due to different missense mutations in the D3 domain are multimerization defects associated with an additional secretion defect (increased FVIII:C/VWF:Ag ratio) and/or clearance defect (increased VWFpp/Ag ratio). Ristocetine Induced Platelet Agglutination (RIPA) is decreased in VWD 2M, increased in VWD 2B , normal in VWD 1, normal in mild to moderate 2A, but decreased in pronounced VWD 2A, 2C and 2D. Dominant VWD 2A caused by mutations in the A2 domain have decreased VWF:RCo/Ag and VWF:CB/Ag ratios due to proteolytic loss of large VWF multimers. Dominant VWD 2B caused by gain of RIPA function mutations in A1 domain have decreased VWF:RCo/Ag and VWF:CB/Ag ratios due to spontaneous platelet-VWF interaction *in vivo* followed by proteolytic loss of large VWF multimers. Dominant VWD 1m caused by mutations in the A3 domain is featured by normal VWF:RCo/Ag ratio and decreased VWF:CB/Ag ratio (2CB) or a decreased VWF:RCo/Ag ratio and normal VWF:CB/Ag (2M). The majority of VWF mutations in the D4 and C1 to C6 result in VWD phenotype 1 secretion defect (SD) with smeary (1sm) or normal (1m) multimers with normal clearance or a minor clearance defect. VWD type 1C (Vicenza) due to heterozygous R1205H/WT mutation in the D3 domain uniformally result in a prounounced FVIII/VWF clearance defect featured by a high VWFpp/Ag ratio and normal FVIII:C/VWF:Ag ratio. Heterozygous S2179F/WT mutation in the D4 domain is featured by pronounced VWD type 1m due to a Secretion Defect (SD) with increased FVIII:C/VWF:Ag ratio in combination with a Clearance (C) defect with increased VWF:pp/Ag ratio.

Keywords: Von Willebrand disease; Von Willebrand factor; ISTH criteria; ECLM classification; VWF gene mutation; Molecular biology; VWF domain; VWF assay; FVIII:C; Ristocetine cofactor; Platelet function analyzer PFA-100

Introduction

We here present a novel focus on the molecular etiology and pathology of von Willebrand factor (VWF) protein behind the International Society of Thrombosis and Haemosiasis (ISTH)

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classification of von Willebrand disease related to mutation location in different domains of the VWF gene. The standard set of von Willebrand factor (VWF) parameters to diagnose and differentiate von Willebrand disease (VWD) type 1 from type 2 include Platelet Function Analyser Closure Time (PFA-CT), FVIII: coagulant activity (FVIII:C), VWF antigen (VWF:Ag), VWF ristocetine cofacor (VWF:RCo), VWF collagen binding (VWF:CB), FVIII binding to VWF (FVIII:BD), Ristocetine Induced Platelet Aggregation (RIPA), analysis of VWF multimers and the responses of FVIII:C and VWF parameters to DDAVP [1-4]. The original description of "Hereditary Pseudohemophilia A" by Erik von Willebrand appeared to be a recessive von Willebrand disease (VWD) type 3 caused by homozygous mutation P812rfs, 2680delC=null mutation in exon 18 [5].

Recessive VWD type 3 disease is caused by homozygous or double heterozygous null/null mutations distributed in any of the VWF gene domains and typically characterized by prolonged bleeding time and APTT, FVIII:C levels below 2%, undetectable VWF: Ag, VWF: RCo and VWF:CB and absence of Ristocetin Induced Platelet Aggregation (RIPA) [2,5-7]. VWD type 3 patients with virtual complete VWF deficiency are homozygous or compound heterozygous for two null alleles (gene deletions, stop codons, frame shift mutations, splice site mutations, and absence of mRNA) in the majority and rarely compound heterozygous for a null allele and a missence mutation or homozygous for a missence mutation. Reports on severe recessive VWD compound heterozygous for a null allele and a missense mutation and homozygous missense or double heterozygous for two missense mutations are associated with very low but measurable FVIII:C and VWF:Ag in particular after DDAVP and should be reclassified as severe recessive type 1 VWD [2,5-7]. Homozygous missense or compound missense/null mutations related to recessive severe type 1 VWD have been indentified in the VWF prosequence D1 and D2 domains, the D4, C1-6 (formerly B1-3, C1-2) and CK domains [6,7]. Missense mutations either homozygous, double heterozygous or associated with a null allele result in severe type 1 VWD caused by mutations restricted to the D1-D2 domains (D47H, S85P, Y87S, D141Y, D141N, C275S, W377C, I427N), to the D4, C1-6 (B1-3, C1-2) and CK domains (P2063S, C2174G, C2362F, N2546Y, C2671Y, C2754W, and C2804Y) [7]. Mild VWD type 1patients with 'Low" VWF levels between 0.30 and 0.60 and PFA-CT values between the upper level of normal and 300 seconds are suspious for carrier state of VWF null or recessive or dominant heterozygous type 1 VWD due to single missense mutations in the D1, D2, D3, D4 and C1-6 domains [7]. Increased FVIII:C/VWF:Ag ratio is related to a Secretion Defect (SD) with restricted response of all VWF parameters to DDAVP. Decreased FVIII:C/VWF:Ag ratio below 0.5 and normal VWF:RCo/VWF:Ag ratios above 0.7 is typically seen in VWD type 2N [1-6]. VWD 2N is a mild hemophilia with normal PFA-CT and VWF function.

Classification of von Willebrand Disease 1980-2019

The classification of dominant VWD type 2 into 2A and 2B started in 1980 with the discovery of heightened interaction of Ristocetine Induced Platelet Aggregation (RIPA) and functional abnormal FVIII/VWF protein in the presence of ristocetine in dominant VWD type IIB (2B), but decreased or absent in dominant VWD dominant IIA (2A) (Figures 1 and 2) [8-11]. Pronounced increase of degraded proteolytic band and triplet structure of each VWF band is the hall mark of VWD type 2A due to mutations (IIA in figures 1 and 2) in the A2 domain and caused by proteolysis of VWF at the VWF cleavage site (1605-1606 A2 domain) due to hypersensitivity of VWF to cleavage protease ADAMATS13 [12-14]. Proteolysis of VWF at the VWF cleavage is also seen to a less extend in normal subjects and in heterozygous Y1584C/WT type 1 VWD patients [9-11,15]. The RIPA gain of gain of function mutation in the A1 domain in VWD 2B results in spontaneous interaction of platelet GPIb-A1 VWF interactions *in vivo* followed by increased proteolysis of VWF at the VWF cleavage (1605-1606 A2 domain) with increase of proteolytic band and triplet structure of each VWF band similar as seen in VWD 2A [9-11,16]. The laboratory phenotypes of VWF parameters in VWD type 2A and 2B are similar characterized by prolonged BT, consistently low VWF:RCo/Ag and VWF:CBA/Ag ratios, absence of high and some of the intermediate VWF multimers with pronounced triple structure

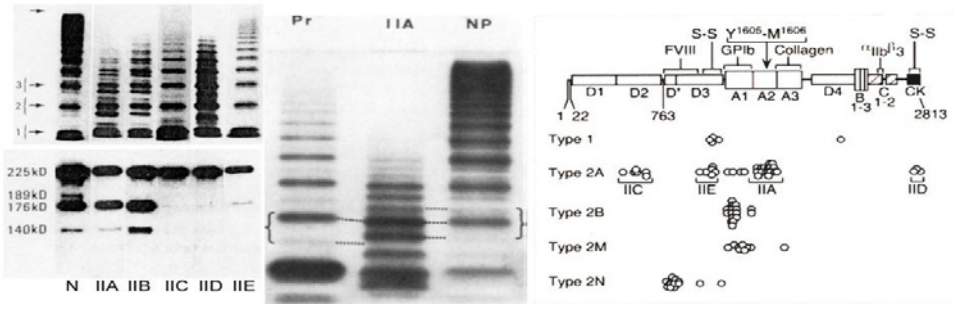


Figure 1: Left: The Ruggeri Zimmerman classification of VWD based on SDS-agarose multimeric analysis of plasma VWF in normal plasma (N) and in VWD type IIA, IIB, IIC, IIE and IID [8-11]. The normal VWF multimeric pattern (N) displays the presence of large multimers and a typical triplet structure of the individual bands. As compared to Normal Plasma (NP) VWD IIA and IIB is typically featured by loss of large multimers with pronounced proteolytic triplet structure of the individual bands due to increased proteolysis of VWF at the VWF cleavage site (1605-1606) in the A2 domain of the VWF gene [12]. Immunoblots of reduced VWF show increase of proteolytic degradation products in VWD type IIA and IIB, and absence of proteolytic degradation products in VWD type IIC, IIE and IID as compared to normal plasma (N) [8-11]. **Middle.** Discovery of recessive VWD IIC (Pt Lane) showing the lack of triplets *vs* IIA with triplet structure of each band (IIA Lane) by Ruggeri et al., [17]. **Right.** The ISTH Classfication of von Willebrand Disease type 2 according to Sadler et al 1994-2006 [19-21] and Meyer et al., 2001 [22] defines all variants of VWD type 2 with lack of large VWF-MM in low SDS resolution gel as 2A including recessive IIC (D2 domain) dominant IIE (D3 domain), IIA (A1 and A2 domains), 2B and 2M (A1 domain) 2N (D'D3 domain) and IID (CK domain).

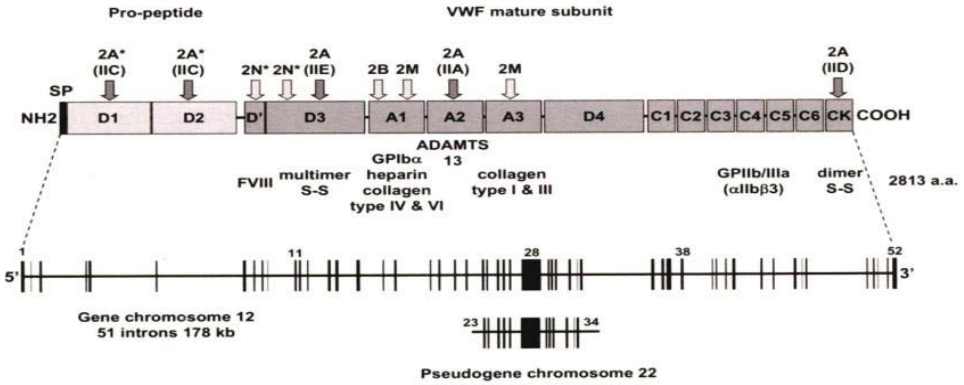


Figure 2: Structure and function relationship of von Willebrand factor gene and pseudogene and the 2017 ISTH classification of von Willebrand Disease (VWD) [25-28]. At time of secretion of proVWF the propetide D1 and D2 domains are split off from the VWF mature subunit subunit at the furin cleavage site 763. The mature VWF subunit circulates bound to FVIII:C and consists of six sequential functional domains including FVIII-binding domain D', multimerization domain D3, the platelet GPIb-alfa and collagen binding domain A1, ADAMTS13 induced VWF cleavage site (1605-1606) domain A2, the ADAMTS13 binding site domains D4, C1-6, the platelet GPIIb/IIIa binding domain C4 and the dimerization domain CK [25-28]. At sites of tissue injury thrombin split off FVIII:C from VWF, and platelet GPIb-alfa and GPIIb/IIIa subsequently interacts with VWF at A1 and C4 followed by cleavage of VWF at 1605-1606 into proteolytic band by ADAMTS13 already bound to VWF secretion from endothelial cells [27,28]. Location of mutations in the VWF gene corresponds to ISTH defined type 2A (including dominant IIA, IIC, IIE, IID showing loss of large VWF multimers, which show characteristic multimer patterns of increased proteolysis in VWD IIA (2A), defective multimerization in VWD IIE (2E), loss of ristocetine induced platelet agglutination (RIPA) in VWD 2M, gain of RIPA function mutation in A1 VWD 2B, defective dimerization defect in VWD IID (2D) and defective multimer assembly leading to secretion/multimerization defect caused by mutation in the D1 and D2 domain in recessive VWD IIC (2C) and recessive severe type 1 (Schneppenheim et al., 2011, Baronciani et al., 2017, Michiels et al., 2017) [25-28].

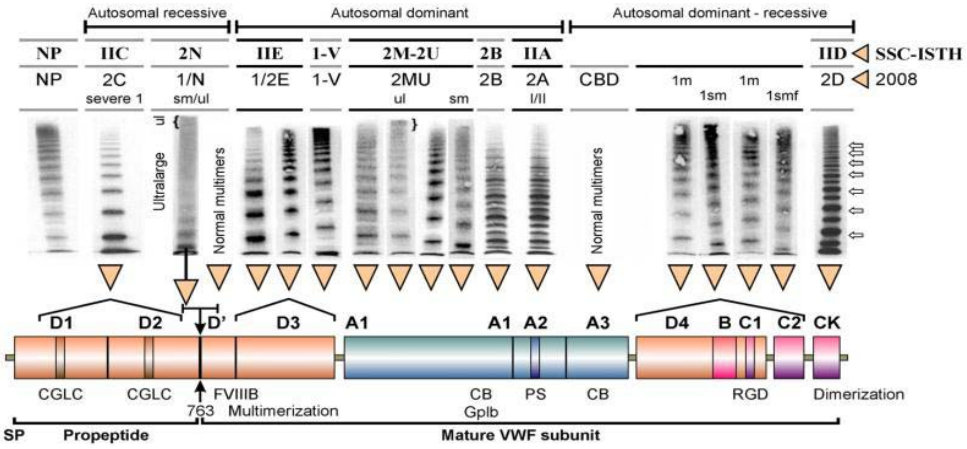


Figure 3: Translation of the 1994-2017 ISTH criteria in figures 1 and 2 into the 2008-2018 ECLM classification for recessive and dominant von Willebrand Disease (VWD) type 2 as defined in table 1. Proteolysis of VWF is a normal event in normal individuals, and increased in type 2A (IIA) and 2B (IIB) VWD showing pronounced triplet structure of each band (Figure 1) as the result of increased proteolysis of large VWF multimers with increase of 176 kDA and 140 kDA VWF fragments (figure 1). Proteolysis of VWF is minimal in type IIC (2C), IID (2D) and IIE (2E) variants with aberrant multimeric structure of individual oligomers lacking the triplet structure of VWF bands (Figure 1) and absence of degraded VWF fragment. Principles to translate the ISTH into the ECLM classification is based on the molecular etiology of VWF multimer pathology in variants of VWD related to mutations in D1, D2, D', D3, A1, A2, A3 and D4-C1-6 domains (Figure 2): in particular VWD severe 1/hemophila in D1, VWD IIC → 2C in D2, VWD 2N in D', VWD IIE → 1/2 E in D3, VWD IIB → 2B in A1, VWD 2M in A1 VWD, VWD IIA 2A in A2, Collagen Binding Defect (CBD) with normal VWF multimers (1m) or smeary VWF multimers (1sm) in D4-C1-6 domains (Gadisseur et al 2009, Budde & Schneppemheim 2011, Michiels et al 2016, 2017, Baronciani et al 2017) [2,15,16,25-28].

of individual VWF bands, and increased VWF degradation products (Figures 1 and 3) [6,9,10]. VWD 2B differs from 2A by increased RIPA in VWD 2B. RIPA is normal in mild and moderate VWD 2A and decreased in pronounced VWD 2A due to gain of RIPA function mutation in the A1 domain the mutated 2B VWF, which spontaneously interact with the platelet GPIb receptor followed by increased proteolysis at the VWF cleavage site 1605-1606 in the A2 domain [16]. Patients with severe VWD 2B respond to DDAVP with thrombocytopenia due to increased interaction of 2B mutated VWF protein and platelet GPIb receptor, which result in *in vivo* platelet aggregates and clumps followed by *in vivo* proteolysis of VWF at the VWF cleavage site in the A2 domain [16]. In 1980 Ruggeri et al., first described a recessive case of VWD IIC (2C) showing that the large multimers of von Willebrand factor in VWD 2C were lacking both from plasma and platelets due to a secretion multimerization defect of VWF from endothelial cells (Figure 1). The large VWF multimers did not appear in the circulation after infusion of DDAVP (1-Deamino-8-D-arginine-Vasopressin) [17]. Mannucci et al nicely confirmed

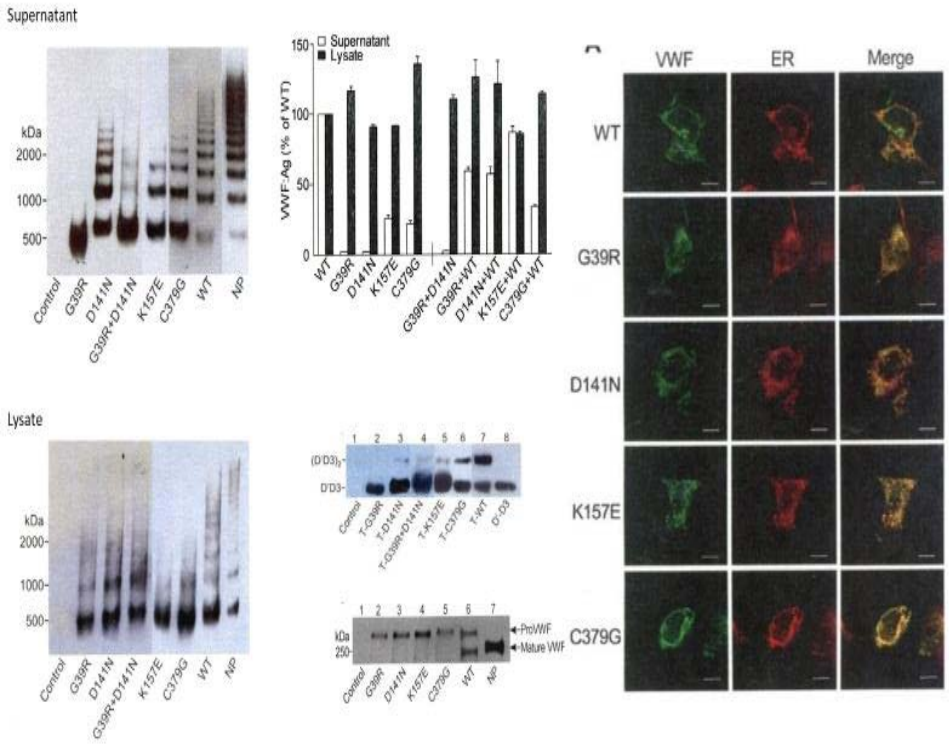


Figure 4: Upper Left, Upper Middle: Expression studies of recombinant (r) rG39R, rD141N and rD141N show hardly any VWF:Ag secretion in the supernatant by recombinant rG39R, rD141N and rG39R+D141N mutants and about 25% of rVWF:Ag was secreted in the supernatant by rK157E and rC379G mutant VWF:Ag as compared to 100% in the supernatant by rWT-VWF:Ag. **Lower Left, Upper Middle:** Expression studies show a bold predominany pro-VWF dimer band in cell lysates but mature VWF multimeric bands were hardly detectable in rG39R, rD141N, rK157G and rC379G compared to rWT-VWF showing a faint proVWF band and normal mature VWF multimeric pattern. **Middle Middle:** The D1 mutants of rVWF show the absence of VWF multimerization in rG39R and rG39R/D141N and a less pronounced multimerization VWF defect in rD141N and rC379G as compared to normal VWF multimerization of rVWF-WT. **Lower Middle:** Lysates (right) show the persistence of proVWF band and supernatants (left) show defective (D'D3) multimerization of rVWF mutants rG39R, rD141N, rK157G mutations in the D1 domain and partially defect D'D3 multimerization of the rC379G mutation in the D1 domain of VWF. **Right:** Immunofluorescence staining in Bovine Endothelial Cell (BOEC) growth studies of rVWF: 75% of rG39R, 85% of rD141N 69% of rK157E and 74% of rC379G was retained in the endoplasmatic reticulum (ER) as compared to 38±9% of recombinant wild type, rWT-VWF was retained in the Endoplasmic Reticulum (ER). The conclusion is that VWD D1 mutants G39R and D141N retain their VWF in the ER and cannot be transported from the ER to the Golgi as demonstrated in BOEC studies, resulting in variable degrees of decreased secretion: G39R and D141n <3% and K157E and C379G about 25%. The D1 mutants of VWF do synthesize VWF and all D1 mutants of VWF show a severe multimerization defect in rG39R and rG39R/D141N and a milder multimerization defect in rD141N and rC379G as compared to rVWF-WT.

the existence of recessive VWD IIC VWD 2C patients showing the absence of large VWF multimers lacking the triplet structure [18].

The ISTH classification of von Willebrand diseases (VWD) is based on the combined use of insensitive von Willebrand factor (VWF) assays VWF antigen (VWF:Ag), VWF ristocetine cofactor (VWF:RCo), Ristocetine Induced Platelet Aggregation (RIPA) and VWF multimeric analysis in a low SDS resolution gel (Figures 1 and 2) [19-22]. The ISTH classification distinguishes VWD type 3, recessive severe type 1, dominant type 1 with values between 0.05-0.30 U/dL), mild Low VWF with values between 0.30-0.60 U/dL [1,23,24]. The ISTH classification distinguishes dominant VWD type 2A (lumping dominant IIA, IIB, IIE and recessive IIC), VWD 2B and a subgroup of 2M showing the loss of large VWF multimers in a low resolution gel (Figure 1). VWF:RCo and RIPA is decreased VWD 2M due to loss of function mutation in the A1 domain, but still retain normal VWF:CB due to the presence of large VWF multimers [1-4]. RIPA is normal in mild and moderate and decreased in pronounced or severe VWD 2A, 2C and 2D due to VWF:RCo deficiency secondary to proteolysis in 2A, and secretion defect in 2C and 2D [1-6]. RIPA is normal in dominant VWD 1 Secretion Defect (SD) and VWF clearance defects (Table 1, Figure 2) [1-6,22-27]. The combined use of VWF collagen binding assay (VWF:CB), RIPA, sensitive VWF multimer analysis and DDAVP challenge test on top of ISTH criteria separates variants of VWD type 1 due to mutations in the D1, A3, D3, D4 and C1-6 domains from VWD recessive 2C and dominant 2A (normal or decreased RIPA), 2M (decreased or zero RIPA, decreased VWF:RCo/Ag ratio, normal VWF:CB/Ag ratio) and 2B (increased RIPA) caused by mutations in the A2, A1 and D2 respectively (Table 1, Figures 2 and 3) [1,6,26-27].

Recessive Severe Type 1 VWD due to Mutations in the D1 Domain of the VWF Gene

Clinical and laboratory characteristics of double heterozygous mutations G39R/D141N, K157E/C1165R in the D1 domain in the study of Yin et al. [29] are featured by recessive severe type 1 VWD (Rec 1) secretion defect and FVIII binding defect (hemophilia A: HA) (Rec-HA, Table 2). Mutations in the D1 domain do not split off VWFpp from proVWF and retain proVWF in the ER, which is associated with severe secretion and intracellular multimerization

Table 1: 2018 ECLM Classification of Von Willebrand Disease: VWD [2,5,6,25-28].

Type 1: VWF:Ag< 0.30 U/mL normal VWF:CB/Ag and VWF:RCo/Ag ratio >0.7	
LowVWF type 1: VWF:Ag levels >0.30-0.60 U/dL due to dominant mutations in the D4, C1-6 domain and carriers of null or missense mutation VWD recessive type 3 and 1 all over the VWF gene (blood group O dependent bleeding phenotype, Michiels et al., [2,5,6,24,28].	
Type 3: VWF:Ag and FVIII:C undetectable or very low double null VWF gene mutation.	
Severe type 1 hemophilia A phenotype due to mutations in the D1 domain (this report)	
Severe type 1 VWD VWF:Ag and VWF:RCo detectable, but increased FVIII:C/VWF ratio due to homozygous or double heterozygous missense mutation for a secretion defect.	
Type 2: Decreased VWF:RCo/VWF:Ag < 0.7 for 2A, 2B, 2C, 2E, 2D and 2M equal to decreased VWF:GPIbR ot VWF:GPIbM < 0.7 with some discrepancies in 2B and 2M	
Recessive von Willebrand Disease: VWD	Domain Fig 2
Recessive severe type 3 double null mutation VWF gene	Double null
Recessive severe or pronounced type 1	Homozygous or Double missense
Recessive severe type 1 VWD-hemophilia A mimicking type 3	D1
Recessive 2N FVIII:c/VWF:Ag ratio <0.5. FVIII-VWF binding defect	D'-D3
Recessive 2C FVIII:C.VWF:Ag increased, secretion mulimerization defect	D2
Dominant von Willebrand Disease VWD	Domain
2E: type 1/2, loss of large multimers, no triplets and increased clearance	D3
2A: Loss of large MM due to increased VWF proteolysis, RIPA N or decreased	A2
2M: Decreased RIPA, VWF:RCo/VWF:Ag ratio, normal VWF:CB/VWF:Ag ratio	A1
2B: Increased RIPA (0.8mg/ml) and thrombocytopenia with VWD type 2	A1
2CBD Collagen type, VWF:RCo/VWF:Ag and VWF:CB/VWF:Ag ratio < 0.7	A3
1m or 1sm	D4 C1-6
2D: dimerization defect loss large MM intervening bands and absence of triplets	CK

Table 2: Laboratory features of Probands (**P**) with recessive severe VWD type 1 associated with haemophilia A due to homozygous or double heterozygous mutations in the D1 domain of the VWF gene in basic research studies: persistence of proVWF due to D1 mutation is associated with secretion of VWFpp dimer, multimerization defect and FVIII binding defect mimicking type 3 VWD [29]. Interpretation of clinical phenotype in table 2A from expression and BOEC studies. **P1**: recessive severe type 1 in G39R/D141N D1D1 mutation associated with severe secretion en multimerization defect of pro-VWF with absence of VWF multimers in plasma due to multimerization defect of pro-VWF that retains in the ER as the explanation of severe secretion defect. **P2**: recessive severe type 1 in K157E/C1165R D1/D3 mutation associated with severe secretion defect and multimerization defect of pro-VWF D1 domain and multimerization defect D3 domain. **P3 and P3B**: C379G/WT dominant 1 secretion and multimerization defect of pro-VWF with lack of large VWF because rC379G/WT is associated with a secretion multimerization defect of pro-VWF that interferes with normal VWF in heterozygous C379G/WT in P3 and P3B.

Family	Member	VWF mutation		WVF:Ag	VWF:RCo	FVIII:C	RCo/Ag	VIII:C/Ag	VWD
Reference			Domain	U/L	U/L	U/L	Ratio	Ratio	
P1	**Proband**	**G39R/D141N D1D1**		**0.01**	**0.02**	**0.02**	**na**	**na**	**Rec 1 HA**
F1	Father	D141N/WT	D1	0.48	0.5	0.74	1	1.5	mild 1 SD
M1	Mother	G39R/WT	D1	0.3	0.36	0.51	1.2	1.7	1 SD
P2	**Proband**	**K157E/C1165R D1 D3**		**0.03**	**0.02**	**0.03**	**na**	**na**	**Rec 1 HA**
F2	Father	K157E/WT	D1	0.87	0.8	0.98	0.92	1.1	Normal
P3	**Proband**	**C379G/WT**	**D1**	**0.08**	**0.06**	**0.13**	**0.75**	**1.6**	**Dom 1 SD**
P3B	**Brother**	**C379G/WT**	**D1**	**0.14**	**0.14**	**0.25**	**1**	**1.8**	**Dom 1 SD**
P4 [31]	**F/63**	**D141Y/null**	**D1**	**<0.01**	**<0.01**	**0.03**	**na**	**na**	**Rec 1 HA**
P5	**F/26**	**C275S/null**	**D1**	**<0.01**	**<0.01**	**0.03**	**na**	**na**	**Rec 1 HA**
M5	Mother	C275S/WT	D1	0.51	0.48	1.24	0.94	2.4	1 SD
Proband		**C370T/C370T**	**D1**	**0.02**	**0.06**	**0.11**	**na**	**na**	**Rec 1 HA**
Proband	**Child**	**W377C/C377T**	**D1**	**0.03**	**0.03**	**0.02**	**na**	**na**	**Rec 1 HA**

defect and no binding of FVIII:C in their homozygous or double heterozygous mutated state. This combined recessive severe type 1 VWD/haemophilia A phenotype (VWD Rec 1-HA, Table 2) is clearly distinct from VWD type 3, severe type 1, VWD type 2C and from dominant type 1 secretion caused by heterozygous C379G/WT in the D1 domain (Table 2). The persistence of proVWF in recombinant VWF (rVWF) of mutated G39R, D141N, K157E and C379G of the D1 domain (Table 2, Figure 4) is associated with the lack of FVIII binding to VWF the D' domain (<5%) and complete lack of multimerization of G39R, D141N and K157E mutants and partial

Table 3: Laboratory features of 3 probands from 3 consanguous families with homozygous R273W/R273W mutation in the D1 domain classified as recessive VWD 1 and mild hemophilia A with increased FVIII:C/VWF:Ag ratios above 3.3 indicative for a severe Secretion Defect (SD).
Five-affected heterozygous R273W/WT family member have well documented mild VWD type 1 [32].

Family	VWF mutation	WVF:Ag	VWF:RCo	FVIII:C	RCo/Ag	VIII:C/Ag	VWD
Member	Domain	U/L	U/L	U/L	ratio	ratio	
Proband AII:1	**R273W/R273W D1**	**0.06**	**0.06**	**0.20**	**1.0**	**3.3**	**Rec 1 SD**
Father AI:1	R273W/WT D1	0.43	0.38	0.60	0.88	1.4	mild 1
Mother	R273W/WT D1	0.45	0.25	0.38	0.55	1.2	mild 1
Proband BII3 R273W/R273W D1		**0.09**	**0.04**	**0.21**	**0.44**	**2.3**	**Rec 1 SD**
Father	R273W/WT D1	0.51	0.45	0.58	0.88	1.1	mild 1
Mother B1:2	R273W/WT D1	0.54	0.42	0.98	0.78	1.4	mild 1
Proband C R273W/R273W D1		**<0.01**	**<0.01**	**0.09**	**na**	**>9**	**Rec 1 SD**
Father	R273W/WT D1	0.47	0.60	0.70	1.3	1.5	

Table 4: Laboratory features of recessive VWD type 2C due to D2 domain mutations in the VWF gene. (Source Michiels et al 2009 [32]).

Mutation/Reference	Age/FM	BT	FVIII:C	VWF:Ag	VWF:RCo	Ag/RCo	VIII:C/Ag	VWF MM
	Family	Minutes	U/mL	U/mL	U/mL	Ratio	Ratio	Domain
Ins405AspPro/null	**39 M**	**>30**	**0.67**	**0.50**	**0.10**	**0.20**	**1.3**	**Rec 2C D2**
Ruggeri 2C	2 children	N	N	N	N	N		Abn (Fig)
A625InsG/null	**19 F**	**>20**	**0.24**	**0.16**	**0.03**	**0.19**	**1.6**	**Rec 2C D2**
G550R/G550R	**II-1 F**	**10.0**	**N**	**1.59**	**0.25**	**0.16**		**Rec 2C D2**
G550R/WT	III-1 F	2.0	N	1.34	0.80	0.60		Abn (Fig)
G550R/WT	III-2 M	6.0 N=<6.0	N	0.86	0.66	0.77		Abn (Fig)
C623W/C623W	**64 F**	**>30**	**0.20**	**0.10**	**<0.10**		**2.0**	**Rec 2C D2**
C570S/C570S	**Boy**	**>20**	**0.12**	**0.05**	**<0.05**	**0.3**	**2.4**	**Rec 2C D2**

lack of of multimerization of the C379G mutant. The expression studies revealed a severe secretion defect and no multimerization of pro-VWF of the rVWF G39R nonsense, D141N missense and G39R/D141N double heterozygous mutants with no multimeriztion of the G39R in the lysates (Figure 4). The missense rVWF D141N, K157E and C379G mutants showed a less severe but pronounced secretion and multimerization defect with a bold pro-VWF protomer as compared to WT rvWF (Figure 4). The conclusions from the expression studies in figure 4 of patients with mutations in the D1 domain (Table 2) are the following : homozygous and double heterozygous mutations in the D1 domain including G39R, D141N, D141Y, K157E, C379G do not split off VWFpp from proVWF that retains in the ER result in a severe secretion defect and loss of D'D3 multimerization with the clinical phenotype of severe VWF and FIII:C deficiency (Rec 1-HA, Table 2). Proband P1 can be diagnosed as severe Rec 1-HA due to G39R/D141N D1-D1 mutation associated with severe secretion and multimerization defect of pro-VWF with absence of VWF multimers in plasma due to multimerization defect of pro-VWF that retains in the ER as the explanation of severe secretion defect. Proband P2 meets the criteria of severe Rec 1-HA due to K157E/C1165R D1/D3 mutation associated with severe secretion defect and multimerization defect of pro-VWF D1 domain and multimerization defect D3 domain. Plasma VWF multimers pattern show the absence of VWF multimers in proband P1 with Rec 1-HA double heterozygous for G39R/D141N, in proband P2 with Rec 1-HA double heterozygous for K157E/C1165R (Table 2) and therefore mimicking type 3 VWD (pseudohemophilia A) (Figure 4) [29]. Two brothers in the study of Yin et al with heterozygous C379G/WT mutated cases in the D1 domain are could be phenotyped as dominant type 1 secretion and multimerization defects with lack of large VWF multimers due to the persistence of pro-VWF. Interestingly, dominant heterozygous rC379G/WT mutated VWD is associated with a secretion multimerization defect of pro-VWF that interferes with normal VWF as clearly demonstrated in two patients with heterozygous C379G/WT in P3 and P3B (Table 2, Figure 4) [29].

Rosenberg et al., [30] described in 2002 two members of a family with clinical symptoms of dominant mild VWD type 1 in mother and daughter with an abnormal VWF multimeric pattern. A marked bold band of VWF dimers in both affected patients with mild dominant VWD type 1 heterozygous for the Tyr87Ser/WT mutation (Figure 5). The predominant dimer VWF band equaled approximately 50% of the VWF antigen, while the remainder of the VWF appeared to multimerize normally. A single missence mutation was detected in exon 4 converting Try87 to a Serine (Tyr87Ser) located in the D1 VWFpp domain. Tyr87Ser resulted in a complete loss of VWF secretion and multimerization but maintenance of normal vesicular storage of VWF in endothelial cells (Figure 5). When a VWF expression vector containing this Tyr87Ser mutation was transferred into COS-7 cells, the expressed VWF mutant consisted predominantly of dimers with nearly complete loss of multimerzation (Figure 5). The platelet binding of rVWF Tyr87Ser *vs* rWT-VWF to ristocetins was 17% and to botrocetin 97% of normal. The FVIII binding of the Tyr87Ser mutatant was decreased to a degree of FVIII binding similar to that of control 2N Arg854Gln mutation. The Try87Ser abnormality

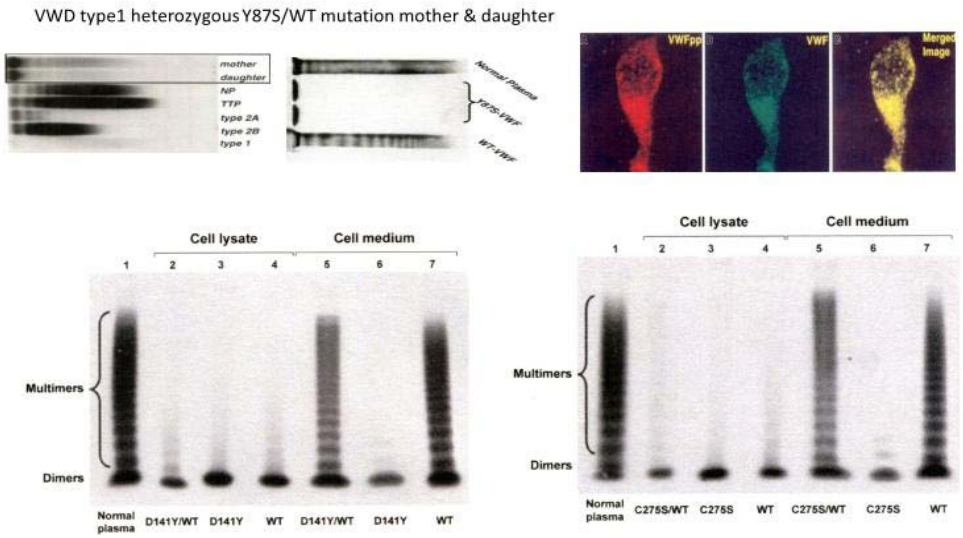


Figure 5: Upper part: Predominance of VWF dimers in dominant mild VWD type 1 heterozygous for the D1 domain mutation Tyr87Ser/WT (left) [30]. Expression studies of rTyr87Ser resulted in a complete loss of VWF secretion and multimerization (middle) but maintenance of normal vesicular storage of VWF in endothelial cells (right) [30]. **Lower part.** Multimeric analysis of rVWF D141Y, rVWF rD141Y/WT and rVWF WTS (left) and rVWF C275S, rVWF C275S/WT and rVWF WT from cell lysate and secreted in supernatant (cell medium) expressed in COS-7 cells (Baronciani et al) [31]. The expression studies of the D141Y (left) and C275S (right) missense mutations showed a severe secretion defect of mainly dimers consisting of proVWF while higher molecular weight bands like tetramers and hexamers were barely detectable similar as has been reported in rVWF G39R, rD141N and rTyr87Ser (Figures 3 and 4). The D141Y and C275S mutations produce non cleaved proVWF that can only form dimers and do not bind FVIII:C similar as decribed for G39R, D141N and Tyr87Ser.

affects the latter stages of VWF biosynthesis; the generation of VWF multimers. When the Try87Ser mutation was introduced into a vWF vector, the secreted VWF was nearly exclusively the dimeric unit (very likely proVWF) not followed by multimerization (Figure 5). The Tyr87Ser mutation manifests itself in a dramatic loss of several VWF functions. The dimeric rVWF Tyr87Ser mutant (very likely proVWF) displayed diminished VWF:RCo activity, collagen binding and FVIII binding. The decrease of collagen binding and platelet binding to these mutated dimers is not surprising since it is known that HMWM have a greater predeliction for collagen or for platelets in the presence of ristocetine.

Baronciani et al., reported two recessive VWD cases double heterozygous for D1 domain missense/null mutation D141Y/null and C275S/null who have detectable but very low VWF levels and FVIII:C levels between 0.02 to 0.04 U/mL mimicking recessive VWD type 3 with documented hemarthros in one of them (Table 2) [31]. Expression studies the missense mutation D141Y and C275S showed a severe secretion defect of mainly dimers consisting of proVWF while higher molecular weight bands like tetramers and hexamers were barely detectable (Figure 4) similar as has been reported in rVWF G39R, D141N and Tyr87Ser (Figure 4). The D141Y and C275S mutations consist of non cleaved proVWF that can only form dimers and do not bind FVIII:C similar as decribed for G39R, D141N and Tyr38Ser [29,30]. Homozygotes for the missense mutations W377C/W377C in the propeptide D1 domain have been described as recessive severe type 1 VWD phenotype (Table 2). Expression studies of recombinant W377C showed a severe secretion defect mainly consisting of a bold VWF dimer band (very likely proVWF) and failed to form intermediate and high molecular weigth multimers due to a multimerization defect in recessive severe type 1 with mild hemophilia A. In contrast, heterozygous C379D/WT mutation in the D1 domain is associated with dominant severe type 1 secretion and multimerization defect (Table 2, Figure 4) [30].

The laboratory features of three probands from three consanguous families with homozygous R273W/R273W mutation in the D1 domain could be classified as recessive VWD 1 and mild hemophilia A with increased FVIII:C/VWF:Ag ratios above 3.3 indicative for a severe Secretion Defect (SD) [32]. Five affected heterozygous R273W/WT family members have well documented mild VWD type 1 (Table 2). Figure 6 revealed no detectable VWF multimers in undiluted plasma as compared to normal undiluted plasma in two homozygous R273W/R273W cases (AII:1 and BII:3), but a typical VWF 2C multimeric pattern became evident when the plasma contained 0.5 U/dL VWF:Ag and electrophoresed on 2% SDS-agarose gels in two homozygous R273W/R273W utated probands AII-1 and BII:3 (Figure 6A) consistent with pronounced secretion and multimerization defect similar as seen in recessive 2C VWD [32] (Table 3).

Recessive VWD Type 2C due to Mutations in the D2 Domain of the VWF Gene

Autosomal recessive VWD type 2C is rare (8 reported probands), and caused by homozygosity for a missense mutation or double heterozygosity of a null allele and missense mutation in the D2 domains (exon 11 to 16) of the VWF propeptide that catalyses the multimerisation in the D3 domain at the N terminus of mature VWF (homozygous N528S, G550R, C623W, double heterozygous D437-R422del, F405insNP/null allele, A625InsG/nul26pdelCT) (Table 4) [32]. The laboratory phenotype of recessive type 2C (IIC) VWD is featured by low to normal levels of FVIII:C and VWF:Ag, very low levels for VWF:RCo, and VWF:RCo/Ag ratios, prolonged bleeding times and the absence of large and intermediate VWF

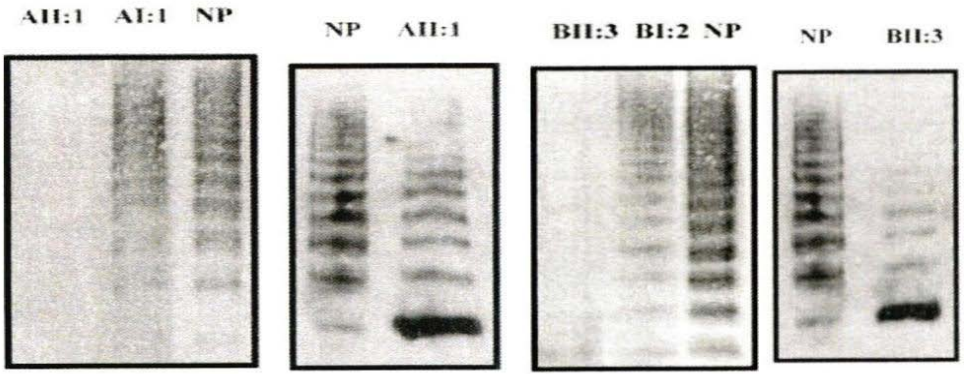
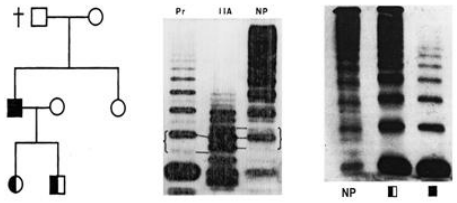


Figure 6A: No detectable VWF multimers in undiluted patient plasma as compared to Normal Plasma (NP) in two homozygous R273W/R273W cases (**AII:1** and **BII:3** in Table 3) diagnosed as severe type 1 VWD secretion mulimerization defect. However these two R273W/R273W homozygous patients showed a typical VWF 2C multimeric pattern, which became evident when the patient plasma contained 0.5U/dL VWF:Ag and was electrophoresed on high resolution 2% SDS-agarose gels in two homozygous R273W/R273W mutated probands **AII-1** and **BII:3.** The lack of large VWF multimers in the two homozygous R273W/R273W patients is consistent with pronounced secretion and multimerization defect similar as seen in recessive 2C VWD (Figure 6B, Table 4) [32].

Clinical Expression and Routine Laboratory Data

	Bleeding symptoms	Ivy bleeding time	VIIIC	VIIIR:Ag	VIIIR:RCo
		min		*U/dl*	
Normal	—	<9	>50	>50	>50
Propositus (Male)	Moderate	>30	67	50	10
Daughter	None	9	95	95	60
Son	None	6	120	120	96



ID	BT [min]	VWF: Ag [IU/ml]	RiCof [IU/ml]	VWF: CBA [IU/ml]	F VIII:C [IU/ml]
I 1	3.5	2.09	1.45	1.44	> 1.00
II 1	10.0	1.59	0.25	0.29	> 1.00
III 1	2.0	1.34	0.80	0.79	> 1.00
III 2	6.0	0.86	0.66	0.57	> 1.00
Normal range	< 6.0	0.6–1.6	0.6–1.6	0.6–1.6	0.7–1.4

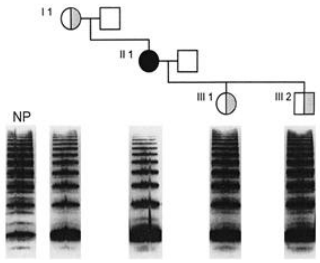


Figure 6B: Left: Analysis of VWF multimers of the VWD 2C patient due to the double heterozygous Ins405AspPro/null mutation (Table 4) showed a typical 2C pattern of defective multimerization. The mother heterozygous for F404indNP/WT show the typical 2C pattern without a faint intervening band. Multimeric analysis of mutant rVWF released to the medium showed only the fastest moving band (the protomer), a trace of the next oligomer and lack of triplet structure. **Right:** Analysis of VWF multimers of the VWD 2C patient homozygous for G550C/G550C (Table 4) revealed the loss of large multimers, the lack of satellite bands (no triplets) and a pronound protomer (VWF-dimer). The VWF multimeric pattern of the affected heterozygous G550C/WT family member as compared to NP and the homozygous case revealed a pattern in between showing the presence of a pronounced VWF-dimer and lower intensity of satellite band as compared to NP.

multimers, increase of a bold VWF-dimer band due to a pronounced multimerization defect (Figure 6B) [32].

Recessive VWD 2N due to Noncysteine or Cysteine Mutations in the D' Domain of the VWF Gene

A decreased FVIII:C/VWF:Ag ratio (<0.5) due to a FVIII binding defect is diagnostic for recessive VWD 2N showing normal VWF multimers, VWF:RCo/Ag and VWFpp/Ag ratios [1-6]. FVIII binding defects in VWD 2N non-cysteine mutations R854Q and R816W in the D' domain of VWF gene have no influence on synthesis, storage, secretion and multimerization of VWF. Homozygous non-cystein R854Q/R854Q mutation and of R854Q double heterozygous with non-cysteine E787K, T791M and R816W mutations in the D' domain result in a mild FVIII binding defect (FVIII:BD) featured by decreased FVIII:C/VWF:Ag ratio around 0.30, moderate hemophilia A and normal bleeding time, PFA-CT, VWF function and multimers. The FVIII:BD is markedly decreased (less than 10%) in VWD 2N due to E787K, T791M, R816W, 869 and C1060 either homozygous or double heterozygous with a null allele. Mutations in the C1 and C2 domain in FVIII gene have no influence on synthesis, storage, secretion and multimerization of VWF [33,34]. The VWD type 2N cysteine mutations C788R/Y; Y795C and C804F in TIL'; C858C/F in E' are associated with aberrant multimerization, poor secretion and reduced FVIII binding to VWF [34]. Homozygous R760W/R760W (D2 domain), and R788/R788 (D' domain) induce a pronounced secretion and multimerization consistent with recessive VWD 2C in which a mild FVIII binding defect (FVIII:BD) of about 0.35 did not contribute to the severity of bleeding phenotype of recessive VWD 2C [34]. The combination of R854Q and R760 in the D'D2 domains produce VWD type 2N with a smeary pattern of VWF multimers due to a mixture of normal VWF and of proVWF. Heterozygous R763/WT Furin cleavage site mutation results in VWD type 1 with a smeary VWF multimers and VWD 2N double heterozygous for R854Q/

Table 5A: Laboratory features in a Belgian family with dominant VWD type 1E or 2 E caused by W1120S/WT mutation in the D3 domain of the VWF gene. (Observations by Vangenechten, Michiels and Gadisseur).

Family	Mutation	FVIII:C	VWF:Ag	VWF:GPIbM	VWF:CB	VIII:C/Ag	GPIbM/Ag	CB/Ag
		U/dL	U/dL	U/dL	U/dL	ratio	ratio	ratio
Proband	W1120S/WT	0.45	0.29	0.34	0.26	**1.55**	1.17	0.90
Mother	W1120S/WT	0.39	0.23	0.25	0.26	**1.70**	1.09	1.13
Sister	W1120S/WT	0.72	0.34	0.21	0.19	**2.12**	0.62	0.56
Sister	WT/WT	0.99	1.07	1.00	1.35	0.92	0.93	1.26
Aunt	W1120S/WT	0.46	0.90	0.18	0.53	**0.51**	0.20	0.59
Uncle	W1120S/WT	0.61	0.32	0.20	0.28	**1.91**	0.63	0.88
Aunt	W1120S/WT	0.49	0.25	0.16	0.20	**1.96**	0.64	0.80
Uncle	WT/WT	0.90	0.63	0.54	0.54	1.42	0.86	0.86

Table 5B: Response to DDAVP of PFA-CT, FVIII:C and VWF parameters in a case of dominant VWD 2E caused by the C1190R/WT mutation in the D3 domain (See VWF multimers figure 10D). Observations by Vangenechten, Michiels and Gadisseur.

DDAVP	PFA EPI	PFA ADP	APTT	FVIII:C	VWF:Ag	VWF:RCo	**VWF:RCo/**	VWD
(exercise)	CT s	CT s	Sec	U/mL	U/mL	U/mL	**Ag ratio**	type
Before	>300	>300	40	0.27	0.40	0.17	**0.43**	2 E
after 1 hr	87	76	30	3.12	1.60	1.04	**0.65**	2 E
2 hrs	134	56	32	1.32	1.26	0.80	**0.63**	2 E
4 hrs	219	114	32	1.17	1.00	0.76	**0.76**	1 E
6 hrs	229	119	33	1.10	0.82	0.68	**0.83**	1 E

Normal values commercialized PFA Closure Times (PFA-CT) collagen/EPI 95-160 seconds and collagen/ADP 70-110 seconds

R763 show a smeary VWF multimeric pattern due to a mixture of normal VWF and pro-VWF protein [34]. The homozygous C1060R/C1060R mutation in the D3 domain, and the double heterozygous D879N/null and C1060R with R854Q or non-sense (null) mutations in particular are associated with a hybrid 2N/E phenotype [34].

Phenotype of Classical Dominant VWD Type 1/2E due to Mutations in the D3 Domain

Vangenechten, Michiels & Gadisseur studied a Belgian family with dominant VWD type 1E case due to the heterozygous W1120S/WT mutation in the D3 domain (Table 5A). The proband suffered from a mild intracerebral bleeding at the age of 4 months from which he completely recovered. Tonsillectomy under prophylactic treatmen with Hemate-P was uneventful. He is known with easy bruising and subcutaneous hematomas since early childhood. At age of 11 years the laboratory findings were: Ivy bleeding time >15 minutes. PFA100-col >300 s, PFA-epi >300 s, normal RIPA, FVIII:C 0.70, VWF:Ag 0.72, VWF:RCo 0.21 and VWF:CB 0.19 U/ml diagnosed as VWD 2E with a typical 2E multimeric pattern in the Laboratory of Budde. His mother had a life-long history of spontaneous easy bruising, hematomas after minor injury, prolonged menarche of 14 days duration followed by iron deficiency microcytic anemia during adolescence.

Gadisseur, Michiels & Gadisseur assessed the response to DDAVP of PFA col/epi, PFA col/ADP closure times (CT), APTT, FVIII:C, VAF:Ag and VWF:RCo in a case of VWD 2E caused by the heterozygous mutation C1190R/WT in the D3 domain (Table 5B). DDAVP induced a complete transient correction of PFA CT, and 3 to 4 fold increase of VWF parameters followed by shortened half life time for VWF:Ag of less than six hours, which will predict increased VWFpp/VWF:Ag ratio above 2 (Table 5B). Such complex responses to DDAVP of VWF:Ag, VWF:RCo and FVIII:C followed by shortened half-life times of FVIII/VWF:Ag complex and prolongation PFA-CT values at VWF:RCo levels of around 0.75-0.80 point to the complex mechanism of the quantitative and qualitative functional defects of the C1190R mutated VWD type 2E (Table 5B).

The FVIII:C/VWF:Ag ratio is around 1 in VWD type 1 and in normal individuals with both blood group O and Non-O. The capacity of FVIII binding sites over VWF subunits on a molecular basis is 1:50 and independent of the size of VWF multimers. In quantitative VWD type 1 with VWF:Ag levels below 0.50U/dL of normal (range 0.10 to 0.50 U/dL) due to selective secretion defects, the ratio of FVIII:C/VWF:Ag will increase to above 1.4 or around 2.0 in VWD type 1 secretion defect (SD, Figure 7) [1,25-44]. Increased FVIII:C/VWF:Ag ratio in VWD type 1 and 2 refers to a VWF secretion defect (SD, Figure 7) can best be documented by a restricted response of VWF:Ag compared to a good response of FVIII:C after DDAVP. The first description of classical autosomal dominant type 1 VWD with high penetration of moderate bleeding symptoms was caused by D3 domain located heterozygous missense mutation C1130F/WT in one family and C1149R/WT in another family [35]. These two D3 domain located missense mutations interfere with the normal VWF subunits coded by the normal VWF allele causing a defective intracellular multimerisation and degradation of VWF leading to a secretion defect of the VWF by endothelial cells [35]. The heterozygous C1130F/WT and C1149G/WT mutations cause a dominant type 1 VWD featured by low VWF levels of <0.20U/ml and the presence of all sizes of VWF multimers in SDS low resolution gel, and an increased FVIII:C/VWF:Ag ratio of 2.0 to 2.3 consistent with a secretion defect of VWF [35]. Castaman et al described 3 unrelated families with dominant VWD 2E due to heterozygous

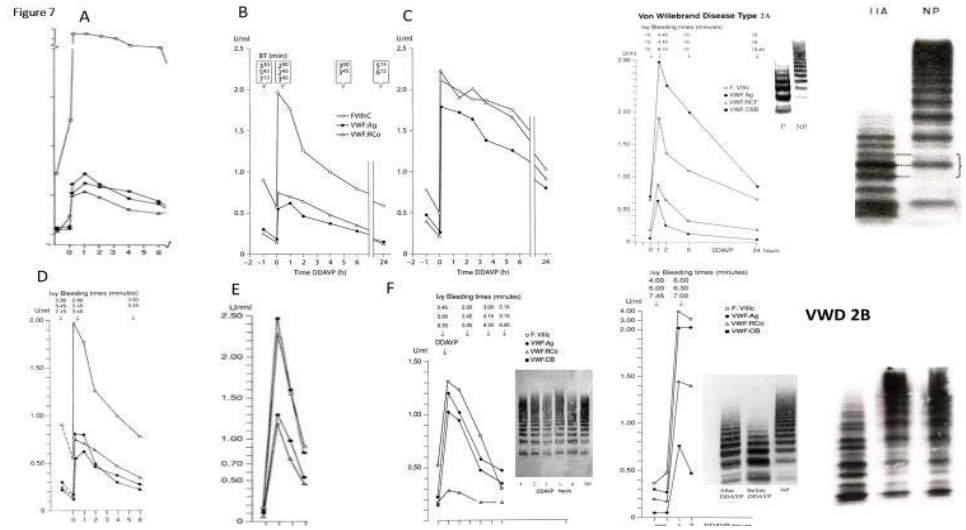


Figure 7: Detection of secretion, clearance and functional defects by analysis of FVIII/VWF responses to DDAVP in VWD type 1 secretion defect (A), secretion/clearnce defect (B and D) pseudo-VWD (C) Vicenza clearance defect (E), VWD 2M (F), VWD 2A (upper right) and VWD 2B (lower left) [1].
A: Restricted response to DDAVP of all VWF:Ag, VWF:RCo and VWF:CB parameters compared to FVIII:C (high FVIII:C/VWF:Ag ratio) followed by normal half-life times is consistent with dominant VWD type 1 secretion defect (SD). **B:** Restricted response of VWF:RCo compared to normal responses of VWF:Ag and FVIII:C followed by shortened half-life times of all FVIII/VWF parameters is consistent with mild clearance defect in moderate dominant VWD type 1. **C:** Normal responses of FVIII:C and VWF:Ag, VWF:RCo and VWF:CB parameters followed by normal half-life times of all parameters is typical for pseudo-VWF in bloodgroup O and absence of VWF mutation. **D:** Restricted responses of VWF parameters ascompared to FVIII:C followed by shortened half-life times of VWF parameters and FVIII:C indicating VWD type 1 with combined Secretion Defect (SD) and increased Clearance (C). **E:** Good responses of FVIII:C, VWF:Ag, VWF:RCo and VWF:CB followed by very short half-life times of the FVIII/VWF complex is diagnostic for Clearance (C) defect in Vincenza type VWD. **F:** Poor response of VWF:Ag, VWF:RCo and VWF:CB compared to FVIII:C (high FVIII:C/VWF:Ag ratio) followed by shortened half-life times of FVIII/VWF parameters is typical for combined secretion and clearance defect in VWD 2M showing a complete normal VWF multimeric pattern before and after DDAVP.
Right upper 2A and right lower 2B VWD: Restricted response to DDAVP of VWF:CB and VWF:RCo followed by shortened half-life times as compared to normal high responses of VWF:Ag and FVIII:C in severe type 2A and in 2B. The VWF multimers in 2A and 2B show the loss of large and increase of small VWF multimers in the Rotterdam method [1], and loss of large vWF multimers with increased triplet structure of intermediate and small VWF bands in the Budde method [25,26,48]. The VWD 2B VWF multimers stem from one of the affected VWD 2B family carrying the R1306W mutation.

C1130F/WT mutation and typical features of pronounced dominant type 1 VWD with VWF values below 15U/dl and increased FVIII:C/VWF:Ag ratios (Table 6) [36]. *In vitro* expression studies indicate that the recombinant C1130F and C1149R mutations cause intracellular retention of VWF and secretion of VWF monomers and dimers consistent with a multimerization defect with the absence of large multimers and the absence of triplet structure of individuals bands [37] The clinical reports of Castaman et al confirmed that patients with heterozygous VWF mutations in the D3 domain have variable laboratory features of dominant VWD type 1 or type 2 (Table 6) with a typical 2E VWF multimeric pattern in low and medium resolution gels (Figure 3). This VWF 2E pattern clearly differs from recessive 2C in that there is no intervening band (Figure 3). Expression studies of mutant rVWF clearly showed a secretion-multimerization defect in VWD type 1/2E caused by the heterozygous mutations R1130F/WT and W1144G/WT in the D3 domain [37].

Casana et al reported the association of dominant mild type 1 VWD with increased FVIII:C/VWF:Ag ratios indicating a secretion defect (VWD type 1SD) in 7 members of one family with heterozygosity of T1156M/WT in the D3 domain of the VWF gene (Table 6) [38]. *In vitro* experiments demonstrated that the recombinant T1156M mutation causes intracellular retention of VWF and secretion of VWF monomers and dimers consistent with a multimerization defect with the absence of large multimers [38]. Heterozygosity for T1156M/WT in two studies is consistent with dominant mild type 1 VWD with normal VWF multimers i a low SDS resolution gel. Compound heterozygosity for the mutation T1156M in the D3 domain of the VWF and a null allele (Q2470X) described by Lethagen et al., [39] was associated with severe recessive VWD with type 2E VWF multimeric pattern showing the lack of large multimers, no triplet structure and no increase of VWF degradation (autosomal recessive VWD type 2E). James et al published a very interesting case of dominant VWD phenotype 1 with the lack of large VWF:g multimers and absence of triplets (2E) with the heterozygous G1180C/WT mutation in the D3 domain [40].

Analysis of the ISTH defined laboratory phenotype based on the restricted diagnostic set of insensitive VWF parameters in 20 VWD patients caused by 20 different mutations in the D3 multimerization domain in the collaborative study of Schneppenheim, Michiels et al., [41] were VWD type 1E or 2E (Table 7). At least seven cases had increased FVIII:C/VWF:Ag ratios indicative for a Secretion Defect (SD) when the ECLM criteria are applied (Table 1, Figure 3). The heterozygous mutations R976C/WT and C1091C/WT in the collaborative study of Schneppenheim Michiels et al show normal values for FVIII:C and VWF (Table 7) and do not meet the ISTH criteria of VWD. The heterozygous L1278R/WT mutation in table 7 is located in the A1 domain and revealed typical features of VWD 2M. The C1227R/Cys2283Arg in table 7 is a typical case of recessive severe type 1 VWD. The heterozygous L1278R/WT mutation in the A1 domain is a typical case of VWD 2M according to the ECLM

Table 6: VWF and FVIII:C parameters and Simplate Bleeding Times (BT) in five Dutch and Italian families with dominant pronounced VWD type 1/2 E multimerization and Secretion Defect (SD) as demonstrated by increased FVIII:C/VWF:Ag ratio in 11 affected member in 5 families carrying the C1130F/WT mutation (Castaman et al., [36]).

Family		VWF:Ag	VWF:RCo	FVIII:C	RCo/Ag	VIII:C/Ag	BT	VWD type
C1130F/WT		IU/dL	IU/dL	IU/dL	Ratio	Ratio	Minutes	ISTH/ECLM
Proband 1		6 17	<6 9	12 28	na 0.53	2.0 1.6	20	2 E SD
Mother		7 17	<6 10	15 20	na 0.59	2.1 1.2	15/20	2 E SD
Proband 2		9 15	<6 10	10 29	na 1.9	1.1 1.9	20	1 E
Father		6 18	<6 9	9 22	na 2.0	1.5 1.2	18	1 E
Proband 3		6 12	<6 10	15 31	na 0.83	2.5 2.6	20	1 E SD
Sister		7 14	<6 10	15 31	na 0.71	2.1 2.2	20	1 E SD
Father		5 14	<5 11	15 32	na 0.78	3.0 2.1	nt	1E SD
Proband 4		5 12	<6 12	12 29	na 1.0	2.4 2.4	14/20	1 E SD
Daughter		7 14	<6 9	10 29	na 0.64	1.4 2.1	nt	2 E
Son		8 15	<6 9	15 30	na 0.60	1.9 2.0	15	2 E SD
Proband 5		6 21	< 6 9	12 35	na 0.43	2.0 1.7	20	2 E SD
Mother		9 20	<6 12	15 33	na 0.60	1.7 1.7	18/20	2E SD
Normal range		47 to 165	51 to 188	52 to 173			<7.5	
Mutation	FVIII:C	VWF:Ag	VWF:RCo	VWF:CB	RCo/Ag	CB/Ag	VIII:C/Ag	WD type
MCMDM	U/dL	U/dL	U/dL	U/dL	ratio	ratio	ratio	**ISTH ECLM**
C1130R	0.13	0.17	0.10	0.06	0.58	0.35	0.76	2 E
C1130R	0.14	0.12	0.07	0.07	0.58	0.58	1.16	2 E
C1130R	0.15	0.22	0.13	0.08	0.59	0.36	0.68	2 E
C1130G	0.08	0.10	0.10	0.26	1.0	2.6	0.80	1 E SD
C1130F	0.10	0.08	0.10	0.06	1.25	0.75	1.25	1 E
C1130F	0.22	0.08	0.04	0.06	0.50	0.75	2.75	2 E SD
C1130F		0.13	0.11		0.84			1 E
W1144G	**0.24**	**0.31**	**0.12**	**0.12**	**0.39**	**0.39**	**0.77**	**2 E**
W1146C		0.14	0.13		0.93			1 E
Family **[38]**	FVIII:C	VWF:Ag	VWF:RCo	RCo/Ag	VIII:C/Ag	VWD type		
T1156M/WT	U/dL	U/dL	U/dL	Ratio	Ratio	**ISTH ECLM**		
II-1	0.80	0.30	0.27	0.90	2.6	1 E SD		
II-3	0.70	0.37	0.29	0.78	1.9	1 E SD		
III-4	0.75	0.34	0.27	0.79	2.2	1 E SD		
III-6	0.62	0.29	0.17	0.60	2.1	2 E SD		
III-8	0.59	0.27	0.17	0.63	2.2	2 E SD		
IV-1	0.68	0.37	0.32	0.86	1.8	1 E SD		

classification (Table 1, Figure 3) diagnosed by the VWF VWD Research Laboratory Antwerp.

The Role of VWFpp/VWF:Ag Ratio to Assess Clearance Defects in VWD Type 1, 2 E and 2M

After splitting proVWF into VWFpp and mature VWF during synthesis and storing of VWF in endothelial cells, the VWF propeptide (VWFpp) remain non-covalently bound to mature VWF bound to FVIII:C as a prerequisite to store VWF in Weibel-Palade Bodies (WBP) in endothelial cells for regulated release [42,43,44]. After release of VWF from WPB in plasma VWFpp and mature large VWF multimers dissociate and circulate with half life times of 2.3 hours for VWFpp and 8-12 hours for FVIII-VWF:Ag complex. Concentrations of VWFpp, FVIII:C and VWF:Ag are set as 1U/dL in normal plasma. In steady state situations, the ratio of VWFpp to VWF:Ag in plasma is by definition equal to 1.0. The rationale behind the use of the VWF:pp/Ag ratio is that in all variants of VWD type 1 and type 2 the half-life of VWF:pp is normal, whereas the half-life of VWF:Ag is shortened by different mechanism including clearance resulting in increased VWFpp/Ag ratios above 2.0. Increased VWF:Ag/FVIII clearance (C) is prominent in VWD Vincenza type 1C (Figure 7E). Increased clearance of the VWF:Ag is prominently seen seen in VWD

Table 7: 2010 update on 20 VWD 1/2E patients due to 20 different missense mutations in the D3 multimerization vWF domain as the cause of VWD 2 E multimerization defect in te study of Schneppenheim, Michiels et al., [41].

Exon	FVIII:C	VWF:Ag	VWFRCo	VWF:CB	VIII:C /Ag	RCo/Ag	CB/Ag	ISTH
Mutation	U/mL	U/mL	U/mL	U/mL	Ratio	Ratio	Ratio	ECLM
22 **V956A**	0.62	0.31	0.25	0.37	2.0	0.81	1.19	1 E **SD**
22 R976C	0.85	1.29	Nt	1.04			0.81	Normal
25 C1091R	nt	1.20	Nt	1.20			1.00	Normal
25 A1105D	nt	0.14	0.11	0.13		0.79	0.93	1 E
25 W1120S	**0.72**	**0.34**	**0.21**	**0.19**	**2.1**	**0.62**	**0.56**	**2 E SD**
25 R1121M	nt	0.23	Nt	0.20			0.87	1 E
25 C1126F	nt	0.18	Nt	0.7			0.36	2 E
26 C1130R	nt	0.17	Nt	0.10			0.59	2 E
26 C1130W	nt	0.22	Nt	0.08			0.36	2 E
26 Y1146C	0.31	0.20	0.07	0.16	**1.6**	0.35	0.79	2 E **SD**
26 C1149Y	0.25	0.17	0.07	0.05	**1.5**	0.42	0.30	2 E **SD**
26 C1153Y	0.56	0.89	0.40	0.55	0.63	0.45	0.55	2 E
26 C1169W	0.17	0.11	0.06	nt	**1.5**	0.52		2 E **SD**
26 G1172C	0.45	0.25	0.15	0.14	**1.8**	0.60	0.56	2 E **SD**
26 C1173F	0.61	0.19	0.10	0.08	**3.2**	0.53	0.42	2 E **SD**
26 C1173F	0.54	0.35	0.32	0.23	**1.5**	0.93	0.65	**1 E SD**
27 C1190R	0.56	0.41	0.14	0.24	1.4	0.34	0.59	2 E
27 C1190Y	**0.81**	**1.20**	**0.48**	**0.21**	**1.5**	**0.40**	**0.18**	**2 E DDAVP**
27 D1195V	nt	0.43	Nt	0.38			0.88	1 E
28 C1227R*	nt	0.03	Nt	0.01			na	2 E
28 L1278P	nt	0.59	Nt	0.31			0.52	2 M
28 L1278R	**0.26**	**0.20**	**0.02**	**0.21**	**1.3**	**0.10**	**1.05**	**2M**
C1227R*/ pCys2283Arg	0.06	0.03	0.05	0.01	na	na	na	Recessive 1

2E and 2M patients with mutations in the D3 and A1 domain as derived from the MCDMD-VWD-1 study (Figures 8 and 9) [42-44]. VWD patients Vincenza type 1C caused by heterozygous R1205H/WT mutation in the D3 domain show a normal or near normal secretion of the VWF:Ag/FVIII:C complex and a rapid clearance of VWF:Ag, FVIII:C and VWF:RCo after DDAVP within one hour which results in a pronouced increased VWFpp/Ag ratios around 10 (Figure 8) diagnostic for VWD 1C Vincenza type (heterozygous R1205H/WT mutation). In the Haberichter studies using a restricted set of FVIII:C, VWF:Ag, VWF:RCo, VWFpp asays, low resolution VWF multimeric analysis and DDAVP challenge test all VWD patients with the heterozygous mutation W1144G/WT are diagnosed as pronounced type dominant VWD 1 according to the ISTH criteria associated with a pronouced Clearance (C) defect as documented by increased VWFpp/Ag ratios above 2.1 (Table 8) [46,47]. The majority of the ISTH defined dominant mutated type 1 patients due to the heterozygous W1144G/WT had prominent increase of FVIII:C/VWF:Ag ratios indicative for an additional Secretion Defect (SD) on top of a multimerization defect of the type 2E VWF multimers lacking large multimers and absence of triplet stucture. Using FVIII:C, VWF:Ag, VWF:RCo, VWFpp asays, low resolution VWF multimeric analysis and DDAVP challenge test in the Haberichter study all dominant VWD patients with the heterozygous mutation S2179F/WT are diagnosed as pronounced type dominant VWD 1 with increased Clearance (C) as documented by increased VWFpp/Ag ratios above 2.1 (Table 8) and with increased FVIII:C/VWF:Ag ratios indicative for a Secretion Defect (SD) (Table 8) [46,47].

VWD 2A patients due mutations in the A2 domain show increased proteolysis of circulating VWF protein caused by hypersensitity to ADAMTS 13, which result in shortened half-life times of VWF:RCo and VWF:CB but only slightly decreased half-life times of VWF:Ag. Consequently, VWD 2A patients due the heterozygous V1499E/WT mutation in the A2 domain with decreased VWF:RCo/Ag and VWF:CB/Ag ratios due to increased proteolysis of VWF and near normal half life time of VWF:Ag after DDAVP resulted in a slight increased of the VWFpp/Ag ratio (VWF V1499E Gouda mutation, (Table 9 [45]). VWD patients due to heterozygous mutation in the D1 and D2 domain showing a Secretion Defect (SD) but normal Clearance (C) indeed do show a restricted responce of VWF parameters to DDAVP, do have decreased valus for VWFpp with normal VWFpp/Ag ratios indicative for a secretion defect (Figures 8&9).

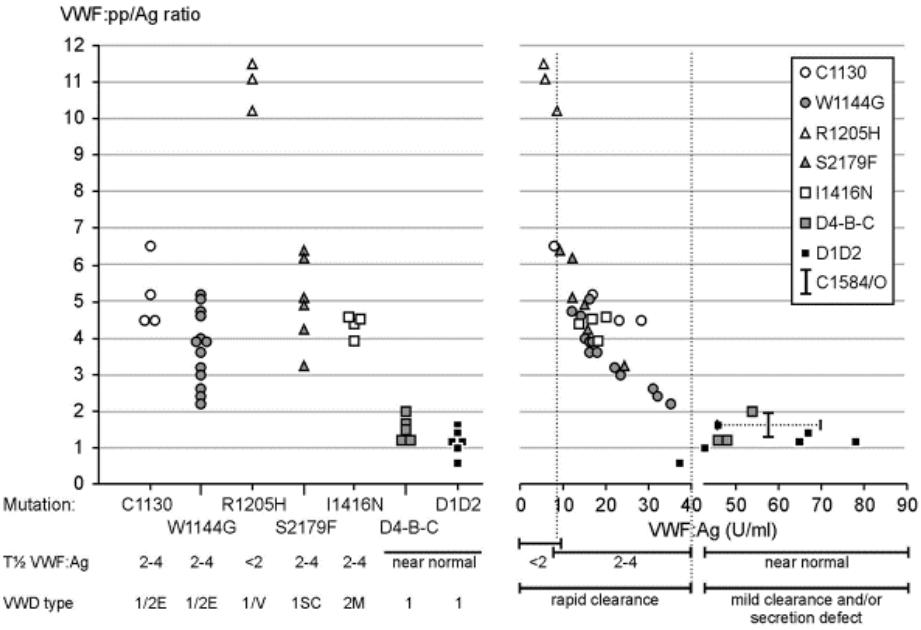


Figure 8: Results of VWFpp and VWF:Ag studies related to VWF:Ag half-life times after DDAVP challenge from European and USA reports on dominant VWD type 1/2 E and Vincenza due to mutations in the D3 domain, 2M associated with I1416N, mild type 1 VWD due to mutations in the D1, D2, and D4-BC domains[50,] and from a third study on the C1584Y mutation in de A2 domain collected from Haberichter studies outside the MCMDM-1 VWD study [6,46,47].

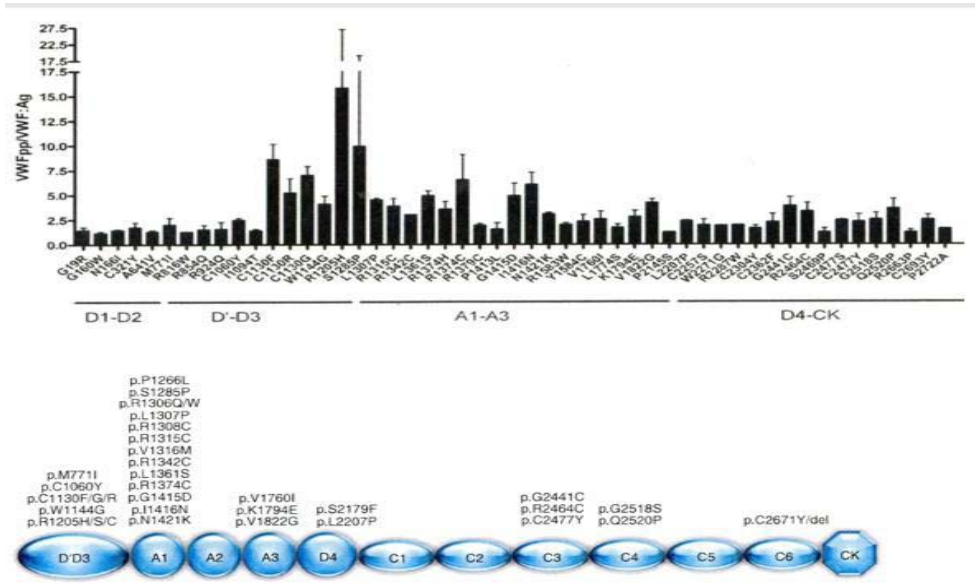


Figure 9: Upper part: Results of VWFpp/VWF:Ag ratios for individuals heterozygous for a single missense mutation in the D1-D2, D'D3, and D4-CK domains of the vWF gene in the MCMDM-1 VWD study in patients with heterozygous mutated VWD mild type 1, Low VWF, type 1 E/2 E, 2M, 2A in the D1, D2, D', D3, A1 and A2 domains and in VWD labeled as 1m and 1sm in the D4, C1-6 domains [42]. VWD patients heterozygous for the mutations in the D1, D2 and D'D3 FVIII binding domain G19R, G160W, N166L, C312Y, R816W, R854W, R924G (SNP), C1060 and C1094T, have completely normal VWF:RCo/VWF:Ag ratio or even below the normal range normal of Blood group O or Non O normal controls. The C1060Y mutation is the D3 domain is essential for FVIII binding to VWF. The C1060Y mutation in the D3 domain has no secretion and no clearance defect in expression studies. The MCMDM-1VWD study reported high VWFpp/VWF:Ag ratios in five mutatant in the D3 domain including C1130F, C1130R, C1130G, W1144G, and R1205H (Vincenza type VWD). On top of RIPA loss of function mutation in several VWD 2M patients do show a clearance defect including S1285P, L1307P, R1315C, R1342S, R1374H, R1374C, P1413L, G1414D, and I14446N. Such clearance defects in VWD 2M patients can be predicted from VWF response curves to DDAVP diagnostic for VWD 2M. The A2 domain mutations R1583W, Y1584C, V1760I, L17774S, K1794E and V1822G had slightly increased VWFpp/VWF:Ag ration between 1 and 2. VWFpp/Ag values between 1 and 2 have been reported in 16 family members with VWD 2A due to the 1579 mutation reported in this study (Table 8) and in 8 affected family members with the VWD 2A V1499E Gouda Dutch mutation (Table 9) [45]. The majority of VWD type 1m and 1sm patients due to mutations in the D4 and C1-6 domains have normal or near normal VWFpp/VWF:Ag ratios except slightly increased vallues in G244 C, R2469C and D2520P. **Lower part in blue**. Location of VWF clearance mutations as defined by increased VWFpp/Ag ratios in the European MCMDM-1 VWD study related to D'D3, A1, A3, D4, C3, C4 and C6 domains of the VWF gene [43].

Table 8: Critical analysis and classification according ISTH and ECLM criteria by the combined use of complete set of VWF parameters including VWFpp and VWF multimers of dominant VWD type 1 patients with pronounced deficiency of VWF parameters below 0.30U/dL in the Haberichter studies caused by the heterozygous mutation W1144G/WT in the D3 domain versus the heterozygous mutation S2179F/WT in the D4 domain of the VWF gene described by Haberichter et al., [46,47].

Mutant	Case	FVIII:C	VWF;Ag	VWF:RCo	VIII:C/Ag	RCo/Ag	VWFpp/	T1/2 days	ISTH	ECLM
	O /Non-O	U/dL	U/dL	U/dL	Ratio	Ratio	Ag ratio	VIII *vs* VWF:Ag	Diagnosis	Diagnosis
W1144G	CI-1 A	0.82	0.48	0.43	**1.7**	0.90	**2.1**		1 E **C**	1 E C SD
W1144G	CII-3 O	0.21	0.14	0.16	**1.5**	1.10	**4.6**	1.3 1.4	1 E **C**	1 E C SD
W1144G	CII-4 A	0.51	0.22	0.29	**2.3**	1.30	**2.6**		1 E **C**	1 E C SD
W1144G	CII-6 A	0.37	0.26	0.21	1.4	0.90	**3.0**		1 E **C**	1 E C
W1144G	CIII-3 A	0.63	0.40	0.32	**1.6**	0.80	**2.3**	nt 3.6	1 E **C**	1 E C SD
W1144G	CIII-5 A	0.45	0.23	0.23	**1.9**	1.00	**5.0**		1 E **C**	1 E C SD
W1144G	CIII-6 A	0.41	0.21	0.25	**2.0**	1.18	**3.9**		1 E **C**	1 E C SD
W1144G	CIII-9 A	0.29	0.23	0.26	1.3	1.13	**3.7**		1 E **C**	1 E C
W1144G	DI-1 O	0.23	0.06	0.15	**3.8**	1.80	**5.1**		1 E **C**	1 E C SD
W1144G	DII-1 O	0.18	0.12	0.08	**1.5**	0.70	**3.6**		1 E **C**	1 E C SD
W1144G	DII-2 O	0.22	0.06	0.13	**3.6**	2.10	**4.7**		1 E **C**	1 E C SD
W1144G	DII-3 O	0.28	0.09	0.19	**3.1**	2.10	**3.1**		1 E **C**	1 E C SD
W1144G	DII-4 O	0.16	0.09	0.18	**1.8**	2.00	**4.0**		1 E **C**	1 E C SD
W1144G	DII-5 O	0.35	0.05	0.08	**7.0**	1.60	**3.9**		1 E **C**	1 E C SD
Normal values					<1.4	>0.70	**<2.0**	11-18 8-12	Normal	Normal

Mutant	Case	FVIII:C	VWF;Ag	VWF:RCo	VIII:C/Ag	RCo/Ag	VWFpp/	T1/2 days	ISTH	ECLM
	O /Non-O	U/dL	U/dL	U/dL	Ratio	Ratio	Ag Ratio	VIII *vs* VWF:Ag	Diagnosis	Diagnosis
S2179F	AI-1 O	0.26	0.06	0.06	**4.3**	1.00	**3.2**	1.3 1.0	1m **C**	1m **C** SD
S2179F	AII-1 O	0.33	0.13	0.09	**2.5**	0.69	**5.1**	nt nt	1m **C**	1m **C** SD
S2179F	AII-2 O	0.21	0.07	0.06	**3.0**	0.86	**6.4**	1.9 1.2	1m **C**	1m **C** SD
S2179F	**AIII-1 O**	**0.28**	**0.12**	**0.14**	**2.3**	**1.17**	**4.2**	**2.3 3.1**	**1m C**	**1m C SD**
S2179F	BI-1 O	0.19	0.13	0.12	**1.5**	0.92	**4.9**	1.6 1.4	1m **C**	1m **C** SD
S2179F	BIII-1	0.29	0.15	0.12	**2.2**	0.80	**nt**	nt 1.7	1m **C**	1m **C** SD
Normal vaues					0.70-1.4	>0.70	<2.0	11-18 8-12	Normal	Normal

Diagnostic Differentiation of Classical Dominant Type 1, 2M, 2B and 2A by the Combined use of FVIII:C, VWF:Ag, VWF:RCo, VWF:CB, RIPA, VWF Multimers and DDVP Challenge Test

Van Vliet & Michiels prospectively evaluated the one center large cohort of VWD patients of the Erasmus University Medical Center Rotterdam (1990-2002) the combined use of FVIII:C, VWF:Ag, VWF:RCo, VWF:CB using collagen type I (Van Vliet VWF:CB method modified by Budde), RIPA and the responses of FVIII:C and VWF parameters to DDAVP for the correct diagnosis of mild VWD type 1, severe recessive type 1 and dominant VWD pronounced type 1, 2M, 2A and 2B (Figure 10) [1-4,7]. The 1990-2002 Rotterdam prospective VWF-VWD study consisted of 167 evaluable VWD patients from 94 families and were classified by Michiels as follows [1]: mild to moderate VWD type 1 in probands of 65 families or isolated cases with VWF value between 0.25 to 0.60 U/dL (LowVWF); 10 probands with pronounced dominant type 1 (VWF values 0.05 to 0.30); 10 probands with VWD 2A; 4 probands with VWD 2B; 2 probands with VWD 2N and 2 probands with VWD type 3 [1]. Within these variants of dominant pronounced VWD patients Michiels & Van Vliet could distinguish at least six different types of FVIII and VWF responses to DDAVP (Figure 10). First, rapid increase followed by rapid Clearance (C) of the FVIII/VWF parameters in dominant Vincenza type VWD 1C. Second, high response of FVIII and restricted responses of VWF parameters in dominant VWD type 1 followed by increased clearance of FVIII/VWF labeled as Secretion Clearance (SC) defect. Third, high response of FVIII and restricted response of VWF parameters followed by normal half-life of FVIII/VWF labeled as VWD Secretion Defect (SD) in dominant VWD type 1. Fourth, rapid increase of VWF but restricted as compared to FVIII:C followed by shortened half-life of FVIII/VWF labeled as clearance type in moderate to mild VWD type 1. Fifth, poor response of VWF:RCo and restricted responses of VWF:RCo/VWF:CB/FVIIII followed by shortened half-life typical for VWD 2M. Sixth, good response and normal half life of FVIII:C and VWF:Ag but restricted responses of VWF:RCo and VWF:CB followed by shortened half life times depending on the severity in VWD 2A and 2B defined as protelolytic type of VWD showing loss of large VWF multimers and increased triplet of each band according to Budde et al., [48] and ECLM (Figure 3, Table 1). Characteristic responses of VWF parameters to DDAVP in other subtypes of mild

Table 9: FVIII:C, VWF parameters and VWFpp/Ag ratios in affected members of the Dutch family with dominant VWD 2A due to the heterozygous V1499E/WT Guoda mutation in the A2 domain (VWF multimers are shown in Figure 10B) [45].

Patient	FVIII:C	VWF:Ag	VWF:RCo	RCo/Ag	VWFpp	VWFpp/	VWD type	Mutation
	U/mL	U/mL	U/mL	Ratio	U/ml	Ag Ratio	ISTH MM	
II-1	0.58	0.63	<0.15	0.23	0.76	1.3	2A	V1499E/WT
III-1	0.35	0.41	<0.15	0.36	0.45		2A	V1499E/WT
III-3								
III-4	0.35	0.33	<0.15	0.45	0.40	1.3	2A	V1499E/WT
III-6	0.35-0.46	0.31	<0.15	0.48	0.51	1.6	2A	V1499E/WT
III-7	0.22-0.64	0.22	<0.15-	<<0.68	0.44	2.0	2A	V1499E/WT
III-8	0.42-0.64	0.28	<0.15	0.53	0.54	1.9	2A	V1499E/WT
III-9	0.42	0.24	<0.15	<<0.62	0.38	1.6	2A	V1499E/WT
III-10			0.21					V1499E/WT
IV-2		0.21	0.17					V1499E/WT
IV-8	0.54	0.29	<0.15	0.52	0.43	1.5	2A	V1499E/WT
IV-11	0.49	0.27	<0.15	0.55	0.56	2.1	2A	V1499E/WT
Range						**1.3-2.1**		
III-2	1.02	1.12	1.27	1.13	1.09	1.0	N	WT/WT
IV-1	1.02	1.22	1.48	1.21	0.99	0.8	N	WT/WT
IV-5	0.45	0.67	0.55	0.82	0.73	1.1	N	WT/WT
IV-6	0.83	0.67	0.70	1.04	0.72	1.1	N	WT/WT
IV-9	0.78	0.79	0.60	0.75	0.72	1.2	N	WT/WT
IV-10	0.51	0.63	0.79	1.25	0.77	1.2	N	WT/WT
Normal	0.50-1.50	0.50-1.50	0.50-1.50		0.60-1.40	**0.8-1.2**	N	WT/WT

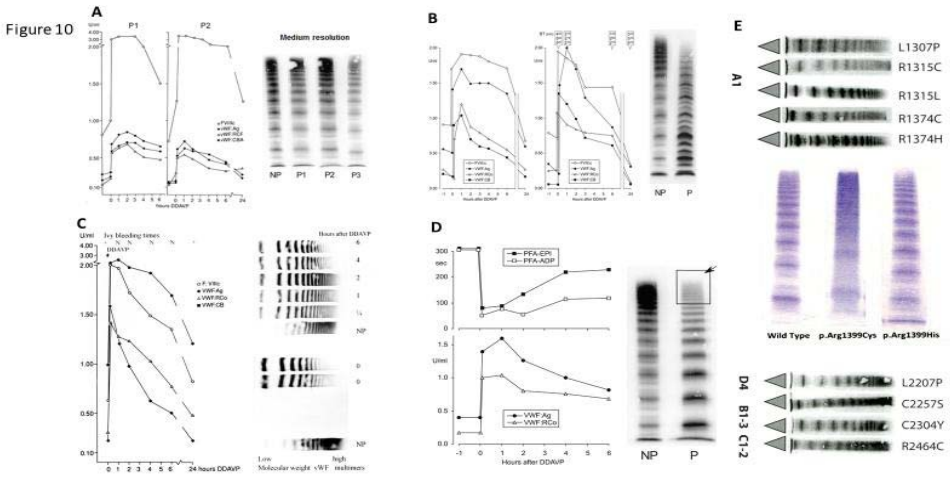


Figure 10: Gold standard ECLM diagnostic differentiation of classical dominant VWD type 1 SD *vs* moderate and severe 2A *vs* 2 E *vs* 2M and 2B by the combined use of FVIII:C, VWF:Ag, VWF:RCO and VWF:CB on yop of responses of VWF parameters to DDAVP and VWF multimeric analysis.
A: Restricted responses of VWF parameters *vs* normal response of FVIII:C to DDAVP followed by normal half-life times of all VWF parameters and FVIII:C diagnostic for VWD type 1 Secretion Defect (SD) in two patients (P1, P2) of one family with normal VWF multimeric pattern in three affected family members (P1, P2 and P3, mutation unknown). **B:** Transient good responses of VWF:RCo and VWF:CB and normal rsponse of VWF:Ag and FVIII:C to DDAVP followed by decreased half life times of VWF:RCo and VWF:CB due to increased proteolysis of VWF but near normal half life times for both VWF:Ag and FVIII:C typical for moderate VWD 2A in two affected members of one family (Gouda Dutch mutation V1499E) showing the loss of large multimers and increased triplets of intermediate and small VWF multimers due to increased proteolysis. **C:** Transient good resonse of VWF:RCo and VWF:CB and good response of VWF:Ag and FVIII:C to DDAVP followed by shortened half life times of the VWF parameters and FVIII:C due to multimerization defect diagnostic for VWD 2E multimerization and Clearance (C) defect (increased VWFpp/Ag ratio (Antwerp Belgian VWD 2E mutation W1120S, Table 6). **D:** Poor response of VWF:RCo and transien good responses of VWF:CB, VWF:RCo and FVIII:C to DDAVP followed by shortened half-life times of VWF:Ag and Factor VIII:C is diagnostic for VWD type 2M associated with an increased Clearence (C) defect (increased VWFpp/Ag ratio, table 10). **E top and middle.** The VWF multimers in VWD 2M patients due to cystein mutations R1315C, R1374C and Arg1399Cys in the A1 domain are smeary in contrast to near normal VWF multimeric pattern in the non-cystein mutations R1315L, R1374H and Arg1399His respectively. **E bottom**: Examples of dominant VWD type 1 patients due to L2207P, 2257C, 2304Y and 2464C mutations in the D4, B1-3, C1-2=D4, C1-6 domains showing a smeary pattern indistinguishable from 2M VWD.

Table 10: VWF missense mutations in the VWF GpIb receptor A1 domain of the VWF gene related to loss of GPPIb RIPA function in VWD 2M or 2U and gain of GPIb RIPA function in VWD 2B as reported in the 2008 ISTH-SSC VWF Online Database updated by Gadisseur & Michiels (Gadisseur et al., 2009) [49].

VWD Type	Exon	Mutation		VWD Type	Exon	Mutation	
1	28	3686T→G	V1229G	2B	28	3946G→A	V1316M
1	28	3692A →C	N1231T	2M	28	3970G→A	G1324S
2U	28	3702T→G	C1234W	2M	28	3971G→C	G1324A
2B	28	3797C→A	P1266Q	2B	28	4010C→T	P1337L
1/2B	28	3797C→T	P1266L	2B	28	4021C→T	R1341W
2B	28	3802C→G	H1268D	2B	28	4022G→T	R1341L
2B	28	3802C→A	H1268N	2B	28	4022G→C	R1341P
2B	28	3814T→G	C1272G	2B	28	4022G→A	R1341Q
2B	28	3814T→C	C1272R	2M	28	4075G→A	E1359K
2A	28	3815G→C	C1272S	2M	28	4085A→C	K1362T
2A	28	3827T→C	L1276P	2M	28	4105T→A	F1369I
2B?	28	3835G→A	V1279I	2U	28	4105T→A	F1369I
2M	28	3835G→A	V1279I	2U	28	4120C→T	R1374C
1	28	3835G→A	V1279I	2U	28	4120CA	R1374S
2M	28	3845T→G	1282R	2U	28	4121G→T	R1374L
2M	28	3854C→T	S1285F	2U	28	4121G→A	R1374H
2M	28	3887T→C	L1296P	2U	28	4135C→T	R1379C
2M	28	3905A→G	D1302G	2U	28	4148T→G	L1383R
2B	28	3912Ins	1304Ins	2A	28	4148T→C	L1383P
2B	28	3916C→T	R1306W	2M	28	4173-4205del	R1392-Q1402del
2B	28	3917G→A	R1306Q	2U	28	4195C→T	R1399C
2B	28	3917G→T	R1306L	2U	28	4215delAAG	K1405del
2B	28	3922C→T	R1308C	2U	28	4263C→G	N1421K
2A?	28	3923G→A	R1308H	2M	28	4273A→T	I1425F
2B	28	3925A→G	I1309V	2M	28	4309G→A	A1437T
2B	28	3929C→T	S1310F	1	28	4339G→C	E1447Q
2B	28	3939G→C	W1313C	2A	28	4373G→A	C1458Y
2B	28	3940G→T	V1314F	2B	28	4378C→G	L1460V
2B	28	3940G→C	V1314L	2B	28	4382C→A	A1461D
2B	28	3941T→A	V1314D	2U	28	4384C→G	P1462A
2U	28	3943C→T	R1315C	2M	28	4399C→T	P1467S
2M	28	3943C→T	R1315C				

VWD patients are not discussed here.

Hermans & Battle produced clearly defined dominant VWD 2 M due to lossof RIPA function caused by mutationsin the A1 domain as a distinct entity, which clearly differ from dominant pronounced VWD type 1 due to a secretion or clearance defect [49]. The genotype/phenotype correlation of RIPA loss of function mutations in the A1 domain with VWF:RCo/Ag ratios <0.60 is clear, whereas the VWF:CB/Ag ratio is normal in VWD 2M and the VWF multimers are normal in a low resolution gel [49]. The response to desmopressin (DDAVP) is poor for VWF:RCo and the response of both VWF:Ag and VW:CB is good in VWD 2M (Figure 10) [49]. Gadisseur et al could label the majority of VWD cases with loss of RIPA function mutations in the A1 domain as 2M or 2U in the 2008 update of the ISTH molecular data base of VWD (Table 10) [50]. Characteristic laboratory features of VWD type 2M include decreased RIPA, a poor response of VWF:RCo and good responses of VWF:CB (collagen type I van Vliet 2002 [1], collagen type I-III Budde [48]) and VWF:Ag and FVIII:C to DDAVP [1,26] and normal, smeary or some loss of large VWF multimers with less resolved triplet structure of each of the multimeric bands in medium resolution gels according to Budde et al., [48,51]. There are four RIPA positive VWD type 1 patients due to heterozygous mutations in the A1 domain (V1229G/WT, N1231T/WT, V1279I/WT and E1447Q/WT and one case of VWD 1/2B caused by the P1266L/WT mutation. Budde et al., demonstrated in his analysis of the MCMDM-1 VWD study [51] that the heterozygous 2M cysteine mutations R1315C/WT and R1374C/WT mutations had a smeary VWF multimeric pattern as compared to near normal VWF

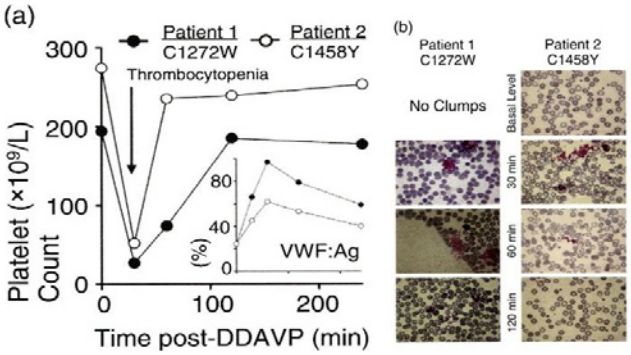


Figure 11: Poor responses to DDAVP VWF parameters in patient 1 with the R1272S mutation and in patients 2 with the R1484Y mutation in the A1 domain in table 11 was associated with transient thrombocytopenia of about one hour paralleled by an increase typical VWD 2B platelet clumps in blood smears. Spontaneous RIPA (0.5mg/mL) was now detected in '*in vitro*' plasma mixing studies at 30 min post-DDAVP infusion consistent with the diagnosis of VWD 2B [54].

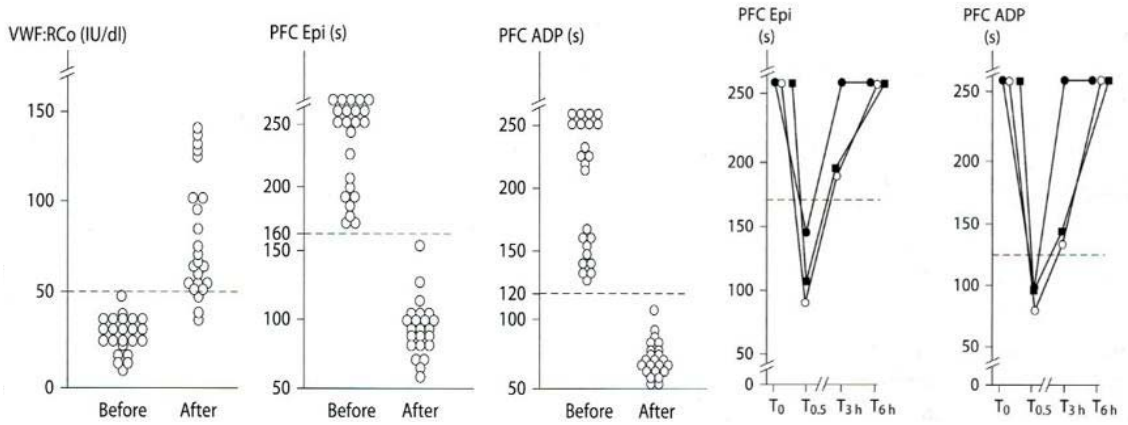


Figure 12: Responses to DDAVP of VWF:RCo and Platelet Analyser Closure Time (PFA-CT) using Epinehrine (Epi) and Adenosine Diahasphate (ADP) cartridges in the study of Fressinaud et al of 34 patients with ISTH defined mild/moderate VWD type 1 (range VWF:RCo 0.14 to 0.50 U/dL) before DDAVP (To) 0.5, 3 and 6 hours (h) after DDAVP. The results show complete correction of the PFA-CT Epi and ADP (left curves) for only a few hours post-DDAVP (right curves) [56,57]. VWD type 1 patients have prolonged PFA-CT ranging from the upper level of normal to 300 seconds (left curves). The response to DDAVP in VWD type 1 induced a transient correction of VWF parameters and PFA-CT in VWD type 1 for one or a few hours (right curves).

multimers in the heterozygous 2M noncystein mutations R1315l/WT and R1374H/WT in the A1 domain [6] (Figure 10). Recently, Fidalgo et al., [52] observed that the heterozygous Arg1399Cys/WT mutation shows smeary VWF multimers as compared with the noncystein heterozygous Arg1399His/WT mutation showing a normal VWF multimeric pattern (Figure 10). Similar findings has recently been confirmed by Boras & Battle in their large cohort of wel defined VWD 2M patients caused by mutations in the A1 domain [53]. In the cohort of VWD 2M VWD mutation reviewed by Gadisseur et al., [49], there are 3 heterozygous mutations R1272S/WT, L1276P/WT and C1460V/WT in the A1 domain, which are labeled as 2A in the 2008 ISTH data base (Table 10) [54,55]. Fischer, Brehm, Schneppenheim and Auton studied the VWD 2A heterozygous mutations in A1 domain p.Cys1272Trp (3816C>G/WT) in patient 1 and the p.Cys1458Tyr (4373G>A/WT) VWD 2A mutant in patient 2 of two unrelated girls (6.3y and 9.6y) with a well documented diagnosis of congenital type 2A VWD (Figure 11) [55]. The two unrelated girls presented with low VWF antigen VWF:Ag; 22% and 27%) and undetectable VWF ristocetin cofactor activity (VWF:RCo) and VWF collagen binding (VWF:CB) without prior evidence of thrombocytopenia or *in vitro* hyper responsiveness to low dose ristocetin (0.5 mg/mL). Hassenplug et al demonstrated that the recombinant R1272S did not show hypersensitivity to ADAMTS13 [14], which prompted Dr Michiels to address the key question on the true nature of the heterozygous R1272S/WT mutation as either VWD 2B rather than 2A. DDAVP treatment in the study of Fischer, Brehm, Schneppenheim and Auton, induced a very short transient thrombocytopenia in which platelet counts decreased 30 min post-DDAVP infusion from normal counts from 194 x109/L to 27x109/L in heterozygous patient 1 (R1271s/WT) and from 274 x109/L to 52x109/L in heterozygous patient 2 (C1458T/WT) with complete restoration of platelet counts to normal in both patients at 120 min (Table 11 is related figure 11). The transient thrombocytopenia of about one hour was paralleled by an increase in median platelet volume, and typical VWD 2B platelet clumps were observed in blood smears (Figure 11). Spontaneous RIPA (0.5mg/mL) diagnostic for VWD 2B was now detected in *'in vitro'* plasma mixing studies at 30 min post-DDAVP infusion. DDAVP induced transient minor increases in VWF:CB, factor VIII (FVIII:C), and VWF:Ag were evident, but VWF:RCo activity remained constant and unimproved as a function of time (Table 11, Figure 11). These observations confirm that the heterozygous mutations C1272/WT and

Table 11: Response to DDAVP of platelet count and von Willebrand factor (VWF) parameters, FVIII:C, activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT) in VWD patients with the heterozygous mutation Cys1272Trp/WT and Cys1485Tyr/WT in the A1 domain of the VWF gene [55]. The transient asymptomatic thrombocytopenia and poor responses of VWF:CB and VWF:RCo after DDAVP with no correction of the VWF multimers confirm that the heterozygous mutations at C1272 and C1458 are mislabeled as 2A has to be reclassified as VWD type 2B (Berlin variant) as documented in figure 11 [55].

Time post-DDAVP(min)	Basal	30	60	120	240
Patient 1,p.Cys1272Trp, c.3816C G,6.3y					
Platelets(*10^9(/L)	194	27*	74*	185	177
Mean Platelet volume (fL)	11.6	13.2	13.1	11.1	11
VWF:Ag(%)	24	66	97	79	59
FVII:C(%)	23	53	52	47	35
VWF:CB(%)	<5	11	10	7	<5
VWF:RCo(%)	<5	<5	<5	<5	<5
aPTT(s)	47.6	38.1	36.9	37.3	43.8
PT(%)	94	93	89	93	92
Patient 2,p.Cys1458Tyr,c.4373G A, 9.6y					
Platelets(*10^9(/L)	274	52*	35	239	253
Mean Platelet volume (fL)	10	12.745	10.1	9.6	9.5
VWF:Ag(%)	24	45	62	53	40
FVII:C(%)	34	181	85	64	44
VWF:CB(%)	<5	22	9	<5	<5
VWF:RCo(%)	<5	<5	<5	<5	<5
aPTT(s)	51.6	43.7	43.3	46.6	50.4
PT(%)	72	68	67	68	69

Values of <5 are below the limit of detection. *Thrombocytopenia.

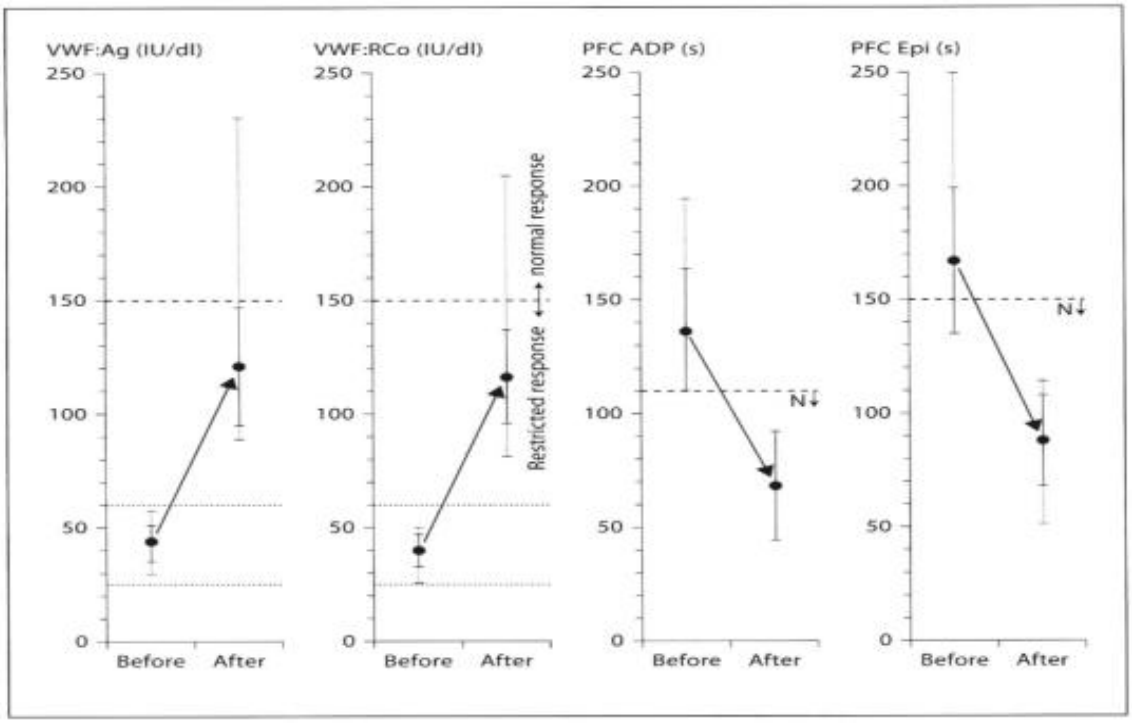


Figure 13: Result of responses to DDAVP of VWF:AG, VWF:RCo and Platelet Analyzer Closure (PFC ADP and Epi) times before DDVAP and one hour rafter DDAVP (0.3µg/kg) in 24 patients with VWD type 1 with normal VWF multimers (mild type 1 VWD LowVWF) in the study of Franchini et al., [58]. Means (black dots) + SD and ranges. Broken line upper range of normal. This study demonstrates that LowVWF VVWD patients with VWF levels between 0.30 and 0.60 U/dL is associated with prolonged PFA-CT between the upper limit of normal and 250 seconds and that DDAVP induces transient corrections of decreased VWF parameters and prolonged PFA-CT closure times to normal one hour after DDAVP in LowVWF VWD type 1 patients.

Table 12: Laboratory parameters in patients with von Willebrand disease type 2A, 2B, 2N, type 3 and VWD type 1 classified according to the 1994 ISTH classification of VWD in the study of Fressinaud et al., [56] and reclassified more strictly according to the Rotterdam modification of the ISTH classification proposed by Michiels & Van Vliet in 2002 [1].

Patients			VWF:		Ratio		VWD type	BT	PFA-100 CT seconds			
VWD type SSC-ISTH		Ag	Rco	Rco/Ag		reclassified 2002 Michiels		ADP		Epi		
Type 1 mild												
1			48	39	0.81		1 mild		10	136		**137**
2			40	39	0.98		1 mild		8	147		173
3			34	38	1.12		1 mild		6	154		202
4			62	38	0.61		1 mild		8.5	148		164
5			39	36	0.92		1 mild		18	193		**159**
6			45	35	0.77		1 mild		6	141		184
7			37	34	0.92		1 mild		6	144		183
8			35	34	0.97		1 mild		16	210		>250
9			38	34	0.89		1 mild		5	145		218
10			45	34	0.76		1 mild		6	138		180
11			42	31	0.74		1 mild		5.5	127		174
12			38	30	0.79		1 mild		8	129		191
13			35	28	0.8		1 mild		18	197		>250
14			29	28	0.97		1 mild		6	170		>250
15			48	27	0.56		1 mild		3.5	141		219
Type 1 or 2 moderate												
16			59	27	0.56		1 or 2		>20	>250		>250
17			31	26	0.84		1 moderate	7	>250		>250	
18			38	26	0.68		1 moderate	17	>250		>250	
19			28	22	0.79		1 moderate	15	>250		>250	
20			23	22	0.96		1 moderate	12	>250		>250	
21			39	22	0.56		2		17	181		>250
22			30	22	0.73		1 moderate	15	181		>250	
23			38	16	0.42		2		8.5	197		>250
24			24	15	0.62		1 moderate	6.5	>250		>250	
Type 1 severe												
25			24	14	0.58		1 or 2 severe	>20	>250		>250	
26			44	13	0.33		2 severe		>20	>250		>250
27			26	12	0.46		2 severe		>20	>250		>250
28			44	13	0.3		2 severe		>20	>250		>250
29			23	10	0.43		2 severe		12	>250		>250
30			12	10	0.83		1 severe		9	>250		>250
31			15	10	0.67		1 severe		>20	>250		>250
32			27	10	0.37		2 severe		11	>250		>250
33			12	9	0.75		1 severe		4	>250		>250
34			29	9	0.31		2 severe		8.5	>250		>250
35			9	0	1		1 severe		>20	>250		>250
36			9	5	0.55		1 or 2 severe	7.5	>250		>250	
Type 2A												
3 patients			75-92	23-43	0.27-0.57		2A mild		8.5-18	>250		>250

7 patients			30-74	<3-43	0.07-0.57		2A		>20	>250		>250
Type 2B												
2 patients			51-61	15-17	0.25-0.33		2B		14->20	>25-		>250
Type 3												
4 patients			<1	<3	-		3		>20	>250		>250
Type 2N												
2 patients			67-83	54-78	0.81-0.94		FVIII:BD		4-5.5	81-94		<150
Hemophilia A	FVIII:C											
12 pts		<1-	57-	54-	0.80-		-		-	63-119		87-138
27	200	164	1									
Hemophilia B	FIX											
2 patients		1.5-3	74-190	58-186	0.78-0.89		-		-	60-109		<130
Normal values		**56-207**	**58-209**	**>0.60**		**-**		**<8.5**	**<120**		**<160**	

C1458T/WT are mislabeled as 2A and has been reclassified by Fisher et al as VWD type 2B (VWD Berlin) [55]. Under a normal life, no stress, or stimulated situations VWD Berlin present as phenotypically VWD 2A according ISTH without the use of a DDAVP challenge test, but after a DDAVP challenge test classified as VWD 2B accompanied by transient thrombocytopenia and not associated with TTP like symptoms due to de-aggregation of platelet clumps by ADAMTS13 mediated increased proteolysis of VWF in VWD 2B.

The Role of PFA-CT, DDAVP Trial and Mutation Analysis in VWD Type 2 Versus Lowvwf VWD

In 1999 Fressinaud et al., [56] evaluated the performance of the PFA-100 closure times (PFA-CT) in 60 ISTH defined patients with VWD type 1 and 2 [19]. The Fressinaud study included 36 patients with ISTH defined VWD type 1, 24 patients with VWD 2A, 2B, 2N, and 3, and in 14 patients with hemophilia. The VWD patients in table 12 could be reclassified by Michiels using the 2002 Rotterdam critera [1] as mild VWD type 1 in 15 (VWF:RCo 28-39IU/dl), moderate VWD type 1 or 2 in 9 (VWF:RCo 15-27 IU/dl) and severe VWD type 1 or 2 in 12 (VWF:RCo 5-14 IU/dl). All patients with severe VWD type type 1 or 2, 2A, 2B and 3 had prolonged PFA CT unmeasurable above 250 seconds in the majority (Table 12). Mild VWD type 1 patients had normal to marginally prolonged BT and prolonged PFA CT with values between the upper limit of normal and 250 seconds indicating the superiority of PFA-CT above the less sensitive classical BT measurement. These observations lead to the conclusion that the PFA-100 is clearly superior to that of BT for the dectecion of VWD. A normal PFA-CT may exclude all variants of type 1, 2 and 3 VWD except 2N. PFA CT Epi and CT ADP were normal in VWD 2N and in hemophilia patients (Table 12).

Fressinaud et al., [57] subsequently evaluated the PFA-100 closure times (PFA-100 CT) in the manegement of 23 mild to moderate type 1 VWD patients with DDAVP. Before DDAVP infusion, the level of VWF:Ag ranged from 15 to 56 (mean 33±9) IU/dl and that of VWF:Rco from 14 to 49 (29±8) IU/dl. All VWD patients had prolonged PFA-100 CT Epi *vs* ADP above 250 seconds in 13 *vs* 8 patients and between the upper limit of normal to 250 seconds before DDAVP in 8 and 25 VWD patients respectively (Figure 12). The PFA CT returned to normal one hour after DDAVP. There was no further follow-up except in 3 cases showing only transient correction of CT for only a few hours (Figure 12). Such transient corrections of PFA-100 are typically seen in patients with mild to moderate VWD type 1/2E, mild to moderate 2M, 2M-Vicenza, mild 2A and in various variants of dominant VWD type due to a secretion and/or rapid clearance defect.

Franchini et al., evaluated in 2002 the PFA-CT in 24 patients with mild type 1 VWD with baseline VWF values between 0.25 and 0.50 U/dL (LowVWF) irrespective of ABO blood group [58]. The PFA-CT were slightly to moderately prolonged but strongly prolonged in only a few and transiently corrected to normal after DDAVP (Figure 13) [58]. Such strongly prolonged PFA-CT transiently corrected to normal similar as seen in VWD type 2E or moderate 2M or 2A. The VWD type 1 patients in the Franchini et al study had clearly defined LowVWF phenotype with VWF:RCo levels between 0.20-0.50 U/dL associated with slight to moderate prolongation of PFA-CT between the upper level of normal to 300 seconds [58].

Michiels & Van Vliet observed in 2002 three types of DDAVP reponses of FVIII:C and VWF parameters in a cohort of 24 patients with mild LowVWF type 1 VWD with VWF values between 0.25 to 0.60 U/dL (LowVWF)1. First, the responses to DDAVP of the VWF parameters in the proband with mild type 1 VWD and VWF parameters around 0.25 to 0.30 U/dL was restrictive reaching values to around 0.80U/dL and this was followed by shortened half-life times of VWF parameters (T ½ VWF:Ag 5.8 hours and VWF:RCo 4.8 hours) indicative for VWD type 1 secretion clearance type [1]. Second, LowVWF patients with values of FVIII:C, VWF:RCo and VWF:CB between 0.30 to 0.60 U/dL had resricted responses of VWF:AG and VWF:RCo as compared to normal responses to DDAVP of FVIII;C followed by shortened half life times of FVIII and VWF levels indicative for increased clearance of VWF frequently seen in VWD type 1E, 2E and 2M VWD patients. Third, LowVWF patients with values of FVIII:C, VWF:RCo and VWF:CB between 0.30 to 0.60 U/dL had completely normal responses to DDAVP of FVIII;C and all VWF parameters to above 2.0 U/dL followed by normal half life times of FVIII and VWF levels up to 24 hours after DDAVP were diagnosed as pseudo-VWD (Figure 7C) [1].

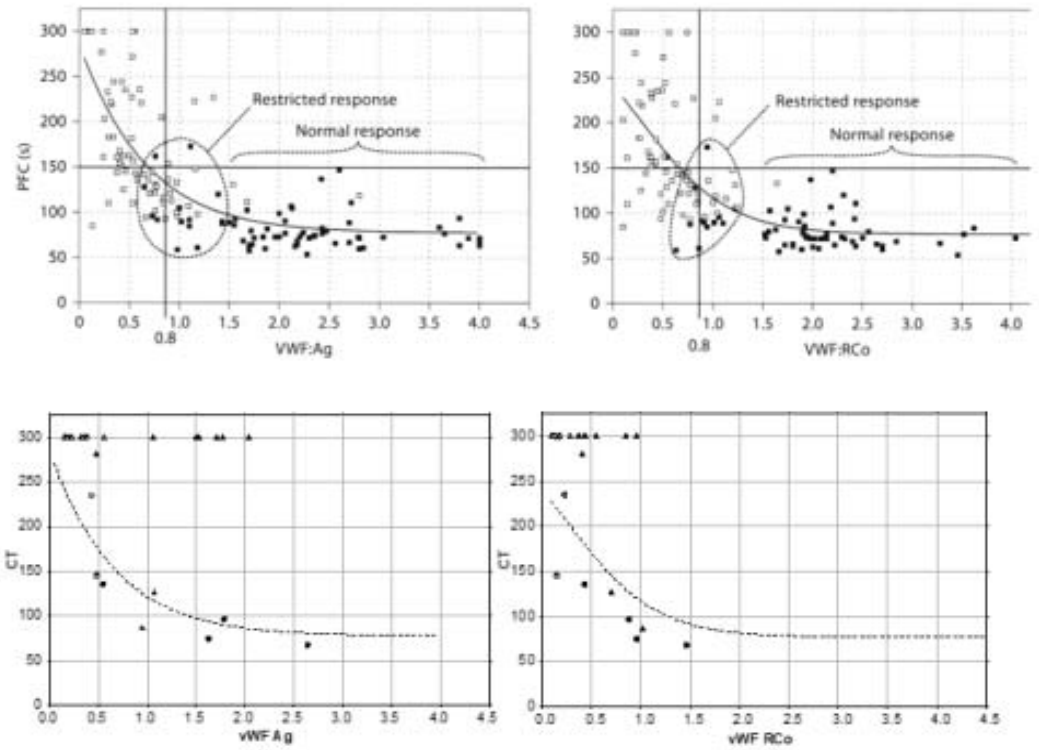


Figure 14: Upper panel. The responses to DDAVP of PFA-100 closure time PFC(s) versus VWF:RCo and PFC(s) versus VWF:RCo in VWD type 1 patients PFA CT before () and 1 hour after (Y) DDAVP infusion in the 2008 study of Van Vliet, Kappers, Leebeek & Michiels [59]. The PFA CTs normalised (<150 sec) in the majority of the patients with mild VWD type 1 (LowVWF). The responses to DDAVP of VWF:RCo to DDAVP showed two clusters: those with restricted responses of VWF:RCo values up to maximal 0.70 and 1.5 U/ml after DDAVP (restricted responders similar as in the Franchni study) versus those with maximal values of VWF:RCo to above 1.5 to above 2.0U/ml after DDAVP (normal responders). Within this group of good responders one hour post-DDAVP there are two subgroups, one with shortened VWF half-life times in cases with mild VWD type 1 and those with normal FVIII-VWF half-life times in cases of pseudo-VWD related to blood group O (Michiels et al., 2002). The response of FVIII:C one hour after DDAVP is two to three times higher that of VWF:Ag and VWF:RCo in Obligates Carriers (OC) of a null allele from parents with VWD 3 and in OC of a missense mutation from Obligate Carriers (OC) from parent with recessive severe type 1 or 2C.
Lower panel: The response to DDAVP in VWD type 2 patients PFA CT before DDAVP (type 2A or 2B Δ, N=10; type 2M ❍ N=2) and 1 hour after DDAVP (type 2A or 2B ▲ N=10; type 2M ●) in the 2008 study of Van Vliet, Kappers, Leebeek & Michiels [59]. Twelve VWD type 2A or B with lack of large VWF multimers had PFA-CT above 300 seconds of whom 5 vases showed a transient correction of PFA-CT after DDAVP. Two VWD 2 M patients with normal VWF multimers had moderate prolongation of PFC-CT beween normal and <300 sec and showed a complete but transient correction to normal after DDAVP.

Between 1998 and 2008 Van Vliet, Kappers, Leebeek & Michiels prospetively evaluated dose responses levels of PFA-CT, VWF and FVIII before and one hour after DDAVP in patients with mild LowVWF type 1 VWD (n=70, VWF range 0.25-0.60 U/dL) who received an infusion of DDAVP (0.3µg/kg) [1,59]. In this study VWF:Ag was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) and VWF:CB by an EIA using collagen type 1 (Figure 14) [1,59]. VWF ratio (VWF:CB/VWF:Ag) was used as a surrogate measure of the multimer distribtution [1,59]. VWF:RCo was determined by measuring the rate of aggregation of fixed platelets induced by ristocetin and patient plasma (von Willebrand factor) with the PAP-4 aggregometer (Biodata) [1,20,23]. Platelet Analyzer Closure Time (PFA-CT) was assessed by using the Platelet Function Analyser (PFA-100® Dade-Behring, Marburg, Germany). The DDAVP responses of PFA-CT, VWF:Ag and VWF:RCo before and after DDAVP in VWD type 1 as shown in figure 14 reveal that pre-treatment values of FVIII -VWF parameters ranged from 0.30 to 0.60 U/dL in the majority of LowVWF patients (VWD type 1, open symbols). The PFA CTs before DDAVP were normal (<160 seconds) or slightly increased in between 160 to 300 seconds but usually less than 300 seconds in the majority and prolonged above 300 seconds in only a few cases of VWD type 1 (figure 14). The LowVWF type 1 VWD patients treated with DDAVP demonstrate a good dose-response with correction of the PFA CTs (closed symbols, figure 14) with normalization of the PFA CTs (<150 sec) in the majority of the patients with VWD type 1. Normalization of the PFA-100 closure time (<150 seconds) was reached at VWF functional levels of about 0.70U/ml VWF:CB whereas the minimal closure time around 80 sec as the maximal effect of DDAVP response of VWF was obtained at VWF functional levels between 2.0 and 2.5 U/ml. This appears to us a key phenomenon showing a correlation between VWF:CB ranging from 0.70 to 2.00 U/dL with correction of the PFA-CT to normal from 150 to 80 seconds indicating that LowVWF type 1 VWD patients are physiologically associated with slightly to moderately increased PFA-CT between 150 to 300 seconds. The present study demonstrated slight to moderate prolongations of PFA-CT in LowVWF type 1 VWD patients due to heterozygous mutations in the D1, D2, D', A3 (Table 13) and in some mutations in the A1 and A2 domain (Y1584C) with VWF between 0,30 and 0,50, and to a less extend also in LowVWF between 0.40 to 0.60 U/dL in individuals with the absence of a causative mutation in the VWF gene

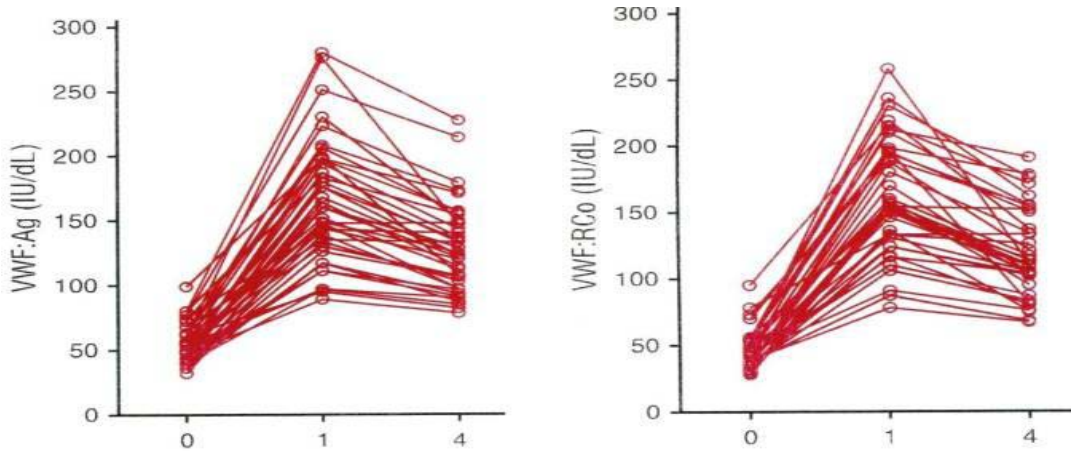


Figure 15: Lavin et al selected a subgroup of 40 LowVWF patients with mild VWD type 1 (range of VWF parameters 0.30-0.50 U/dL) in whom plasma VWF:Ag and VWF:RCo were measured before, and after 1 hour and 4 hours post-DDAVP [60]. The VWF response curves to DDAVP in the Lavin study show three variants of VWD type 1: VWD Secretion Defect (SD), VWD Clearance (C) defect, and pseudo VWD showing normal responses of FVIII:C and VWF followed by normal half lifetime of FVIII:C and VWF parameters.

Table 13: Classification of VWD patients due to heterozygous mutations in the C1 to C6 domain of the VWF gene according to ECLM criteria using the results of FVIII:C and VWF measurements in the Budde MCMDM study of VWD type 1 due VWF mutations in the D4 C1-6 domains [51].

Mutation/	FVIII:C	VWF:Ag	VWF:RCo	VWF:CB	RCo/Ag	CB/Ag	VIII:C/Ag	VWD type
Domain	U/dL	U/dL	U/dL	U/dL	ratio	ratio	ratio	
L2270P/C1	0.71	0.26	0.19	0.35	0.73	1.35	**2.7**	1 SD sm
C2257S/C1	0.70	0.29	0.42	0.30	1.45	1.03	**2.4**	1 SD sm
C2304Y/C1	0.42	0.20	0.19	0.37	0.95	1.85	**2.1**	1 SD sm
C2362F/C2	0.88	0.35	0.34	0.39	0.97	1.11	**2.5**	1 SD sm
G2441C/C3	0.61	0.22	0.20	0.26	0.91	1.18	**2.8**	1 SD sm
C2464C/C3	0.32	0.25	0.25	0.37	1.00	1.48	**1.7**	1 SD sm
C2464C/C3	0.52	0.33	0.30	0.50	0.91	1.52	**1.7**	1 SD sm
C2477Y/C4	0.68	0.38	0.33	0.43	0.87	1.13	**1.8**	1 SD sm
C2477S/C4	0.58	0.28	0.40	0.31	1.43	1.11	**2.1**	1 SD sm
Q2520P/C4	0.48	0.19	0.27	0.19	1.42	1.0o	**2.5**	1 SD sm
Yadegari et al. [62]								
C2085Tyr/D4	0.42	0.22	0.13		0.78		**1.91**	1 SD sm
C2327Trp/C1	0.22	0.13	0.07		0.63		**1.69**	2 M sm
C2619Tyr/C5	nt	0.37	0.43		0.70		nt	1
C2676Phe/C6	0.84	0.39	0.30		0.77		**2.15**	1 SD

on DNA testing (Table 13). Normalisation of the PFA-100 closure time (<150 seconds) was reached at VWF levels of about 0.75U/ml.

The response to DDAVP of VWF:RCo to DDAVP in VWD type 1 in the Rotterdam study showed two clusters (figure 14) [59]. First, LowVWF VWD patients with restricted responses with values of VWF:RCo up to maximal 0.50 and 1.5 U/ml are consistent with the diagnosis of restricted response to DDAVP in VWD type 1. Second, mild VWD type 1 showing DDAVP responses of VWF:RCo to values above 2.0U/mL are labeled as good to complete responders to DDAVP in patients (individuals) with LowVWF, who in fact do not have VWF deficiency and can readily be labeled as pseudo-VWD (Figure 7C) [1,4]. Sadler calculated in 2003 that most diagnoses of mild VWD type 1 (LowVWF) are mainly false positive VWD labeled as pseudo-VWD by Michiels in 2002 [1,23]. Sadler reasoned that in the general population 25% have one or two mild bleeding (clinically mild but insignificant) and 2.5% are low plasma VWF indicating that 0.25x 0.025=0.6% individuals in the general population with a correct diagnosis of LowVWF type 1 VWD VWF and mild bleeding manifestations in particular when associated with blood group O [23]. LowVWF mild VWD type 1 with mild bleeding symptoms and VWF:Ag, VWF:RCo and VWF:CB are featured by VWF parameters between 0.30 to 0.60 U/ml and normal ratios for VWF:RCo/Ag and VWF:CB/Ag with no or mild bleeding symptoms, no family history of bleeding [1-4,7].

Lavin et al., [60] studied a large cohort of 126 LowVWF type 1 VWD patient with VWF values in the range between 0.30-0.50 U/dl

as defined by the NHLBI and the UK HCDO [61]. By this selection criterion the majority of LowVWF type 1 VWD patients were women (122/126=89%) and the median median age of enrollment was 38,8 years very suggestive for an "acquired reproductive organ" related bleeding in young adult females with no childhood bleeding history. Bloodgroup O was more common, 89% compared to the normal population, 55% as could be expected in LowVWF type 1 VWD patients with mild bleeding. Lavin et al analysed the possible underlying background of this cohort of 126 LowVWF patients diagnosed as mild VWD type 1 with normal VWF:RCo/Ag ratios using the NHLBI/HCDO inclusion criteria. Concomittant hemostatic coagulation defects or platelet functional defeats were recorded in 12 cases. Both plasma FVIII:C and VWF:Ag levels were significantly reduced (mean 76U/dl for FVIII:C, data for VWF:Ag not shown) in the 126 LowVWF VWD type 1 patients compared to normal controls (mean FVIII:C 105U/dl, data for VWF:Ag not shown) [61,62]. The FVIII:C/VWF:Ag ratios were slightly increased in LowVWF patients (1.3) as compared to controls (1.07) indicating a secretion defect in those Low VWF patients with FVIII:C/VWF:Ag ratios above 1.4 [1,6]. The plasma VWFpp levels and VWFpp/Ag ratios above 3 were clearly increased in 8 cases (VWFpp/Ag above 2 in 18 cases=14%), strongly indicative for a pronounced clearance defect, which most frequently are due to mutation in the D3, A1 domains andsomtimes in the A3, D4, C3 domains [42-44]. Lavin et al., [60] did not check the mutation in those eight cases with increased VWFpp/Ag ratios as the underlying molecular cause of a pronounced VWF clearance defect. Only 40% of the LowVWF type 1 VWD patients were found to have a damaging mutation of the VWF gene (WT/null mutaton in 7, WT/2N in 2, loss of function mutations in the A1 domain in 18, Tyr1594Cys mutation in the A2 domain in 11, and WT/missense mutation in the D4-C1-6 domain in 5 cases. Lavin et al selected a subgroup of 40 LowVWF patients in whom plasma VWF:Ag and VWF:RCo were measured before, and after 1 hour and 4 hours post-DDAVP (figure 15) [60].The response curves of VWF:Ag and VWF:RCo to DDAVP in the Lavin study (Figure 15) show that the cohort LowVWF patients contain all three variants of VWF Secretion Defect (SD), Clearance (C) defect (indicated by arrow in figure 15) and pseudo VWD (Figure 7C) as defined by Michiels et al in the Rotterdam VWD study [1]. It has to be stated here that VWD type 1 patients with normal VWF multimers (VWD type 1m) due to mutations in the D4-C1-6 domain in the European MCMDM-1VWD study due to mutations L1774S, K1794E*, C2304Y*, R2313H, G2518S*, Q2544X*, C2693Y, and P2722A in the C1 to C6 domains, have mild VWD type 1 disease, are autosomal dominant or mild with variable penetrance of beeding manifestations (star indicates secretion defect, Table 13) [51], which could be confirmed recently in two studies [52,59]. VWD type 1 VWD patients in the European MCMDM-1 VWD study with abnormal multimers (VWD type 1sm) included of heterozygous mutations in the D4 and C1-6 CK domains V1822G*, L2207P*, C2257S*, C2304Y*, C2362F*, G2441C*, R2464C*, C2477Y*, C2477S*, and Q2520P* and usually present with mild to moderate dominant VWD type 1, and a smeary pattern of abnormal VWF multimers (star indicates secretion defect, Table 13). The majority of VWD 1m and 1sm mutations in the D4 and C1-6 have increased FVIII:C/VWF:Ag ratios around or above 2 (indicated by an astrix) indicating a Secretion Defect (SD) and a restricted response of VWF parameters to DDAVP.

The Platelet Function Analyzer Closure Times (PFA-CT) are strongly prolonged (>250 or >300 sec) in recessive VWD type 1, recessive 2C due to homozygous or double heterozygous mutation in the D1 and D2 domains, and in dominant 2A, 2B, 2E, 2M and 2D due to heterozygous mutations in the A1, A2, A3 and CK domains. PFA-CT are slightly to moderately prolonged with values between normal and 250 or 300 seconds in mild VWD type 1 patients due to heterozygous/WT mutations in the D1, D2, D', A3, D4 and C1-6 domains and in some mutations located in the A1 (P1266L/WT) and A2 (Y1584C/WT) domains. PFA-CT are slightly prolonged in LowVWF carriers of a null/WT or missense/WT mutation ranging from 0,30 and 0,50 U/dL. PFA-CT is normal or near normal in LowVWF between 0.40 to 0.70 U/dL with the absence of a causative mutation in the VWF gene.

Conclusion

In conclusion, the proposed translation of the ISTH criteria in figure 1 and 2 into the European Clinical, Laboratory and molecular (ECLM, Table 1, Figure 3) classification of VWD in the present study will facilitate improved diagnostic performance, better targeted treatment options of bleeding manifestations, and proper clinical orientation in precision medicine and personal VWD management counseling and prevention of bleedings particularly in risky situations like trauma and surgery for bleeding prevention.

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