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Review Article

Von Willebrand Disease (VWD): Diagnostic Differentiation of Pseudo, Mild, Moderate to Severe VWD Type 1 and 2 by a DDAVP Challenge Test on Top of the ISTH Classification

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Abstract

A complete set of FVIIII:C and von Willebrand factor ristocetine cofactor, collagen binding and antigen (VWF:RCo. VWF:CB, VWF:Ag) and Ristocetine Induced Platelet Agglutination (RIPA) and VWF multimeric analysis in a low resolution gel is mandatory to diagnose all variants of Von Willebrand Disease (VWD) according to ISTH (International Society on Thrombosis and Haemotasis) criteria. The response to DDAVP of VWF parameters is normal in pseudo-VWD, and mild VWD type 1 Bloodgoup O, but restrictive in carriers of recessive type 1 and 3 VWD. The response to DDAVP is restricted in pronounced VWD type 1 and 1/2E, transiently good in mild type 2A group II, good for VWF:CB but poor for VWF:RCo in VWD 2M, poor for VWF:RCo and VWF:CB in 2A group I, 2B, 2C and 2D, very poor in recessive VWD severe type 1m and absent in VWD type 3. VWF multimers in a low resolution gel are normal in VWD type 1 and 2M, but RIPA is normal in dominant VWD type 1 and decreased in dominant VWD type 2M.

Dominant VWD 1/2E due to mutations in the D3 domain of VWF result in defective multimerization, defective secretion and/or increased clearance of VWF. The triplet structure of each band and loss of large VWF multimers is charateristic for VWD type 2A and 2B due to increased proteolysis of each VWF band. Mild to moderate VWD 2A group II patients have normal FVIII:C and VWF:Ag, decreased VWF:RCo and VWF:CB, normal RIPA and transient correction of BT, FVIII:C, VWF parameters, and large multimers for a few hours post-DDAVP. Severe VWD 2A group I patients have low VWF:Ag and very low levels for VWF:RCo and VWF:CB, no RIPA and poor response of functional VWF:RCo and VWF:CB. VWD 2B is featured by loss of large VWF multimers due to increased proteolysis caused by increased interaction of platelets and mutated VWF.

Keywords: Von willebrand disease; Von willebrand factor; ADAMTS13; DDAVP; Von willebrand factor assays; Von willebrand factor multimers; Von willebrand factor mutations

Introduction

The Von Willebrand Factor (VWF) mediates the adhesion and aggregation of platelets to the subendothelium at sites of vascular injury and therefore plays a crucial role in the earliest stages of primary hemostasis. The VWF is the carrier protein of coagulation Factor VIIII (FVIII) in the circulation. The hemostatic potency of VWF depends on the degree of multimerization with the largest multimers being most hemostatically effective in platelet adhesion and aggregegation, thereby securing primary hemostasis as measured by the bleeding time and PFA-100 closure times.

Congenital Von Willebrand Disease (VWD) is a hereditary life-long bleeding disorder caused by a quatitative type 1 or a functional type 2 VWF deficiency or the absence of VWF and FVIII in type 3 VWD labeled pseudohemophilia by Erik von Willebrand [1,2]. The present evaluation describes the use of FVIII and the Von Willebrand Factor (VWF) assays VWF antigen (VWF:Ag), VWF ristocetine cofactor (VWF:RCo), VWF collagen binding (VWF:CB), VWF multimers, Ristocetine Platelet Aggregation (RIPA) and responses of FVIII and VWF parameters to DDAVP to correctly diagnose type 1 and type 2 VWD as manadory in routine daily practice when the ISTH (International Society on Thrombosis and Haemostasis) criteria are applied [3-5].

Bleeding manifestations

A standardized questionaire for bleeding history of suspected VWD include epistaxis, cutaneous bruises and hematomas, minor wounds, tooth extraction, bleeding after minor and major trauma and surgery and menarche and menorrhagias for women [6]. Each possible bleeding event is scored as 1 point when present and no action, as 2 points when present with medical attention and as 3 points when pronounced with intervention. A score of less than 2 points excludes a bleeding tendency. One clear cut bleeding even and a score of more than 2 points is a clear indication for laboratory screening in

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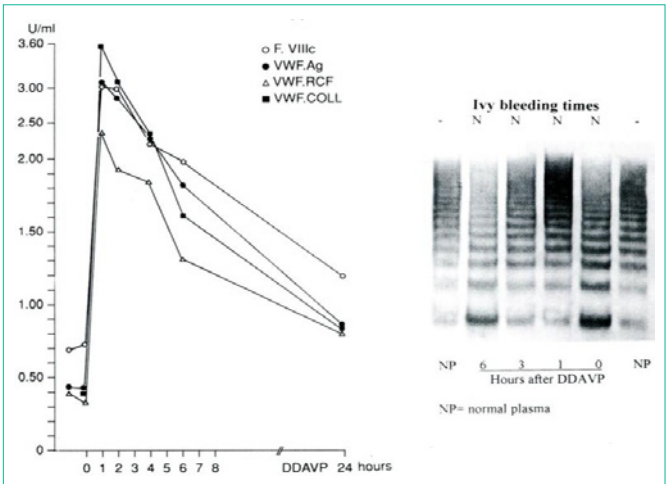

Figure 1: Completely normal responses of FVIII:C and all Von Willebrand Factor (VWF) parameters to DDAVP from around 0.4 U/mL to high levels of 2.5 to 3.0 U/mL followed by normal half life times of VWF Antigen (Ag) Ristocetine Cofactor (RCF) and Collagen Binding (COLL) activities consistent with the diagnosis of pseudo-Von Willebrand Disease (pseudo-VWD). This case underwent major orthopedic surgery under the cover of DDAVP (0,3 ug/kg iv) and no bleedings were noted in the perioperatve and postoperative period [2].

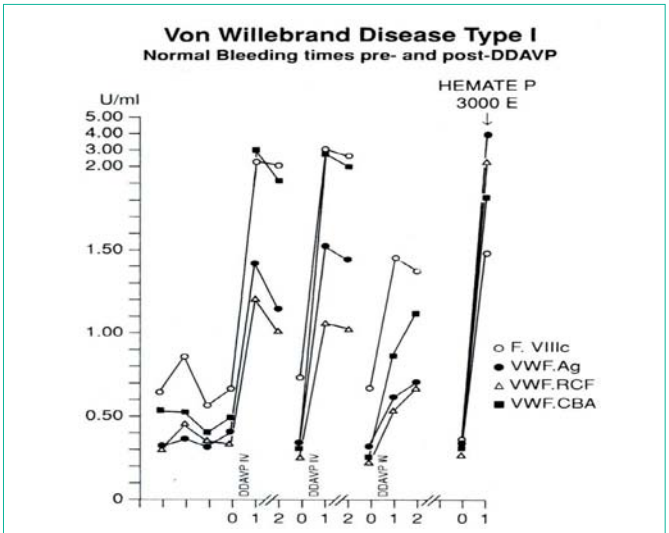

Figure 2: FVIII and Von Willebrand Factor (VWF) parameters in mild VWD type 1 with normal Ivy bleeding times, normal VWF multimers and increased FVIII/VWF:Ag ratio before and after DDAVP with restricted responses of VWF parameters as compared to FVIII to DDAVP, which is diagnostic for mild VWD type 1 in a young adult woman with easy bruisability and profuse menstruations and anamnestically no overt bleeding manifestations in childhood.

search for a bleeding defect. The sensitivity, specificity and negative predictive vaues of a bleeding score of more than 3 in male and 5 in female are 65%, 99% and 99.6% respectively. If less restricive cut-off had been choosen (a score higher than 2 in males and females), the sensitivity and specificity for diagnosis of carriers of VWD would have been 78.5% and 86.9% respectively. In our experience one clear cut prolonged bleeding episode oe event with attention (score 2) or with intervention (score 3) is suspcious for a bleeding diathesis. A positive bleeding score of VWD specific bleeding manifestations during childhood in particular bleeding after umbilical cord loss, after the first menarch and after minor or major trauma or surgery are indicative for a congenital VWD bleeding disorder.

In this report bleeding severity in patients with VWD were graded as very mild, mild moderate and severe according to Dr Eikenboom et al (Leiden University Medical Center) [7].

Very mild: only one or or two unclear minor bleeding symptoms of minor clinical significance.

Mild: one or two obvious symptoms such as recurrent epistaxis, profuse menstruations, or frequent hematomas, which usually do not require treatment. This is usually mild VWD type 1 (Figures 1 and 2).

Moderate: more than two bleeding symptoms for which FVIII/VWF concentrate transfusion was needed because of abnormal bleeding after surgery, trauma or both, orhas bled for more than 24 hours after tooth extraction. A moderate bleeding type is usually recognized in childhood in dominant or recessive VWD type 2 and pronounced type 1.

Severe: the VWD patient has severe or moderate pseudohemophilia, hemarthrosis, muscle bleeding, and a need for prophylactic FVIII/VWF concentrate.

Laboratory diagnosis and ISTH classification of VWD

The laboratory methods of platelet function, FVIII:C VWF parameters and DDAVP challenge test are described in great detail [2]. The laboratory diagnosis of VWD is based on decreased values of FVIII, VWF parameters and the results of RIPA, VWF:RCo/Ag ratio, VWF:CB/Ag ratio, FVIII/VWF:Ag ratio and subsequently classified according to the following criteria [1-5,7].

VWD type 1 is a quantitative VWF deficiency below the level of normal with equally decreased values of VWF:Ag, VWF:RCo, VWF:CB, a normal VWF:RCo/Ag or VWF:CB/Ag ratio and normal VWF multimers. A decreased FVIII/VWF:Ag ratio below 0.50 is indicative for a FVIII binding defect on VWF (VWDtype 2N). An increased ratio of FVIII/VWF:Ag above around 2.00 in VWD type 1 after DDAVP is indicative for a secretion defect as one of the mechanisms of decreased VWF parameters.

VWD type 2 is a qualitative VWF deficiency with normal or decreased values for FVIII and VWF:Ag, and much lower values for the functional VWF parameters VWF:RCo and VWF:CB with decreased ratios (<0.60) of VWF:RCo/Ag and VWF:CB/Ag and loss of large VWF multimers. VWD type 2 can be subclaasified as 2A with normal or decreased RIPA, 2B with increased RIPA, and 2M with selective loss of VWF:RCo but normal VWF:CB and multimers [1-5].

The spectrum of VWD type 1 and type 2: one center experience 1990-2000

Michiels & Van Vliet analysed between 1992 and 1997 275 index cases of patients coded as VWD irrespective of blood O or non-O in the Academic Medical Center Rotterdam with a referral region of about 2 million inhabitants [2]. The first group of 128 (46.5%) index patients had VWF antigen and functional levels around 40% to 60%, a very mild bleeding tendency, no family history, normal Ivy bleeding times, usually showed normal responses of VWF and FVIII:C to DDVAP when tested on indication, do not have von

Table 1: Good responses of von Willebrand factor (VWF) parameters to DDAVP in 24 cases with mild von Willebrand disease (VWD) type 1. Mild type 1 VWD patients are subdivided in those with completely normal VWF reponses and normal half life times of VWF":Ag and VWF:RCo can be diagnosed as pseudo-VWD (Figure 3) and those with restricted responses of VWF to DDAVP followed by shortened half life times of VWF;RCo and VWF:CB (Figure 2).

Case	Before DDAVP T = 0						Within 15 Minutes after DDAVP					
	vWF: FVIII u/mL	Ag u/ml	Rcof u/ml	CBA u/ml	Rcof/Ag Ratio	CBA/Ag Ratio	vWF: Ag u/mL	Rcof/Ag Ratio	CBA/Ag Ratio	T1/2 VWF. RCo/Ag. CB/ Ag		
1	0.3	0.21	0.15	0.13	0.75	0.65	2.27	0.92	1.67	9.1	10.2	4.2
2	0.29	0.24	0.2	0.17	0.8	0.71	1.65	0.8	2.2	8.5	7	4
3	0.81	0.5	0.35	0.34	0.76	0.68	2.58	0.78	1.22	9.3	10.1	5.2
4	0.78	0.52	0.48	0.49	0.92	0.94	2.74	0.78	1.59	10	8.3	5.8
5	0.65	0.6	0.54	0.48	0.92	0.8	2.76	0.76	1.75	11	8.2	6.4
6	0.98	0.43	0.29	0.3	0.67	0.7	2.55	1.19	1.78	14	9.4	8.4
7	0.3	0.37	0.39	0.36	1.05	0.97	3.18	1.19	1.23	12	10	5.6
8	0.38	0.38	0.32	0.26	0.84	0.68	2.19	0.98	1.05	11	8.9	6.1
9	0.82	0.56	0.48	0.75	0.86	1.27	1.96	1.11	1.59	8.7	9.7	5.1
10	0.83	0.51	0.45	0.56	0.88	1.1	1.28	0.69	1.34	10	7.4	6.3
11	0.69	0.54	0.57	0.56	0.97	0.95	2.02	1.05	1.25	12	7.8	6.9
12	0.82	0.6	0.52	0.34	0.87	0.57	1.15	1.11	1.03	13	9.4	8.3
13	0.65	0.51	0.45	0.56	0.88	1.1	1.28	0.69	1.34	11	7.4	6.3
14	0.58	0.47	0.44	0.47	0.94	1	2.01	0.65	1.61	9.3	8.7	5.4
Mean	**0.63**	**0.46**	**0.4**	**0.41**	**0.86**	**0.86**	**2.12**	**0.91**	**1.48**	**9.4**	**8.7**	**6**
15	0.3	0.25	0.27	0.12	1.08	0.48	2.48	1.08	1.29	4.6	3.4	2.7
16	0.24	0.21	0.13	0.17	0.62	0.81	1.74	1.15	2.28	4.5	3.6	1.8
17	0.81	0.46	0.32	0.53	0.69	1.15	2.45	0.7	1.49	5.5	6.7	3.9
18	0.78	0.46	0.35	0.63	0.76	1.37	2.14	1.2	2.25	7.4	4.9	5.2
19	0.85	0.49	0.52	0.31	1.06	0.63	3.71	0.77	1.38	3.9	4.5	4.2
20	0.98	0.62	0.45	0.67	0.72	1.08	2.41	1.22	1.7	4.5	4	4.9
21	0.3	0.33	0.27	0.17	0.82	0.51	2.25	1.41	2.14	70	6.6	3.5
22	0.46	0.33	0.22	0.24	0.67	0.73	1.64	0.81	1.33	6.5	6.4	6.8
23	0.82	0.23	0.19	0.13	0.83	0.57	2.08	1.34	1.8	75	3.8	3
24	0.89	0.4	0.32	0.34	0.8	0.85	1.46	0.83	2.09	70	4.4	2.9
Mean	**0.64**	**0.38**	**0.3**	**0.33**	**0.8**	**0.81**	**2.23**	**1.05**	**1.87**	**5.8**	**4.8**	**3.9**
Mean all cases												
vWF: FVIII U/m	**Ag u/mL**	**RCo 3x u/mL**		**CB 2x u/mL**	**RCo 3x/ Ag Ratio**	**vWF CB 2x/Ag Ratio**	**Ag u/ml**	**RCo 3x/ Ag Ratio**	**vWF: CB 2x/Ag Ratio**			
0.64	0.43	0.36		0.38	0.84	0.85	2.16	0.97	1.6			

Willebrand disease and were diagnosed as pseudo von Willebrand [4,10]. The other 167 index patients of 94 families were diagnosed as ISTH defined VWD and classified as mild VWD type in 65 families, severe dominant VWD type 1 in 10 families (of which 2M in 3), type 2A, 2B and 2N in 10, 4 and 2 families and recessive type 3 in 3 families respectively [3]. Out of the 65 families with mild VWD 1 we prospectively investigated the DDAVP responses of the probands or index cases of 24 families (Table 1) with VWF levels between 20 and 60% [2]. The diagnosis of mild VWD type 1 was based on a personal bleeding history and decreased values for FVIIII, VWF:Ag VWF:RCo and VWF:CB with normal rations for VWF:RCo/Ag and VWF:CB/Ag. Bleeding manifestation in the proband of 24 index family cases (Table 1) were usually mild and occasionally moderate. The Ivy bleeding times were usually normal or only slightly prolonged in only a few cases. The values for VWF:Ag VWF:RCo and VWF:CB were between 0.20 and 0.60, with normal ratios for VWF:RCo/Ag (0.62-1.08) in all and normal ratios for VWF:CB/Ag in 21 of 24 cases of mild VWD type 1 (Table 1). DDAVP has been administered intravenously to each of the 24 idex cases with mild VWD type 1 (Table 2).

The responses to DDAVP on the VWF parameters in the 24 probands with mild VWD type 1 was normal showing a 2 to 10-fold increase of VWF:Ag and VWF:RCo. The VWF:RCo/Ag ratio remained normal (0.65-1.19) after DDAVP in all 24 VWD type 1 patients. The majority of VWD type 1 patients showed significantly higher peak values of vWF:CB immediately after DDAVP, which resulted in significantly higher VWF:CB/Ag ratios (1.4-2.8) after

Table 2: DDAVP trial (0.3 ug/kg in 100 ml physiological saline intravenously over 30 minutes) used at the Goodheart Institute, Rotterdam 1992-2002 in the Dutch cohort of VWD patients to calculate the recovery and half life times of FVIII:C and VWF parameters for improved diagnosis and charaterization of VWD patients [2,3].

Blood sample DDAVP	After DDAVP at 15 minutes	After DDAVP 1 hour 2	4	6	12	24 hours
Ivy BT	+	-	+	-	-	+
PFA-100	+	+	+	+	+	+
RIPA	+	+	+	+	+	+
FVIII:C	+	+	+	+	+	+
VWF:Ag	+	+	+	+	+	+
VWF:RCo	+	+	+	+	+	+
VWF:CB	+	+	+	+	+	+
VWF:MM	+	+	+	+	+	+
VWF						
Propeptide	+	+	+	+	+	+

Table 3: Clinical manifestations, laboratory features and responses of VWF parmeters to DDVAP in probands of six patients with pronounced VWD type 1.

Proband	Age	Gender	History of Bleeding Manifestations						
1	22 y	M	Mild: mild epistaxis and bruisability after trauma						
2	45 y	F	Mild: occasional mild epistaxis, easy bruising after trauma and mild to moderate menorrhagia, bleeding after dental extraction						
3	41 y	F	Mild: asymptomatic in childhood, bleeding after dental extraction at age 11, and mild menorrhagia since adolescence; daughter mild bruising and brother mild bruises and bleeding after tonsillectomy						
4	28 y	M	Moderate: since early childhood recurrent epistaxis often *severe* and lasting for hours and tamponade at one occasion, spontaneous bruises						
5	24 y	F	Moderate: since childhood recurrent epistaxis, bruises, and bleeding after tonsillectomy and pronounced menorrhagia; no response of DDAVP on wound bleeding after trauma						
6	49 y	F	Autosomal dominant hemophilia-like bleeding tendency featured by occasional hemarthrosis, recurrent hematomas and muscle bleedings, and recurrent mucocutaneous bleeding since early childhood						
Proband/	**BT**	**FVIIIC u/mL**	vWF: Ag u/mL	**RCo u/mL**	**CB u/mL**	**Ratio VWF CB/Ag**	**RIPA**	**Response to DDAVP**	**VWF MM**
1	N	0.19	0.09	<0.10	0.12	1.3	N	decreased	N
2	+	0.56	0.17	0.16	0.13	0.8	N	decreased	N
3	+/N	0.67	0.19	0.14	0.21	1.1	N	decreased	NT
Daughter	+/N	1.26	0.18	0.23	0.16	0.9	N	decreased	NT
Brother	N	0.82	0.21	0.17	0.27	1.3	N	decreased	NT
4	+	0.52	0.23	<0.20	0.16	0.7	decr	abnormal	Near N
5	+	0.41	0.19	<0.10	0.13	0.7	decr	abnormal	Near N
6F	N	0.15	0.07	<10	0.07	1	N	abnormal	N
Son	N	0.19	0.08	<10	0.06	0.8	N	abnormal	N

+ = slightly prolonged, N = Normal, decr = decreased, NT = Not Tested

DDAVP. The group of 24 proband of VWD type 1 could be separated in a group of 14 proband with normal biological half life times of the VWF parameters and a group of 10 probands with dereased half life times of VWF parameters (Table 1). In such cases the levels of FVIII:C and VWF parameters were still in the normal range 24 hours after DDAVP on the basis of which we classified them as pseudo-VWD (Figure 1) [2]. Three patients with mild VWD type 1 and a mild bleeding history showed a normal to very good response to DDAVP (Figure 1) and therefore underwent major surgery including laparoscopic cholecytectomy, uterine extirpation and orthopedic surgery under the cover of one dose DDAVP (0.3 ug/kg intravenous preoperatively and on day 1. No bleedings were noted in the perioperative and postoperative period.

The responses to DDAVP of the VWF parameters in the 8 out of 24 probands in Table 1 with mild type 1 VWD had VWF parameters between 0.25 and 0.50 U/mL was restrictive responses to DDAVP reaching values around and somewhat above 1.0 U/mL $\pm$ 0.5 U/mL (Figure 2). In a case of symptomatic mild VWD type 1 featured by bruises and moderate menorrhagia since adolescence, we compared the effect of DDAVP Intravenous (IV), 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[2]. DDAVP intranasal and one dose of 3000 Units Hemate-P (40 U/kg, Figure 2). DDAVP showed a restricted response of VWF:Ag and VWF:RCo to values around 1.5 U/mL and normal response of VWF:Ag and VWF:CB to values between 2.0 and 3.0 U/mL., whereas the responses of DDAVP intranasal were rather poor as compared to DDAVP IV and Hemate-P (Figure 2) [2].

In 2003, Sadler confirmed the 2002 Rotterdam concept of Michiels & Van Vliet [2] in distinguishing pseudo-VWD versus

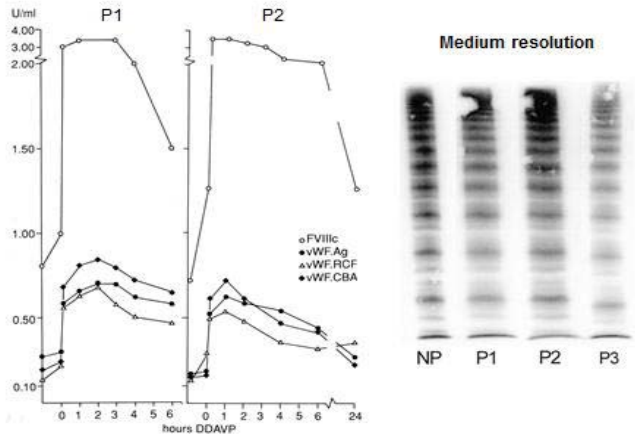

Figure 3: DDAVP responses in 2 cases of pronounced type 1 VWD Secretion Defect (SD) showing high FVIII/VWF:Ag ratio before and after DDAVP with normal VWFmultimers (VWF MM) and restricted decreased response to DDAVP of all Von Willebrand Factor (VWF) parameters in two members of one family (proband and her brother, proband 3 in Table 3). Medium resolution gel multimeric analysis according to Budde [14,15].

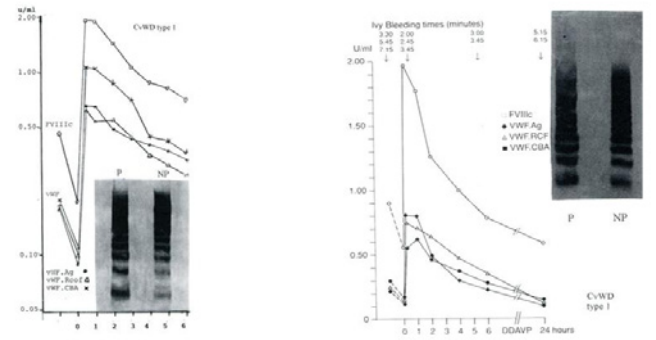

Figure 4: Pronounced VWD type 1 with Von Willebrand Factor (VWF) parameters around 0.15 U/mL with normal VWF multimers and increased FVIII/VWF:Ag ratio indicative for reduced secretion of VWF antigen (VWF:Ag): VWD type 1 secretion defect = VWD 1SD (Table 2, proband 1 and 2). Multimeric analysis according to Michiels & Van Vliet 2002 [2].

mild, moderate and severe VWD. Sadler calculated that most diagnoses of mild VWD type 1 are false positive VWD [8]. In the general population 25% have one or two mild bleeding (clinically insignificant) and 2.5% low plasma VWF indicating that 0.25x 0.025 = 0.6% individuals in the general population have a chance of low VWF and bleeding just by chance. Patients with mild VWD type 1 with levels for VWF:Ag, VWF:RCo and VWF:CB between 0.30 to 0.60 U/ml and normal ratios for VWF:RCo/Ag and VWF:CB/Ag usually present with no or mild bleeding symptoms, no family history of bleeding and normal BT and normal PFA-100 closure times on repeated occasions. We have labeled this category of false positive VWD type 1 as pseudo-VWD featured by very mild VWF deficieny and normal responses to DDAVP of VWF parameters and FVIII:C [3]. The ratio of FVIII/VWF:Ag ratio is around 2.00 after DDAVP in mild VWD type 1 due to heterozygous nonsense mutation /wild type (WT/nonsense carriers type VWD type 3) and in VWD type 1 due to heterozygous WT/ missense mutation in the D1, D4 and C1 to C6 domain of f VWD (Budde et al. European MCMDM-1VWD study).

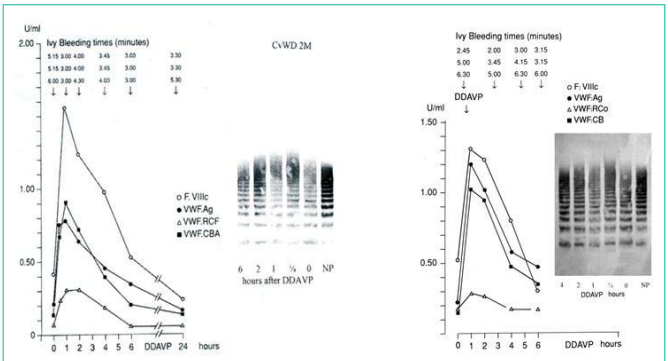

Figure 5: Poor response of VWF:RCo to DDAVP and fairly good responses to DDAVP of FVIII:C, VWF:Ag and VWF:CB in 2 cases with pronounced VWD 2M with normal VWF multimers before and after DDAVP (cases 4 and 5 in Table) showing normal VWF multimeric pattern in low resolution gel according to Michiels & Van Vliet) [2]. Interestingly the half life times of FVIII:C, VWF:Ag and VWF:CB are shortened both cases of pronounced VWD 2M indicative for an additional rapid clearance (C) defect of the FVIII-VWF complex.

Blood group is a significant determinant of plasma VWF concentration. In the study of Gill *et al.* [9], 456 normal individuals with blood group O had the lowest VWF:Ag levels (mean 0.75 U/ml with a ±SD of 0.356-1.57) followed by blood group A (mean 1.06±2SD 0.48-2.33) than blood group B and AB with completely normal VWF levels. About one third of individuals with blood group O, only a few with blood group A and hardly any with blood group B and AB have VWF levels below 0.60 U/ml. Of 114 patients diagnosed as type 1 VWD in the study of Gill et al, blood group O was found in 77%, blood group A in 18%, blood group B in 4%, and AB in 0%, whereas the frequency of these blood groups in the normal population was significantly different: 45%, 45%, 7%, and 3% respectively [9]. These data strongly indicate that a large proportion of patients diagnosed as mild type 1 VWD have decreased concentration of structurally normal VWF in the range of 0.25 to 0.60 U/ml with normal FVIII:C/VWF:Ag and VWF:RCo/Ag ratios on the basis of blood group O.

Autosomal dominant VWD type 1: diagnostic differentiation from VWD 2M

The probands of six families from the Rotterdam cohort of VWD patients (1990-2000) had pronounced autosomal dominant VWD type 1 with VWF levels between 0.07 and 0.20 U/mL (Table 3) [2]. Correct typing of pronounced VWD type 1 by the VWF:RCo/Ag ratio is problematic because of the low sensitivity of the VWF:RCo assay at VWF:Ag levels below 0.20. The VWF:CB/Ag ratio was normal in all cases of pronounced VWD type 1 (Table 3). On top of normal VWF:RCo/Ag and VWF:CB/Ag ratios RIPA is normal in VWD type 1 and decreased in VWD 2M (Table 3).

The six probands with pronounced VWD type 1 or 2M all had a bleeding history since early childhood and a normal or slightly increased Ivy bleeding time, but showed a decreased response to DDAVP of VWD parameters was decreased in VWD type 1 (Figures 3 and 4) and abnormal in VWD 2M (Figure 5). Probands 1 and 2 in Table 3 with pronounced VWD type 1 had a mild to moderate bleeding tendency already in early childhood, normal RIPA, a normal to slightly prolonged Ivy bleeding time, and a a normal VWF multimeric pattern, but a decreased response of all VWF parameters

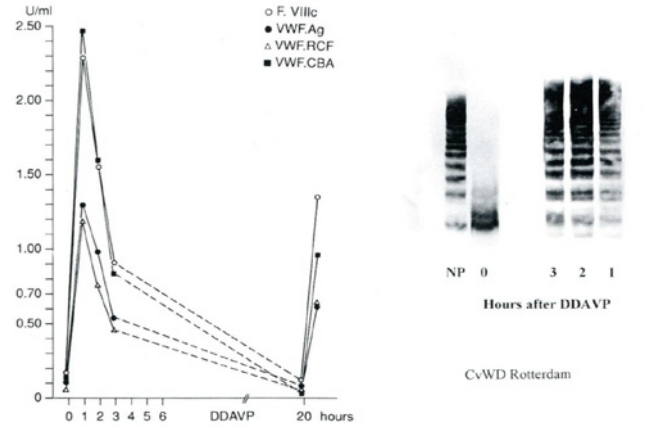

Figure 6: Pronounced to severe VWD type 1 with very good responses of FVIII and VWF:Ag with appearance of ultralarge VWF multimers post-DDAVP, good but restricted responses of VWF:RCo and VWF:CB one hour post DDAVP followed by very rapid clearance of FVIII and all VWF parameters labeled as VWD type 1 clearance defect: VWD 1C Rotterdam with hemophilia like bleedings since early childhood very suggestive for VWD Vincenza type 1C. Low resolution multimeric analysis according to Michiels & Van Vliet 2002.

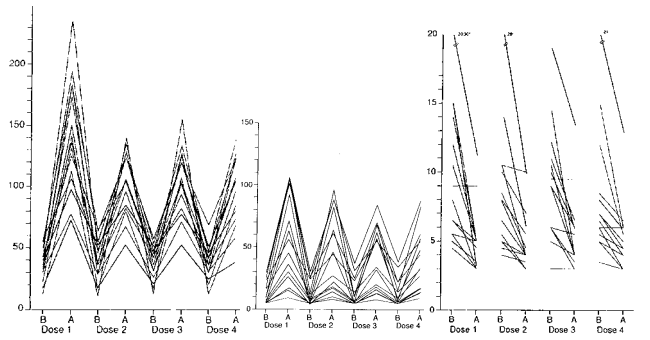

Figure 7: Results of DDAVP intravenously on FVIII:C (left), Von Willebrand factor ristocetine cofactor (VWF:RCo, middle) and Simplate bleeding times (right) in 15 patients with variable degrees of VWD type 1 and type 2M in the study of Mannucci et al. (Table 4) [10]. Normal to restricted responses to DDAVP of FVIII:C (U/dl, left), and restricted to poor responses of VWF:RCo (U/dl, middle) and partial correction Simplate bleeding times (right, normal value ≤7 minutes) before and directly after DDAVP (dose 0.3 ug/kg) with a time interval of 24 hours on four consecutive days (DDAVP dose 1, 2, 3 and 4 on day 1, 2, 3 and 4 respectively) [13]. These data clearly indicate that DDAVP is not therapeutically effective in pronounced VWD type 1, 2M and severe 1 (Table 4).

to DDAVP, suggesting a secretion defect of VWF from endothelial cells. The increased high FVIII/VWF:Ag ratios before and after DDAVP in three cases of pronounced VWD type 1 (Figures 3 and 4) is indicative for a secretion defect [2].

Proband 3 in Table 3 is a 44-year-old woman, her daughter and her brother with pronounced autosomal dominant VWD type 1 (Figure 3). They were diagnosed as pronounced VWD type 1 according to normal ratios for VWF:RCo/Ag and VWF:CB/Ag and normal RIPA (Table 3). All three presented with mild to moderate bleeding symptoms since early childhood. The responses to DDAVP were identical in all three patients and featured by completely normal response of FVIII:C , but a decreased restricted subnormal response to all VWF parameters with normal ratios for VWF:RCo/Ag and VWF:CB/Ag post-DDAVP indicating a secretion defect of VWF from endothelial cells: VWD type 1SD (Figure 3).

The probands 4 and 5 had overt VWD with a mild to moderate bleeding tendency in particular after trauma and surgery, a decreased RIPA anda slightly prolonged Ivy bleeding time grade 1 (Table 3). The response to DDAVP of VWF:Ag and VWF:CB was rather good, but the response of VWF:RCo to DDAVP was poor (Figure 5). The half life times of FVIII:C, VWF:Ag and VWF:CB post-DDAVP were significantly shortened, indicative for a rapid clearance of the FVIII-VWF complex. The VWF multimeric pattern of proband 4 and 5 are normal in low resolution gel before and after DDAVP (Figure 5). These laboratory findings in proband 4 and 5 are consistent with the diagnosis of symptomatic VWD 2 defined by Michiels in 2002 as VWD 2M [2-5]. VWD 2M is typically featured by decreased RIPA, normal VWF multimers before and after DDAVP, decreased response of VWF:RCo to DDAVP and reasonable good responses of FVIII, VWF:Ag and VWF:CB to DDAVP followed by rapid clearance of the FVIII-VWF after copmlex indicating an additional clearance defect in VWD 2M (Michiels).

Two members of three generations within one family (grandmother, mother and son Table 3) had a well documented autosomal dominant hemophilia-like bleeding tendency featured by occasional hemarthrosis, recurrent hematomas after trauma and muscle bleeding,and recurrent mucocutaneous bleeding since early childhood. Laboratoruy investigations were consistent with severe type 1 VWD with normal ratios for VWF:RCo/Ag and VWF:CB/Ag, normal RIPA on repeated testing. The response to DDAVP one hour post-DVT showed normal responses of FVIII;C and VWF:CB one hour post-DVT, but restricted responses of VWF:Ag and VWF:RCo followed by short half life time of all parameters consistent with rapid clearance of the FVIII-VWF complex. A second DDAVP test

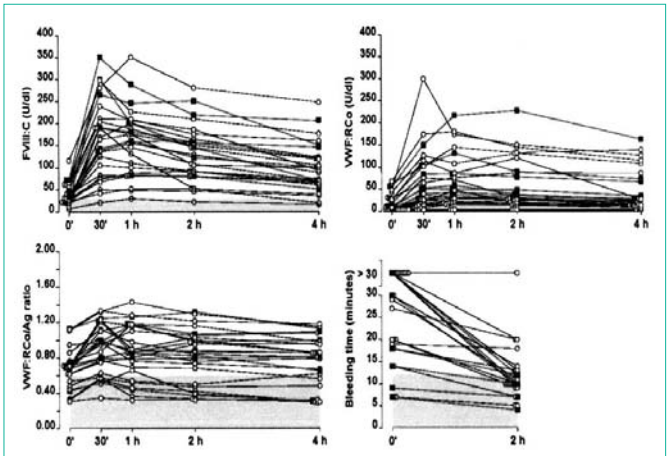

Figure 8: Response to DDAVP in 26 ISTH defined Von Willebrand Disease (VWD) type 1 patients [12]. The responses of FVIII:C to DDAVP were good in about 8 to 10 cases, but restrictive or even poor or non-responsive in more than half of type 1 VWD patients. These results indicate various degrees in severity of ISTH defined type 1 VWD patients. Among VWD type 1 patients there is a group of mild VWD type 1 with a good response of VWF:RCo, normal VWF:RCo/VWFAg ratio before and after DDAVP and complete correction of VWF parameters and bleeding times after DDAVP. There is another well recognized group of severe type 1 VWD patients with no or very poor response to DDAVP of VWF:RCo, decreased VWF;RCo/Ag ratio before and after DDAVP and no or partial correction of bleeding time 2 hours after DDAVP [10].

Table 4: Baseline laboratory data in 15 patients with von Willebrand disease diagnosed as type 1 according to Mannucci et al. in 1985 [10] and reclassified according to 2009 ISTH criteria/Antwerp [2,5.11].

Patient	Age/Sex	FVIII:C	VWF:	VWF:	RCo/Ag	Simplate BT	VWD ISTH
		(U/dl)	Ag	RCo	ratio	minutes (≤7)	Reclassified
1	36/M	18	12	<6	0.5	15	severe 1
2	36/F	22	11	9	0.81	7	1
3	29/F	30	58	29	0.5	5	mild 1 or 2
4	55/M	31	8	<6	<0.75	15	1
5	53/M	3	20	<6	<0.30	8	severe 1
6	47/M	33	18	7	0.39	12	2M
7	32/F	36	27	<6	0.22	8	2M
8	32/F	40	15	<6	<0.40	20	2M
9	24/F	42	23	23	1	14	1
10	30/M	42	16	6	0.38	11	2M
11	42/F	42	24	29	1.26	5	1
12	19/M	50	15	25	1.66	7	1
13	55/F	55	33	16	0.48	5	mild 1 or 2
14	63/M	57	24	7	0.29	6	2M
15	48/M	12	12	9	0.75	5	severe 1

Table 5: 2010 update on VWD 2E (2A/IIE) due to missense mutations in the D3 multimerization VWF domain and related phenotype of VWF parameters in 57 patients with a VWD 2E multimeric pattern [15].

Exon	nt	aa	VWF:Ag	VWF:CB	Ratio CB	VWF:RCo	Ratio RCo	FVIII:C
22	2867T>C	V956A	0.31	0.37	1.19	0.25	0.81	0.62
22	2926C>T	R976C	1.29	1.04	0.81		ND	0.85
25	3271T>C	C1091R	1.2	1.2	1	-	ND	-
25	3314C>A	A1105D	0.14	0.13	0.93	0.11	0.79	-
25	3359G>C	W1120S	0.34	0.19	0.56	0.21	0.62	0.72
25	33623>T	R1121M	0.23	0.2	0.87			
25	3377G>T	C1126F	0.18	0.07	0.36	-	-	-
26	3388T>C	C113OR	0.17	0.1	0.59	-	-	-
26	3387C>G	C1130W	0.22	0.08	0.36	-	-	-
26	3437A>C	Y1146C	0.2	0.16	0.79	0.07	0.35	0.31
26	3446G>A	C1149Y	0.17	0.05	0.3	0.07	0.42	0.25
26	3458G>A	C1153Y	0.89	0.55	0.62	0.4	0.45	0.56
26	3507T>G	C1169W	0.11	-	-	0.06	0.52	0.17
26	3514G >T	G1172C	0.25	0.14	0.56	0.15	0.6	0.45
26	3517T>C	C1173R	0.19	0.08	0.42	0.1	0.53	0.61
26	3518G>T	C1173F	0.35	0.23	0.65	0.32	0.93	0.54
27	3568T>C	C119OR	0.41	0.24	0.59	0.14	0.34	0.56
27	3569G >A	C1190Y	0.94	0.18	0.19			
27	3583G>T	D1195Y	0.43	0.38	0.88	-	-	-
28	3679T>C	C1227R	0.03	0.01	0.33	-		-
28	3833T>C	L1278P	0.59	0.31	0.52	-	-	-
28	3833T> G	L1278R	0.2	0.21	1.05	0.02	0.1	0.26

nt = nucleotide change, aa = amino acid change, Ag = VWF:Ag, CB = VWF:CB, RCo= VWF:RCo.

20 hours later showed pronounced restrictive responses of FVIII, VWF:CB, VWF:Ag and VWF:RCo. Multimeric analysis of VWF from plasma showed one strong double band of low molecular weight VWF before DDAVP and complete correction after DDAVP of the

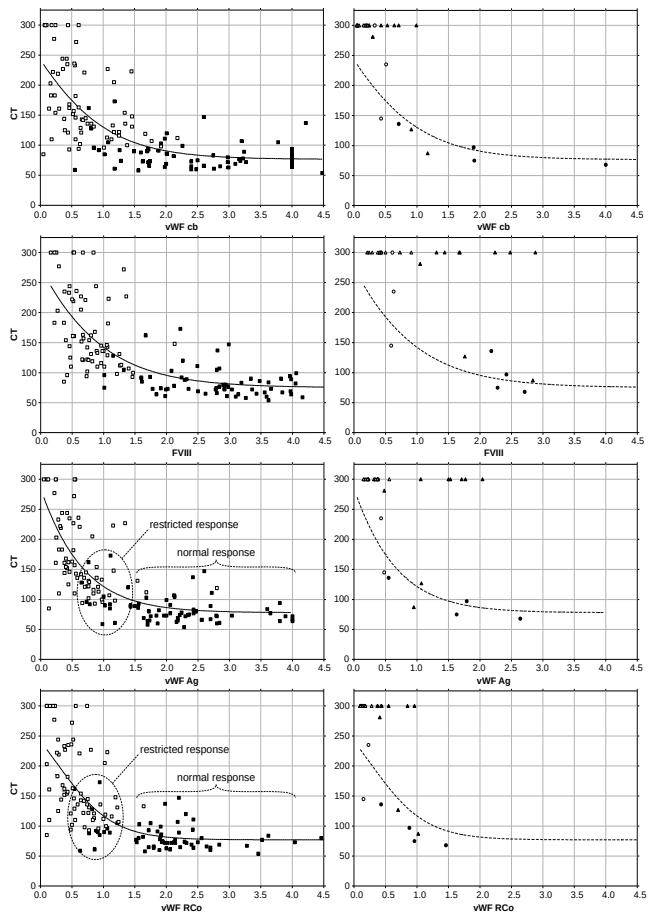

Figure 9: Dose-response relation between PFA-100 closure time (PFA CT) and FVIII-VWF.Left: VWD type 1 patients PFA CT before (□) and 1 hour after (■) DDAVP infusion. Right: VWD type 2 patients PFA CT before (type 2 ∆, type 2M ○) and 1 hour after DDAVP (type 2 ▲, type 2M ●) [13]. The PFA CTs normalised (<150 sec) in the majority of the patients with VWD type 1. Normalisation of the PFA-100 closure time (< 150 sec) was reached at VWF levels of about 0.75 U/ml. The minimal closure time (about 80 sec) or the maximal effect of von Willebrand factor was obtained at a level between 2.0 and 2.5 U/ml. The responses to DDAVP of VWF:RCo to DDAVP showed two clusters: those with restricted responses with values of VWF:RCo up to maximal 0.50 and 1.2 U/ml are consistent with restricted response to DDAVP in VWD type 1 versus those with maximal values of VWF:RCo to above 1.5 to 4.5 U/ml indicating complete normal responses to DDAVP in patients (individuals), who in fact do not have VWF deficiency.

VWF multimeric pattern to normal with the presence of ultralarge VWF multimers at 1 to 3 hours post-DDAVP. We have labeled this case as severe autosomal dominant FVIII-VWF deficiency Rotterdam due to rapid clearance (C) of the FVIII-VWF complex consistent with VWD 1C associated with a hemophilia-like bleeding phenotype since early childhood.

DDAVP responses of VWF parameters in severe and mild type 1 VWD

In 1992, Mannucci *et al.* evaluated the response of DDAVP (0.3 ug/kg) before and one hour after DDAVP with an interval of 24 hours on 4 consecutive days to a group of 15 patients with VWD type 1 (Table 4) [10]. The Bleeding Time (BT) before and after DDAVP were performed in 14 patients with VWD and prolonged in 8 cases (Table 4, Figure 8 right) [9]. The prolonged BT became normal in 6/8

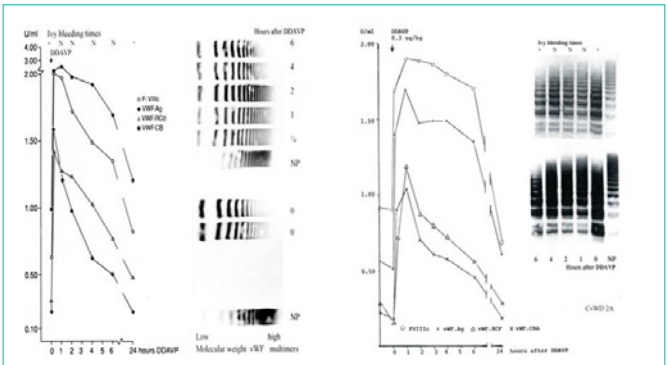

Figure 10: Left VWD 2^{E} (2A/IIE): completely normal response of FVIII:C and VWF:Ag, but restricted responses of VWF:RCo and VWF:CB followed by shortened half life times (about 6 hours), transient correction of Ivy bleeding time (BT) transient reappearance of some of the large VWF MM but no correction of the 2E VWF MM pattern in a case VWD 2E (mutation C1190). VWF MM analysis according to Michiels & Van Vliet 2002.
Right VWD 2A (2A/IIA): responses of FVIII and Von Willebrand Factor (VWF) parameters and Ivy bleeding times in a moderate VWD type 2A showing a transient good tresponse of functional VWF:RCF and VWF:CB to about 1 U/mL followed by short half life times due to increased proteolysis of VWF multimers and normal responses of FVIII and VWF:Ag followed by normal half life times. There was a transient correction of bleeding times and VWF multimers for about 4 hours post-DDAVP. Low resolution gel multimeric analysis according to Michiels & Van Vliet, 2002 [2]. (VWD 2A (IIA) Mutation V1499E).

but became prolonged again after 24 hours (Figure 7 right). The BT became normal directly after DDAVP in 6/8 on day 1, day 2 and day 3, and in 7/9 on day 4. There were 3 severe type 1 VWD patients in Table 4 with low values for FVIII:C and VWF parameters and a very poor response of VWF:RCo (Figure 7 middle). This type of response as has been described in recessive severe type 1 VWD patients. At least 5 patients in Table 4 can readily be reclassified as VWD type 2M based on decreased VWF:Ag and very low VWF:RCo with RCo/Ag ratios of less than 0.50 when the SSC-ISTH criteria of 1994 and 2006 are applied [2-5]. The response to DDAVP in these type 2M patients is restrictive (not reaching the lower limit of normal, less than 0.60 U/dl) for VWF:Rco (Figure 7 middle), whereas the response of FVIII:C is reasonable normal reaching 2 or 3 times higher to values between 1.0 to 1.5 U/dl (Figure 7 left). The decreased ratios of VWF:RCo/VWF:Ag ratios in type 2M VWD patients before DDAVP did not correct after DDAVP thereby confirming the diagnosis VWD type 2M [2,5,11]. The responses of FVIII to DDAVP was reasonable good (Figure 7, left) and much better than the responses of VWF:Ag to DDAVP (Figure 7 middle).

Federici *et al.* investigated in 2004 the responses to DDAVP in 26 pronounced, moderate and mild type 1 VWD patients (Figure 8) [12]. After DDAVP FVIII:C and VWF:RCo increased in the majority of type 1 VWD patients. The majorty of VWD type 1 showed a restricted response of VWF:RCo not reaching normal values needed for normal hemostasis (Figure 8). There were 7 type 1 patients who had ratios below the lower limit (0.6 according to SSC-ISTH criteria) before and after DDAVP (Figure 8) indicating type 2VWD functionally non-responsive to DDAVP. After DDAVP there was a subset of VWD type 1 patients with normal VWF:RCo/VWF:Ag ratio consistent with VWD type 1, and a subset with decreased VWF:RCo/VWF:Ag ratio consistent with type 2 VWD indicative for VWD 2M (Figure 8).

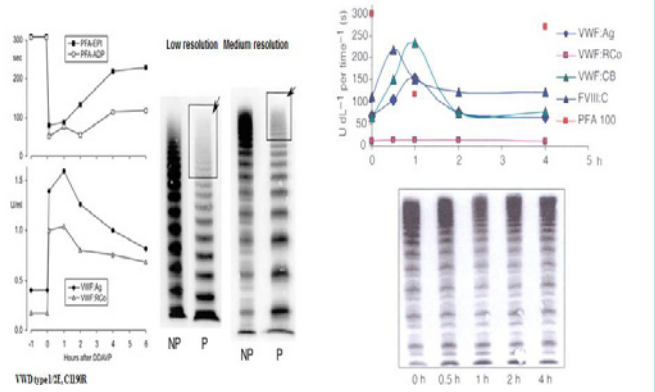

Figure 11: Left VWD 2A/IIE, mutation C1190R): Transient correction of PFA-100 closure time and restricted increase of VWF parameters from around 0.20-0.40 U/mL to around 1.0 U/mL. In VWD type 2 E VWF multimeric pattern is charaterized by a lack or relative decrease of large multimers and the absence of th outer sub-band of the normal triplet structure. Medium resolution gel according to Budde [17,18].

Right VWD 2M: poor response of VWF:RCo to DDAVP, normal VWF multimers before and after DDAVP and good responses of FVIII, VWF:Ag and VWF:CB followed by shortenend half-lfe time indicating rapid clearance defect of the FVIII-VWF complex on top of loss of VWF:RCo function in VWD 2M[17]. Medium resolution gel according to Budde [16.,17]. For comparison figure 12 show characteristic responses of the VWF multimeric structure to DDAVP in a healthy control (left) and in VWD 2E (right) in a medium resolution gel according to Budde [17,18].

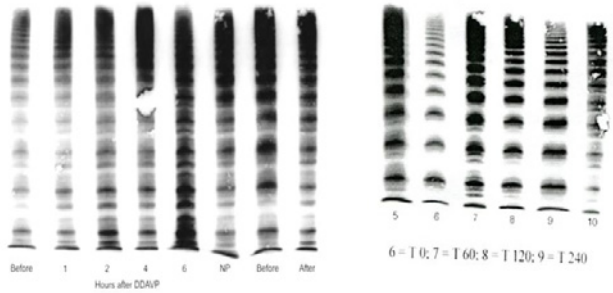

Figure 12: Response of Von Willebrand multimeric pattern in a normal healthy control (left) and a typical case of VWD 2E (2A/IIE, right) to DDAVP before and after 1, 2, 4 and 6 hours post-DDAVP. The normal healthy control showed increase of triplet structure a few hours post-DDAVP reflecting increased proteolysis of large vWF multimers in a medium resolution according to Budde [17,18].

Prospective evaluation of DDAVP responses in VWD type 1 and type 2

Between 1998 and 2008 Van Vliet *et al.* prospetively evaluated the responses of PFA, VWF and FVIII before and one hour after DDAVP (0.3 µg/kg) in patients with VWD type 1 (n=70) and type 2 (n=14: 8 type 2A, 2 mild type 2 and 4 type 2M) [13]. In this study VWF:Ag was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) and VWF:CB by an ELISA using collagen type 1 [2,13]. VWF ratio (VWF:CB/ VWF:Ag) was used as a surrogate measure of the multimer distribution [11]. VWF:RCo was determined by measuring the rate of aggregation of fixed platelets induced by ristocetin and patient's VWF plasma with the PAP-4 aggregometer (Biodata) [2,13]. Closure time was assessed by using the Platelet Function Analyser (PFA-100® Dade-Behring, Marburg, Germany) [13]. The dose responses of PFA, VWF and FVIII before and one hour after DDAVP in 70 VWD type 1 are shown in Figure 9, left.

Pre-treatment values of FVIII and VWF parameters in 70 patients labelled as VWD type 1 (open symbols, Figure 9) ranged from severe, moderate and mild VWF deficiency to normal values. The PFA Closure Times (CTs) were normal or in the upper region of normal (<300 sec) in the majority and prolonged (>300 sec) in only a few cases of VWD type 1 (Figure 9 left). The VWD type 1 patients treated with DDAVP demonstrate a good dose-response with correction of the PFA CTs (closed symbols, Figure 9 left) in all VWD type 1 patients. Normalisation of the PFA-100 closure time (< 150 sec) was reached at functional VWF levels of 0.75 U/ml and above.

The responses to DDAVP of VWF:Ag were not significantly different from FVIII (Bonferroni's Multiple comparison test) (Figure 9) [13]. The response of VWF:RCo to DDAVP showed two clusters (Figure 9 lower left): First, those with restricted responses with values of VWF:RCo up to maximal 0.50 and 1.2 U/ml are consistent with restricted response to DDAVP in VWD type 1 as seen our previous study (Figure 2) [2]. Second those with maximal values of VWF:RCo to above 1.5 to 4.5 U/ml indicating complete normal responses to DDAVP in patients (individuals), who in fact do not have VWF deficiency. Michiels & van Vliet have labeled this category of false positive VWD type 1 as pseudo-VWD (Figure 1) [2].

Van Vliet, Kappers, Leebeek and Michiels evaluated between 1998 and 2008 the responses to DDAVP of PFA, VWF parameters and FVIII before and after DDAVP in 24 patients with VWD type 2 (Figure 9 right) [13]. Pretreatment values of functional VWF:RCo and VWF:CB are significantly lower as compared to VWF:Ag and FVIII:C in 24 patients with VWD type 2. The PFA CTs were prolonged (<300 sec) in all VWD type 2 patients except in two (open symbols, Figure 9, right). None of the severe type 2A VWD patients showed a transient correction of the PFA CTs after DDAVP one hou rafter DDAVP (closed symbols, Figure 9 right). In contrast, type 2M or mild type 2 A VWD patients (N = 6) showed a transient correction of PFA CTs to near normal or normal values (<150 sec) one hour post-DDAVP (Figure 9 right). These studies clearly demonstrate that normal responses to DDAVP of PFA CT is dependent on the correction of functional plasma VWF concentration and the VWF:CB/Ag ratio together with correction of plasma VWF multimer distribution with the reappearance of large multimers.

Diagnostic differentiation of VWD 2E vs 2A and VWD 2E vs 2M

Patients with VWD type 2 E (2A/IIE) due to VWF mutations in the D3 domain have laboratory features of dominant VWD type 1 with a type 2E VWF multimeric pattern in low and medium resolution gels [11,14-17]. Schneppenheim, Michiels & Budde described 22 different mutations in the D3 domain of the VWF gene as the cause of VWD type 2E (2A/IIE) phenotype (Table 5) [15]. Most of these 22 mutations affect cysteine residues, 17 of them being novel, all are clustering in the VWF D3 domain mulitmerization domain [15]. An intracellular retention of most mutants and/or a defect of multimerization seem to be the main pathogenic molecular mechanisms and the sensitivity of mutant 1/2E (2A/IIE) VWF to ADAMTS13 proteolysis of mutant VWF was not different from wild-type VWF in a static assay [15]. Among this cohort of 22 VWD 2E (2A/IIE) patients we contributed

Table 6: Dominant VWD type 1 secretion defect (high FVIII/VWF:Ag ratio) caused by S2179F mutation in D4 domain. The high FVIII:C/VWF:Ag ratio is indicative for a secretion defect (SD).

Mutation	FVIII:C Ag %	VWF: RCo %	VWF: VWF:Ag %	VIII:C/ VWF:Ag ratio	VWF:Rco/ ratio	VWD type MM	Reference VWD type
Family 1	33	13	9	2.5	0.7	Type 1SD	Haberichter[19]
S2179F	21	7	6	3.0	0.86		
	28	12	14	2.3	1,2		
Family 2	19	13	12	1.5	0.9	Type 1SD	Haberichter[19]
S2179F	29	15	12	1.9	0.8		
	35	18	10	1.9	0.55		
S2179F	26	6	6	4.3	1.0	1	MCMDM-1[14]

Table 7: Laboratory features of severe dominant VWD 2A (2A/IIA) Group I in 3 unrelated cases (mutation unknown in 2 and S1506L mutation, figure) and mild/moderate VWD of moderate VWD 2A (2A/IIA) group II in 3 related cases of alarge family with the mutation V1499E [27] (Figure 15).

Case/mutation	Sex	BT Minutes	BT after DDAVP	Response to DDAVP	VIII:C U/ml	VWF: (U/ml)			RIPA
						Ag	RCo	CB	
Three cases with severe VWD 2A Group I [21].									
1 unknown	F	>15	3-9	Poor	0.93	0.45	0.13	0.05	decreased
2 unknown	F	>15	4-8	Poor	0.38	0.15	<0.10	<0.05	absent
3 S1506L	F	>15	>15	**Poor**	**0.66**	**0.41**	**0.15**	**0.05**	**absent**
				Figure 17					
Family with mild to moderate VWD 2A group II (mutation V1490E) [21,28].									
Uncle	M	4-10	<4	**Transient**	0.92	0.56	0.28	0.23	Normal
Proband	F	6-10	<4	Transient	-	0.42	0.15	0.18	Normal
nephew	M	3-6	<4	**Transient**	-	1.08	0.38	0.52	Normal
				Figure 16					
Normal		<4	<4	Normal	>0.60	>0.60	>0.60	>0.60	Normal

Table 8: Laboratory features of autosomal dominant VWD type 2 M according to Hermans & Batlle 2009 [29,30].

1.	Very low levels of VWF:RCo in plasma and in defective ristocetine induced platelet aggregation (RIPA).
2.	Decreased VWF:RCo/VWF:Ag ratio, <0.6, in particular after DDAVP.
3.	Normal VWF:CB/VWF:Ag ratio, >0.7 to 1.2, in particular after DDAVP.
4.	Prolonged PFA^{100} (Coll/ADP and Coll/EPI) closure times (CT: >300 seconds).
5.	Near normal to prolonged bleeding times but variable.
6.	Response to DDAVP poor for VWF:RCo, but good for VWF:Ag, VWF:CB and FVIII:C (Figures 5 and 11 right)
7.	Relative decrease of large VWF multimers and lack of triplet structure in a medium resolution gel (Figures 5 and 11 right)
8.	Loss of ristocetine cofactor functions (VWF:RCo and RIPA) due to loss of GPIb function mutations in the A1 domain.

with two novel mutations W1120S and C1190R (Figure 10, left) and W1120S in the D3 domain associated with dominant VWD type 1/2E [15]. The normal VWF multimeric pattern in medium resolution gels displays a typical triplet structure and the presence of large multimers (Figure 3) [16,17]. The type 2 E (2A/IIE) VWF multimeric pattern is characterized by a lack or relative decrease of large multimers and the absence of th outer sub-band of the normal triplet structure consistent with a multimerization defect (Figures 10 and 11 left) [14,15]. VWD 2M typically shows a poor response of VWF:RCo to DDAVP, normal VWF multimers before and after DDAVP and good responses of FVIII, VWF:Ag and VWF:CB followed by shortenend half-life time indicating rapid clearance defect of the FVIII-VWF complex on top of loss of VWF:RCo function of the A1 domain in VWD 2M (Figure 11 right) [17]. For comparison Figure 12 show characteristic responses of the VWF multimeric structure to DDAVP in a healthy control (left) and in VWD 2E (right) in a medium resolution gel according to Budde [16,17].

In VWD 2A (2A/IIA), there is a lack of large multimers with markedly pronounced triplet structure of the outer sub-bands duet o increased proteolysis. The loss of large multimers together with increased triplet subbands representing proteolytic VWF fragments originating from increased cleavage of VWF at the Y1605-M1606 bond are due to mutations in the A2 domain of VWF (Figure 10 right) [15,16]. Response to DDAVP of Von Willebrand multimeric pattern in a normal healthy control (Figure 12 left) and a typical case of VWD 2E (Figure 12 right) before DDAVP and after 1, 2, 4 and 6 hours post-DDAVP. The normal healthy control showed transient increase of triplet structure a few hours post-DDAVP reflecting

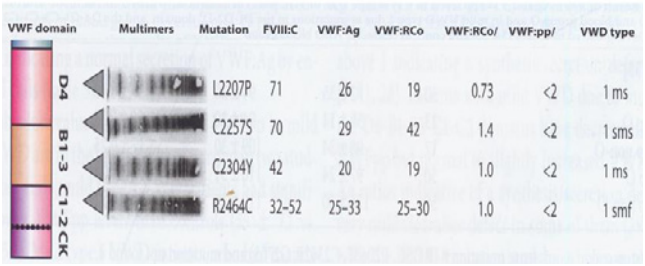

Figure 13. Four heterozygous mutations in the D4, C1 to C6 domains of VWF associated with VWD type 1 and a smeary VWF multimeric pattern according to Budde in th MCMDM-1VWD study [20].

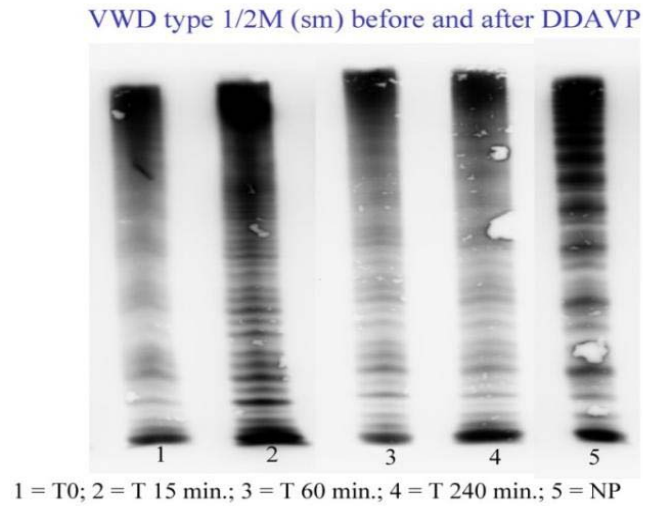

Figure 14: Multimeric pattern (medium resolution gel) of plasma VWF in a type 1 VWD patient with smeary VWF multimers due to a putative heterozygous missense mutation in the carboxyterminal part of the VWF gene (D4, C1 to C6 domains). At time 0, a smeary pattern is evident reflecting a mixture of normal and mutant VWF proteins. After infusion the typical sub-banding appeared, which slowly disappeared at 120 and 240 minutes.

increased proteolysis of large vWF multimers in a medium resolution according to Budde [16-18].

VWD type 1 with normal or smeary VWF multimeric pattern due to muations in the D4 and C1 to C6 domains of VWF

The Canadian and European VWD-1 studies discovered a new category of VWD type 1 due to mutations in the D4 and C1 to C6 domains of the VWF gene. Data from the European MCMDM-1VWD study show two groups of heterozygous mutations in the D4 C1 to C6 domains do show up with either normal multimers (NM, VWD 1m) or abnormal smeary multimers (AbM, VWD 1sm) [11,14]. The group with normal VWF multimers L1774S, K1794E*, C2304Y*, R2313H, G2518S*, Q2544X*, C2693Y, and P2722A have mild VWD type 1 disease, are autosomal dominant or recessive with variable penetrance of beeding manifestations [11,14]. Four of these mutations in the D4 andC1 to C6 domains domains had an increased FVIII:C/VWF:Ag ratio of 2 or more (indicated by an astrix) and manifested complete penetrance of bleeding manifestations indicating a dominant Secretion Defect (SD) as the cause of VWD type 1SD. The group with abnormal multimers of heterozygous mutations in the D4 and C1 to C6 and CK domains V1822G*, L2207P*, C2257S*, C2304Y*, C2362F*,

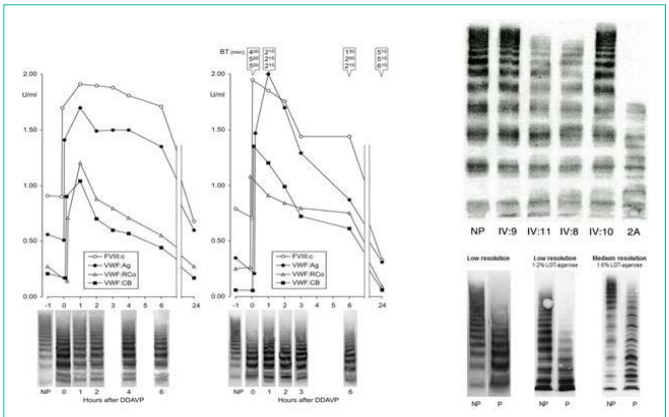

Figure 15: Left. Two related cases of a large family (two brothers) with autosomal dominant mild to moderate VWD type 2A (2A/IIA) group II caused by a novel mutation in the A2 domain (V1499E) [27] show normal response of FVIII:C and VWF:Ag, restricted responses of VWF:RCo and VWF:CB followed by shortened half life times (about 6 hours), transient reappearance of some of the large VWF MM and transient correction of IVY Bleeding Time, but no correction of the 2A VWF MM pattern after DDAVP. VWF MM analysis in laboratory 1 according to Michiels & Van Vliet 2002 [2].
Right. Upper. VWF multimeric analysis of mutant VWF V1499E showing loss of large VWF multimers in 2 afftected family members carrying the V1499E mutant (IV:11 and IV:8) compared tot wo non-affected family members (IV-9 and IV-10) and loss of large and intermediated VWF multimers and typical triplet structure of VWF bands.
Right lower. Comparison of VWF multimeric pattern in low resolution gel according to Michiels & Van Vliet[2] (left) versus Budde in low (middle) and medium (right) resolution gels of the proband with moderate VWD type 2A (2A/IIA, mutation V1499E) showing decrease of large and increase of small VWF MM in low (left and middle) and typical triplet structure of VWF bands (right) diagnostic for increased proteolysis in VWD in severe VWD 2A2 (2A/IIA, mutation V1499E) [21,28].

G2441C*, R2464C*, C2477Y*, C2477S*, and Q2520P* have mild to moderate VWD type 1, and usually smeary pattern of abnormal VWF multimers (Figure 13) [11,14]. Multimeric pattern (medium resolution gel) of plasma VWF in a case of type 1 VWD with smeary VWF multimers due to a putative heterozygous missense mutation in the D4 anC1 to C6 domain is shown in Figure 14. At time 0, a smeary pattern is evident reflecting a mixture of normal and mutant VWF proteins. After infusion the typical sub-banding appeared, which slowly disappeared at 120 and 240 minutes. The majority of mutations in the D4, C1 to C6 and CK domain with normal or smeary VWF multimers have increased FVIII:C/VWF:Ag ratios around or above 2 (indicated by an astrix). The increased FVIII/VWF:Ag ratio predicts a Secretion Defect (SD) with restricted responses of VWF to DDAVP and/or more or less rapid clearance of VWF after DDAVP (VWD type 1 SD/C). Expression studies of mutant VWF will clarify, show, or predict abnormal banding of mutant VWF multimers as the cause of a smeary pattern in heterozygous cases. Such smeary pattern of VWF multimers are more pronounced after DDAVP. Haberichter et al. reported two unrelated families with the heterozygous S2179F mutation in D4 domain (Amino acid 1940-2300) featured by moderate VWD type 1, increased FVIII:C/VWF:Ag ratio, restricted response to VWF to DDAVP followed by rapid clearance of VWF parameters consistent with VWD type 1SD/C (Table 6, Figure 13) [19]. The European MCMDM-1VWD study reported one case of pronounced VWD type 1, normal VWF multimers but high FVIII/VWF:Ag ratio indicative for a secretion defect (Table 6) [14,20]. As

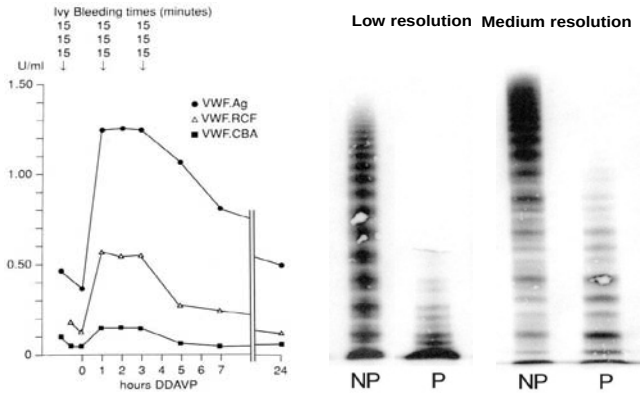

Figure 16: No response of VWF:CB, poor response of VWF:RCo and good response of VWF:Ag is a case of VWD type 2A (2A/IIA) group I due to S1506L mutation in the A2 domain. VWF multimers type 2A (2A/IIA) according to Budde. NP = Normal Plasma; P = Patient VWD type 2A (2A/IIA) group I. VWF multimeric analysis according to Budde [17,18,21].

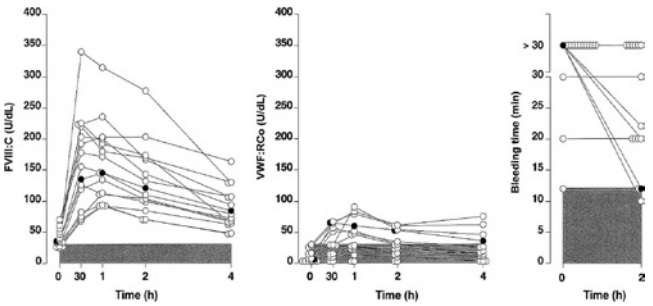

Figure 17: Poor and restricted responses of VWF:RCo as compared to good responses of FVIII:C in 15 patients with VWD type 2A (2A/IIA) with no correction of strongly prolonged bleeeding times after 2 hours DDAVP except in three (Federici et al.) [12]. These observations strongly indicate the need of purified VWF concentrate or VWF-FVIII concentrate with a high ratio of VWF:RCo/VWF:Ag with the presence of large VWF multimers and high a ratio of VWF:RCo/FVIII:C above 2 for the management of bleedings and prevention of bleedings.

compared to mild type 1 VWD the responses of VWF parameters and FVIII:C to DDAVP are decreased (restricted as compared to FVIII = secretion defect) followed by rapid clearance of VWF parameters in pronounced dominant VWD type 1 SD/C caused by S2179F mutation in D4 domain (Haberichter et al.) [19].

VWD 2A group II and I due to mutation in the A2 domain of VWF

The pertinent findings in patients with type 2A (2A/IIA) VWD include prolonged BT, consistently low VWF:RCo/Ag ratio and VWF:CB/Ag ratio, absence of large VWF multimers, pronounced triplet structure of individual bands and increased VWF degradation products due to increased proteolysis (Figures 15,16, Table 7). VWD type 2A (2A/IIA) results from missense mutations in the A2 domain, exon 28, of the VWF gene (C1485Y, L1503P, G1505E, S1506L, F1514C, !524del, V1539E, L1540E, L1540P, S1543F, Q1556R, L1562P, G1579R, L1580V, R1583W, R1597W, R1597G, R1597Q, V1604F, V1607D, G1609R, S1613P, D1614G, I1628T, G1629R, E1638K, L1639P, P1648S, V1665E, G1672R) [21].

The absence of high VWF multimers and increased triplet

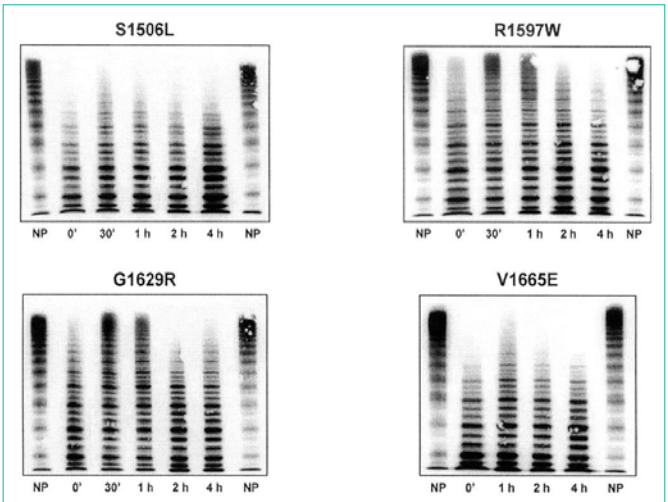

Figure 18: DDAVP induced the re-appearnce of large VWF multimers in the VWD 2A (2A/IIA) type II caused by the mutations R1597W and G1629, but not in VWD 2A (2A/IIA) Group I caused by the mutations S1506L and V1665E in the A2 domain of the VWF gene [12].
During surgery or trauma in type 2A VWD patients and in type 2B VWD patients as well.

structure is the consequence of increased proteolysis of large VWF multimers (Figures 17 and 18). Structural changes within the A2 domain can produce two different characteristic phenotypes of type 2A (2A/IIA) VWD [21]. Expression studies demonstrated that the single missense mutations V1607D, S1506L, L1540P and G1505R resulted in poor or no secretion of high molecular weight due to impaired transport of VWF multimers between the endoplasmatic reticulum and the Golgi complex (so-called VWD 2A group 1 defect) with very likely intracellular proteolysis of large VWF multimers [22-25]. Expression studies demonstrated that at least 5 missense mutations in the A2 domain R1597W, G1505E, E1638K, I1628T and L1503Q result in normal secretion of high molecular weight multimers similar to wild type multimers indicating that subsequent loss of large VWF multimers is caused by hypersensitivity to ADMATS13 induced increased proteolysis in plasma (so-called VWD 2A (2A/IIA) group 2 defect) [22-26]. Interestingly, platelet lysates demonstrated decrease of large VWF multimers for G1505L and S1506L mutants of VWD 2A group 1, but a normal pattern for the G1505E and R1597W mutants of VWD 2A group 2. This simple means that VWF of severe VWD 2A (2A/IIA) group II is already proteolysed in endothelial cells before secretion, whereas VWF in mild to moderate VWD 2A (2A/IIA) group II is secreted as large multimers which after secretion from endothelial are proteolysed due to hypersensitivity to ADAMST13.

Hassenpflug et al. Investigated the impact of mutations in the A2 domain of VWF commonly found in patients with VWD type 2A (2A/IIA) on ADAMTS13-dependent proteolysis of VWF [16]. They used recombinant human ADAMTS13 (rhuADAMTS13) to digest recombinant full-length recombinant VWF (rhuVWF) and a VWF fragment spanning the VWF A1 through A3 domains, harboring 12 different VWD type 2A (2A/IIA) mutations (G1505E, G1505R, S1506L, M1528V, R1569del, R1597W, V1607D, G1609R, I1628T, G1629E, G1631D, and E1638K). With the exception of G1505E and I1628T, all mutations in the VWF A2 domain showed increased specific proteolysis of VWF independent of the expression

Table 9: Distribution of samples from a large retrospective study on diagnosis of VWD diagnosed as VWD type 1, 2A (2A/IIA), 2B (2B/IIB) and 2M based on VWF multimers in a low resolution gel and functional VWF paraleters according to established cut-off values for VWF:CB/Ag and VWF:RCo ratios [34].

Multimer Pattern	Number of Cases	vWF:CB/Ag Ratio	vWF:RCo/Ag Ratio*
All samples (n = 497)	67	≤ 0.5	≤ 0.7
	1	≤ 0.5	>0.7
	112	>0.5	≤ 0.7
	317	>0.5	>0.7
Normal pattern (n = 449)	20	≤ 0.5	≤ 0.7
	1	≤ 0.5	>0.7
	111	>0.5	≤ 0.7
	317	>0.5	>0.7
Type 2A/IIA (n=18)	18	≤ 0.5	≤ 0.7
	0	≤ 0.5	>0.7
	0	>0.5	≤ 0.7
	0	>0.5	>0.7
Type 2 (n =29)	29	≤ 0.5	≤ 0.7
	0	≤ 0.5	>0.7
	0	>0.5	≤ 0.7
	0	>0.5	>0.7
Type 2M (n = 1)	0	≤ 0.5	≤ 0.7
	0	≤ 0.5	>0.7
	1	>0.5	≤ 0.7
	0	>0.5	>0.7

level. RhuVWF harboring the mutations G1505E, M1528V, G1609R, I1628T, G1629E, G1631D and E1638K were similar in expression level with normal multimer distribution consistent with Group II VWD 2A. Due to the lack of proteolytic activity and shear stress in the culture medium both rhuVWF Wild Type (WT) and rhuVWF mutants group II are fully multimerizied in these experimental *in vitro* studies [16]. The rhuVWF mutants were hypersensitive for ADAMTS13-dependent proleolysis *in vitro*. The VWD mutation C1272S resulted in resistance of the recombinant VWF fragment to ADAMTS13-dependent proteolysis and should be diagnosed as VWD 2M and not 2A. The rhuVWF mutants G1505R, S1506L, R1568del, and V1607D resulted in decreased *in vitro* expression of high- and intermediate-molecular weight multimers of VWF [16], consistent with a group I mechanism [22-25]. Proteolytic hyper susceptibility to ADAMTS13-mediated proteolysis of mutant VWF group II *in vitro* closely correlated with the *in vivo* phenotype in VWD 2A (2A/IIA) patients. Increased VWF susceptibility for ADAMTS13 is a constitutive property of classical VWD type 2A/IIA, thus explaining the pronounced proteolytic fragments and loss of HMWM seen in multimer analysis in VWD patients [15-18].

Federici et al. demonstrated a poor response of VWF:RCo as compared to restricted or good response of FVIII:C in 15 patients with VWD type 2A with no correction of strongly prolonged bleeding times except in two (Figure 17) [12]. This study also showed that in patients with severe VWD type 2A (2A/IIA) group I due to the mutations S1506L and V1665E showed poor responses of functional

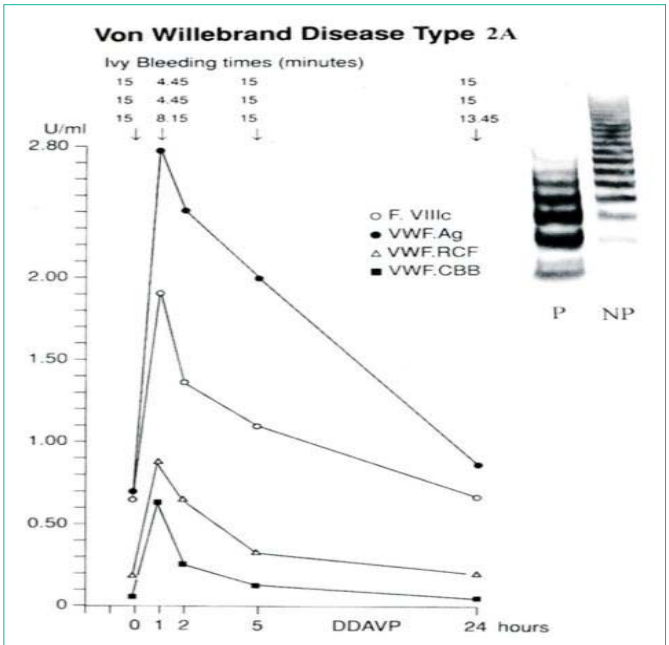

Figure 19: Response of FVIII and Von Willebrand Factor (VWF) parameters in a patient with severe VWD type 2 A (2A/IIA) showing a poor response of functional VWF:RCF and VWF:CB followed by short half-life times due to increased proteolysis of large VWF multimers. There was correction of bleeding time for only one hour post-DDAVP and no correction of VWF multimers. The responses of FVIII and VWF:Ag were normal followed by normal half-life times [2,21].

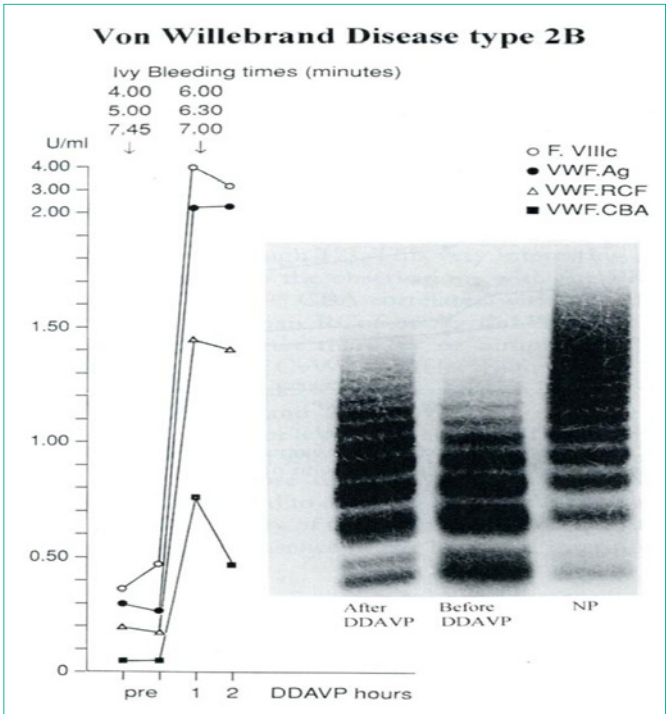

Figure 20: Response of FVIII and Von Willebrand Factor (VWF) parameters in a patient with pronounced VWD 2B (2B/IIB) showing a poor response of functional VWF:RCF and VWF:CB 1 and 2 hours post-DDAVP, no correction of bleeding time and no correction of the loss of large VWF multimers. The responses of FVIII and VWF:Ag were normal followed by normal half -ife times. Low resolution gel multimeric analysis according to Michiels & Van Vliet, 2002 [2].

VWF parameters to DDAVP no reappearance of the large multimers (Figure 18), with persistence of a strongly prolonged BT, whereas in patients with mild to moderate VWD 2A (2A/IIA) group II due to the mutations R1597W and G1629R responded better to DDAVP with transient increase of large multimers (Figure 18) associated with transient with transient correction of BT and transient correction of VWF:RCo to low normal values for 1 to 4 hours (Figure 15) [25]. Severe VWD type 2A (2A/IIA) group I show a typically proteolytic pattern with pronounced triplet before and after DDAVP and in platelet, which strongly suggest that proteolysis of large multimers occurs already intracellular. Mild to moderate VWD type 2A (2A/IIA) group II show transient correction of VWF multimers after DDAVP and normal VWF multimers in platelet indicating that mutant VWF after secretion by endothelial cells in group 2 VWD type 2A (2A/IIA) is proteolysed in plasma due to increased sensitivity to ADAMTS13.

The observations in 1986 by Batlle et al. [26,27] and in 2002 by Michiels et al. [2,5,20] are completely in line with the observed heterogeneity of mild, moderate type 2A (2A/IIA) versus severe type 2A (2A/IIA) VWD with regard to bleeding symptoms, laboratory phenotypes and typical responses to DDAVP. Patients with severe VWD 2A (2A/IIA) group I have low values for VWF:Ag very low or undetectable level for VWF:RCo and VWF:CB, no RIPA at high concentration of ristocetin 1.75 or 2.0 mg/ml and show a minor or poor response of functional VWF:RCo and VWF:CB with no corretion of VWF multimers and bleeding time after DDAVP (Figures 16 and 19). Patients with mild to moderate VWD 2A (2A/IIA) group II are featured by normal or subnormal values for FVIII:C and VWF:Ag, VWF:RCo values >0.20, normal RIPA at ristocetin concentrations of 1.2 or 1.75 mg/ml, and show a complete but transient correction of BT, FVIII:C and VWF parameters, and large multimers for a few hours after DDAVP (Figures 16, Table 7) [20,28].

VWD 2M due to loss of function in the A1 domain of VWF

Autosomal dominant VWD 2M according to Hermans & Batlle in 2009 has been misclassified in the literature as VWD 2A, !B, severe type 1 and 2, 2A Variant and Unclassified (2U) [29]. Dominant von Willebrand disease type 2M, 2A Variant and 2U are variable expressions of one distinct disease entity caused by loss of function mutations in the A1 domain of the von Willebrand factors gene (Table 8) [30]. VWD 2M is featured by poor response of VWF:RCo to DDAVP, normal VWF multimers before and after DDAVP and good responses of FVIII, VWF:Ag and VWF:CB followed by shortenend half-lfe time indicating rapid clearance defect of the FVIII-VWF complex on top of loss of VWF:RCo function in VWD 2M (Figure 5 and 11 right) [17]. For comparison Figure 12 show characteristic responses of the VWF multimeric structure to DDAVP in a healthy control (left) and in VWD 2E (2A/IIE, right) in a medium resolution gel according to Budde [16-18].

The A1 domain (aa 1260-1479) is structurally delineated by a disulfide bridge between Cys1272 & Cys1458. X-ray diffraction studies of the A1 crystal structure revealed a globular shape comprising a central core constituted of 6 hydrophobic α-strands, surrounded by 6 amphipathic α-helices [31,32]. The analysis of naturally occurring loss-of-function mutations (VWD 2M), together with mutagenesis and GPIbα peptide docking studies, have identified a central front groove on the A1 domain next to strand α3, as part of the binding site for GPIbα. VWD type 2M mutations, characterized by loss-of-function for VWF, while maintaining a normal multimerization pattern, primarily cluster around the VWF interaction site with its platelet receptor GPIbα. Two main clusters VWD 2M with loss of function mutations in the A1 domain are located in amino acid (aa) region 1272 to 1302 and in aa1359 to 1437. Three VWD 2M loss of function mutations in aa 1315, aa1324 and aa1462-1467 can be indentified as isolated among 2B VWD gain of function mutations. Several mutations in exon 28 of the A1-domain in patients with VWD type 2M have been described including L1382P, D1277-E78delinsnsl, R1342C, G1415D, I1416N [26,29]. The mutations S1285P L1307 in the MCMDM-1VWD studyl [32] very likely belong to the 2M VWD category. VWD cases compound heterozygous for R924Q-R1315C, P2145S-R1315C, P1266L-R1315C and L1481fs-Y1584C in the European VWD type 1 EU study [31] show predominant features of VWD type 2M. The ISTH registry of VWD has included the R1374C, R1374H, R1374S, R1374L, R1379C, K1405del and P1462A mutations as Unclassified (U) or 2A Variant VWD group. L1382 was labeled as 2U or 2A, and F1369I and R1315C as 2M or 2U [33]. The mutations L1282R, S1285F, L1296P, D1302G, G1324S, G1324A, R1392-Q1402del, E1352K, K1362T, P1475S and P2781S were labeled as VWD 2M [33].

VWD 2B due to gain of GPIb function mutation in the A1 domain of VWF

VWD type 2B (2B/IIB) cannot be distinguished from 2A (2A/IIA) by multimer analysis alone since there multimeric pattern are very similar [16-18]. The combined use of FVIII:C, VWF:Ag, VWF:RCo and VWF:CB and RIPA is of critical importance to differentiate pronounced VWD 1SD, from 2E (2A/IIE) and 2M (Figures 10 and 11) and to differentiate VWD 2A (2A/IIA, Figure 19) and 2B (2B/IIB, Figure 20). The VWF:Ag detects all multimeric forms of VWF equally with no differential sensitivities to different molecular weights species including large, intermediate and small multimers. The VWF:RCo assay detects all large and some of the intermediate vWF multimers in all variants of type 2 VWD and in VWF concentrates. The VWF:CB ELISA assay is sensitive to the presence of large vWF multimers with normal VWF:MM in VWD type 1 and 2M and does better detect the absence of the hemostatically potent large VWF multimers than the VWF:RCo assay in patients with VWD 2A (2A/IIA) and 2B (2B/IIB) (Table 9) [34]. Adcock *et al.* retrospectively studied 497 cases in whom results for VWF multimeric analysis in low resolution gels. VWF:Ag, VWF:RCo and VWF:CB (Gradipore ELISA assay, Corgeni, Westminster CO) were available [34]. Based on VWF:RCo/Ag and VWF:CB/Ag ratios anormal VWF multimeric pattern was seen in 449 and the 48 VWD patients were diagnosed as type 2A (2A/IIA, N=18), 2B (2B/IIB, N=29) with loss of large VWF multimers (Table 9). Twenty one out of 449 samples (4.7%) had normal VWF multimers in low resolution gel whereas VWF:CB and VWF:Ag//RCo ratios were below below <0.5 and 0.7 (Table 9) indicating that low resolution gel is not sensitive enough to pick up minor loss of large VWF multimers.

The distribution of VWF:RCo/Ag ratios for all type 2A (2A/IIA) and 2B (2B/IIB) showed that those classified as type 2A (2A/IIA) the mean VWF:RCo/Ag ratio was 0.48+0.040 and for those samples classified as type 2B (2B/IIB) , the mean VWF:RCo/Ag ratio was 0.35+0.023 [34]. VWD type 2A and type 2B with loss of large VWF multimers show that for those classified as type 2A/IIA the mean

VWF:CB/Ag ratio was 0.21+0.019 and for those classified as type 2B/IIB the ratio VWF:CB/Ag was 0.36+0.025 [34]. The conclusion of this laboratory evaluation using the combination of low VWF resolution gels, VWF:Ag, VWF:RCo and VWF:CB is that all ISTH defined type 2A variants IIA, IIC, IIE, IID VWD as well as all VWD type 2B (2B/IIB) VWD patients show the loss of large VWF multimers in a low resolution gel. Interestingly, VWF:RCo/Ag ratios was below <0.7 in VWD 2A (2a/IIA) and VWF:CB ratios was below <0.5 in both VWD 2A (2A/IIA) and VWD 2B (2B/IIB) again indicating that the vWF; CB assay is sensitive to the loss of large and intermediate VWF:MM as compared to the VWF;RCo assay (Table 9) [34]. The sensitivity and specificity for the diagnosis of VWD type 2A and 2B is 100% with the combined use of VWF:RCo and VWF:CB assays on top of the loss of large VWF multimers in a low resolution gel.

VWD type 2B (2B/IIB) is caused by a gain of function mutation of the GPIb receptor in the A1 domain of VWF [20,35]. The crystal structure of a gain-of-function A1 domain mutant R1306Q in complex with the amino-terminal domain of GPIbα (also containing a gain-of-function mutation) confirmed that the frontal part of A1 constitutes the contact area for GPIbα [31,32]. Two distinct areas of tight interaction were revealed, the first and most extensive contact site being located near the top of A1, the second involving residues near the bottom face of A1. A main cluster of VWD 2B gain of GPIb function mutations (increased binding of VWF-A1 and platelet-GPIbα) are located between aa1304 and aa1341 with two exceptions aa1315 and aa1324 (Table 10). Two minor clusters of VWD 2B mutations are located at aa1268-1272 and aa1460-1462 [29,30,33].

The key criterion for the diagnosis of VWD 2B (2B/IIB) is the loss of large VWF multimers (Figure 20) due to increased protelolysis as the second step after increased interaction of platelets mutated VWF due a gain of function in the A1 domain as reflected by increased RIPA [2,6,21,35]. VWD 2B (2B/IIB) is caused by hyperreactive mutant VWF with increased affinity for the platelet receptor glycoprotein Ib-alpha *in vivo* followed by increased proteolysis of large VWF multimers [34,35]. That process of increased VWF-GpIb-platelet interaction of mutant VWF in VWD 2B (2B/IIB) is associated with *in vivo* platelet clumping cleared from the circulation and thereby complicated by moderate to severe thrombocythopenia [35]. In a cohort of 67 VWD 2B (2B/IIB) patients from 38 unrelated families, Federici & De Groot evaluated the clinical and molecular predictors of thrombocytopenia and the risk of bleeding. Thrombocytopenia was found in 30% at baseline and in 57% after stress conditions in only those VWD 2B (2B/IIB) who did meet the ISTH criteria [35]. Platelet counts were always normal in 16 patients (24%) from 5 familiescarrying the P1266L or R1308L mutation, who usually have a type 1B variant of VWD with normal VWF:RCo/Ag ratios of 0.9 and 0.8 respectively.

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