

Research Article

Combined Use of Rapid Von Willebrand Factor (VWF) Activity, VWF-Propeptide and Classical VWF Assays for Improved Diagnosis of Von Willebrand Disease Type 1, 2N and 2E Due to Mutations in the D1, D2, D', D3 and D4 Domains of the VWF Gene

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Abstract

Introduction: The Brno Antwerp and London VWD investigators used a complete set of Von Willebrand Factor (VWF) assays for the diagnosis and classification of Von Willebrand Disease (VWD) according to European Clinical Laboratory and Molecular (ECLM) criteria (Clinical Applied Thrombosis/Hemostasis 2017; 23: 518).

Aims: The Brno Antwerp London VWD investigators directly compared the rapid von Willebrand factor (VWF) assays VWF:GPIbM and VWF:GPIbR in Von Willebrand Disease (VWD) against the complete set of VWF assays using the ECLM criteria as the gold standard for VWD classification anno 2018. Between 2008 and 2018 we prospectively studied the Brno-Antwerp cohort with VWD type 1, 2N and 2E due to mutations in the D1, D2, D' and D3 domains of the VWF gene.

Methods: The complete set of rapid and classical VWF assays include Platelet Function Analyser Closure Time (PFA-CT) Von Willebrand Factor (VWF) Antigen (Ag), Ristocetine Cofactor activity (RCo), Collagen Binding (CB), Propeptide (pp), Ristocetine Induced Platelet Aggregation (RIPA), the rapid VWF activity assay VWF: GPIbM based on glycoprotein Ib (GPIb) binding to particles coated with G233V and M239V mutants in the absence of ristocetine, the rapid VWF: GPIbR assay in the presence of ristocetine, and the responses to DDAVP of FVIII: C and VWF parameters to pick up secretion and/or clearance defects of VWF.

Results: The VWF: RCo/VWFAg, VWF: GPIbM/VWFAg and VWF: GPIbR/VWF: Ag ratios are completely normal (above 0.7) in all variants of VWD type 1 and Low VWF. The VWF: RCo/VWF: Ag, GPIbR/VWF: Ag and GPIbM/VWF: Ag ratios vary around the cut off level of 0.70 in VWD due to multimerization defect in the D3 domain and therefore diagnosed as either type 1 E or type 2E VWD. Type 1 due to a heterozygous mutation in the D1 domain is featured by persistence of proVWF as the cause of VWF secretion/multimerization and FVIII binding defect mimicking VWD type 3 together with decreased values for VWFpp, VWFpp/Ag ratios. The majority of 22 different missense mutations in the D3 domain are of type 1 or 2 E multimerization defect usually associated with an additional secretion defect (increased FVIII: C/VWF: Ag ratio) and or clearance defect (increased VWFpp/Ag ratio). The majority of VWF mutations in the D4 and C1 to C6 are VWD type 1 SD with smeary (1sm) or normal (1m) multimers with no or a minor clearance defect. The heterozygous S2179F mutation in the D4 domain is featured by VWD type 1 Secretion and Clearance (SCD). The introduction of the rapid VWF:GPIbM or VWF:GPIbR assays as compared to the classical VWF: RCo assay did change VWD type 2 into type 1 in about 10 to 12%.

Conclusion: A complete set of sensitive FVIII: C and the VWF assays VWF: Ag, VWF:GPIbM or GPIbR, VWF: PP and VWF multimer analysis are mandatory for a correct diagnosis and classification of VWD type 1, 2N and 2E due to mutations in the D1, D2, D' and D3 domains of the VWF gene.

Keywords: Von willebrand disease; Von willebrand factor; Antigen

Introduction

The ISTH classification of VWD patients in Europe clearly distinct VWD 2A (lumping IIA, II E, IIC, IID) with loss of large and increased triplet banding of VWF multimers and VWF ratio < 0.7; from 2B with loss of large and increased triplet banding of VWF multimers, VWF:RCo/Ag ratio <0.7 but increased RIPA; 2M with decreased RIPA and normal or smeary vWF multimers, from 2N with FVIII: C/VWF Ag ration <0.5 (Figure 1) [1-12]. The ECLM criteria as the gold standard for VWD classification anno 2018 in Table 1 use a complete set of VWD disease related assays including PFA-CT, VWF: Ag, VWF: RCo, VWF: GPIbM and or VWF: GPIbR, VWFpp, the ratios for FVIII: C/VWF: Ag, VWF: RCo/Ag, VWF: GPIbM/Ag, VWF: GPIbR/Ag, VWFpp/Ag, VWF multimeric analysis in low medium to high SDS resolutaion gel, and detection of the molecular defect (Table 1) [1-12].

In this real field report Michiels, Smejkal, Mayger prospectively analysed the performance of the rapid von Willebrand INNOVANCE activity assay based on GPIb binding to particles coated with gain of function mutants G233V and M239V (VWF: GPIbM assay) in the absence of ristocetin and the rapid VWF: RCo HemoSIL (VWF: GPIbR) in the presence of ristocetin as compared to classical VWF ristocetin cofactor (VWF: RCo) and collagen binding (VWF: CB) ELISA assay in the large Brno cohort of VWD patients as the direct extension of two precious studies by Vangenechten et al. Between 2008 and 2018 we prospectively collected the Brno-Antwerp cohort of VWD patients and measured the rapid and classical VWF assays in 204 patients with suspected VWD from 95 families. VWD was diagnosed according to the ISTH/EAHAD and European Clinical Laboratory and Molecular (ECLM Table 1 Figure 1) criteria for the classification of all possible variants of VWD. Molecular characterization and classification of VWD started in each patient started with the detection and location of the molecular defect to each of the D1, D2, D", D3, A1, A2, A3, D4, C106 and the CK domains of the VWF gene are recently described by Inge Vangenechten (Figure 1) [11,12]. In these studies the Brno-Antwerp VWD investigators applied the complete set of all VWF parameters in one and the same blood sample of the individual patient (Table 1) [11,12]. Standardized molecular characterisation of VWD patients allows precise classification into the correct biological type of VWD, the identification of carriers of VWD type 3 and severe type 1 in familial genetic studies and any other molecular defect in the cohort of 204 cases with high suspicion of having VWD according tot he ISTH/EAHAD criteria. The gold standard approach of the updated really 2020 nor 2018? ECLM classification in Table 1 wil become particularly relevant and even mandatory for genetic counselling for all variant of VWD with high bleeding risk, but particular in severe recessive type 1 and type 3 VWD. Moreover, the 2020 ECLM classification facilitates the evaluation of purified human or recombinant VWF concentrates for targeted prophylactic requirements, targeted treatment options of bleeding manifestations and clinical orientation in precision and personal medicine, particularly in risky situations like trauma and surgery for bleeding prevention.

Methods

Laboratory diagnosis, phenotyping and genetic analysis of vWD patients were performed by Vangenechten et al. (Figure 1 [11,12])

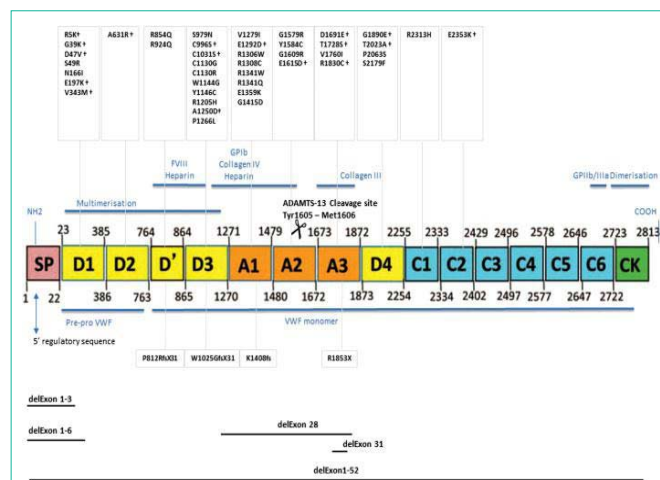


Figure 1: Structure and function of Von Willebrand Factor (VWF) protein and localization of VWF mutations in the Brno cohort of VWD patients (Source Vangenechten et al. [12]).

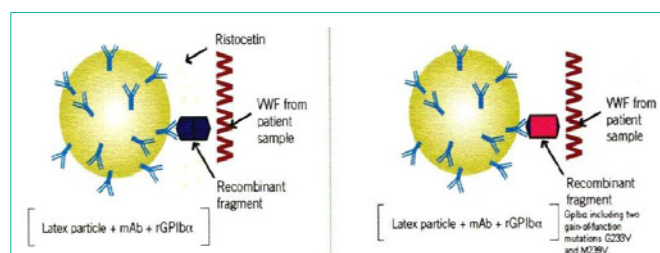


Figure 2: Principle of the HemosIL VWF:GPIbR assay (Werfen, Warrington, UK) and the INNOVANCE[®] VWF: G.PIbM assay (Sysmex UK, Milton Keynes, UK) Source Katarzyna et al. [13].

and principles of rapid von Willebrand activity assays VWF: GPIbM and VWF: GPIbR (Figure 2 Source Mayger et [13]) as described in great detail by Vangenechten et al. [11,12].

Results

Recessive double heterozygous severe type 1 and heterozygous/WT type 1 VWD due to mutations in the D1 domain

The double heterozygous G39R/P812Rfs and G197/P812Rfs and homozygous P812Rfs/P812Rfs due to mutations in the D1 and D' domain are diagnosed as VWD type 3 with detectable FVIII: C and VWF: Ag levels (Table 2). The heterozygous D1 mutations G39R/WT, D47V/WT, S49R, and N166I/WT in the VWF gene could be diagnosed as VWD type 1 or 2 irrespective of blood group (Table 2) had decreased values for VWF: RCo (0.06-0.45), VWFpp (0.15-0.50), the VWFpp/Ag ratios were decreased between 0.63-0.99 and below 1.0 with one exception. The PFA-CT times in heterozygous/WT mutation in the D1 domain were prolonged to strongly prolonged with values above 300 seconds in all (Table 2). VWD type 1 due to a heterozygous mutation in the D1 domain is featured by a secretion/multimerization defect with the persistence of proVWF in rVWF expression studies (Figure 1). Four of 12 VWD type cases with heterozygous D1 mutations had decreased ratios for VWF: RCo/VWF: Ag indicating type 2 VWD, but 12 of 13 cases had normal ratios for VWF: GPIbM/Ag and VWF: GPIbR consistent with type

Type 1: VWF:Ag < 0.30 U/mL normal VWF:CB/Ag and VWF:RCo/Ag ratio >0.7

Low VWF type 1: VWF: Ag levels >0.30-0.60 U/mL 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].

Type 2: Decreased VWF: RCo/VWF: Ag < 0.7 for 2A, 2B, 2C, 2E, 2D and 2M equal to decreased VWF:GPIbR or VWF: GPIbM < 0.7 with some discrepancies in 2B and 2M

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.

Recessive von Willebrand Disease: VWD	Domain
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 multimerization 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: Results of VWD type 3 due to double heterozygous mutations in the D1/D' domain and in heterozygous/WT VWD due to mutations in the D1 domain of the VWF gene. Results of VWD type 3 patients and heterozygous carriers for a D1 Domain mutation.

Mutation	FVIII:C	VWF:Ag	VWF:RCo	GPIbM/R	VWF:pp	RCo/Ag	GPIbM/R	VWFpp/	PFA-CT	ISTH/ECLM
D1 Domain	U/dL	U/dL	U/dl	U/dL	U/dl	CB/Ag	Ag ratio	Ag ratio	EPI ADP	Blood Group
G39R/P812fs	0.03	0.02	.	.	zero	na	na	na	nt	type 3
E197K/P812fs	0.02	0.03	.	.	zero	na	na	na	nt	type 3
P812Dfs/hom	0.03	0.02	.	.	zero	na	na	na	nt	type 3
G39R/WT	0.58	0.31	0.45	nt/nt	0.22	1.45	nt	0.70	nt	type 1 O
G39R/WT	0.55	0.52	0.40	0.47/0.47	0.42	0.77	0.90/0.90	0.80	>300/199	type 1 B
G38R/WT	0.34	0.48	0.30	0.31/0.33	0.33	0.63	0.64/0.69	0.69	>300/nt	type 2 B
D47V/WT	1.14	0.55	0.39	0.40/0.41	0.28	0.71	0.73/0.74	0.51	221/176	type 1 B
D47V/WT	0.52	0.26	0.18	0.20/0.24	0.21	0.69	0.80/0.93	0.81	284/198	type 2/1 O
S49R/WT	0.99	0.81	0.60	0.69/0.84	0.51	0.74	0.85/1.04	0.63	261/118	type 1 B
S49R/WT	0.47	0.45	0.35	0.22/0.37	0.39	0.78	0.74/0.81	0.87	262/252	type 1 O
N166I/WT	0.52	0.39	0.37		0.39	0.95		0.99	>300/196	type 1 A
N166I/WT	0.37	0.15	0.18		0.22	1.20		1.45	212/165	type 1 .
N166I/WT	0.16	0.15	0.06		0.15	0.40		0.89	>300/292	type 2 .
N166I/WT	0.93	0.45	0.37	0.36/0.32	0.34	0.82	0.80/0.71	0.76	>300/161	type 1 B
N166I/WT	1.09	0.43	0.27	0.41/0.43	0.39	0.51	0.75/0.81	0.74	>300/>300	type 2/1 O
E197K/WT	1.83	0.82	0.81	0.78/0.78	0.56	0.99	0.95/0.95	0.68	nt	type 1 A

1 VWD.

The heterozygous G39R/WT, D47V/WT, S49R/WT, N166I/WT and 197/WT mutations are diagnosed as VWD type 1 with decreased VWF: pp values, decreased VWFpp/VWF: Ag ratios below 1.00 (one exception) and have pronounced prolongation of the PFA-CT irrespective of blood group O vs Non-O (Table 2). The E197K/

WT mutation was diagnosed as normal (Table 2). The V343M/WT mutation in the D1 domain reported with dominant mild VWD type 1m (Table 3). The V343M heterozygous/WT mutation in the D1 domain is diagnosed as VWD type 1 with decreased VWF: PP values and near normal pFA-CT (Table 3).

The A631R heterozygous mutation in the D2 domain is diagnosed

Table 3: Results of dominant VWD 1 due to V343M/WT mutation in the D1 and dominant VWD 2C due to A631R/WT mutation in the D2 domain of the VWF gene.

Mutation	FVIII:C	VWF:Ag	VWF:RCo	GPIbM/R	VWF:pp	RCo/Ag	VWF:CB/	VIII/VWF	PFA-CT	ISTH/ECLM
D1 Domain	U/dL	U/dL	U/dl	U/dL	U/dl ratio	CB/Ag	Ag ratio	Ag ratio	EPI ADP	VWD type
V343M/WT	0.46	0.38	0.32	0.32 0.29	0.42 1.10	0.84 1.19	0.85 0.76	1.21	nt	1 M Fig
V343M/WT	0.50	0.38	0.30	0.32 0.31	0.56 1.60	0.86 1.10	0.90 0.87	1.32	168 134	1 M
D2 Domain										
A631R/WT	0.28	0.34	0.11	0.19 0.26	0.44 1.30	0.32 0.68	0.55 0.76	0.82	>301 >300	2C Fig
A631R/WT	0.32	0.34	0.02	0.18 0.18	0.57 1.67	0.06 0.74	0.53 0.54	0.94	nt	2C

Table 4: Results of dominant heterozygous mutated VWD in the D' domain.

Mutation	FVIII:C	VWF:Ag	VWF:RCo	GPIbM/R	VWF:pp	RCo/Ag	GPIIM/Ag	Blood	PFA-CT	ISTH/ECLM
D' domain	U/dL	U/dL	U/dl	U/dL	U/dl Ratio	VIII/Ag r	GPIR/Ag	group	EPI ADP	VWD type
P812rfs/	homozyg	0.03	0.01	nt	nt					3
P812rfs/WT	0.30	0.53	0.63	0.43 0.46	0.45 0.85	1.19	0.81 0.87	nt	nt	1
P812rfs/WT	0.78	0.39	0.31	0.40 0.39	0.55 1.41	0.79	1.03 0.99	O	nt	
P812rfs/WT	0.23	0.32	0.35	0.32 0.33	0.25 0.83	1.09	0.99 1.04	B	nt	1
P812rfs/WT	0.88	0.31	0.29	0.36 0.31	0.29 0.69	0.94	1.18 0.99	O	257 168	1
P812rfs/WT	0.83	0.36	0.23	0.30 0.28	0.23 0.72	0.64	0.82 0.78	O	275 177	21
R812rfs/WT	0.50	0.30	0.21	0.22 0.28	0.37 1.19	0.70	0.74 0.93	O	>300 259	1
P812rfs/WT	0.64	0.42	0.28	0.41 0.32	0.32 0.89	0.67	0.98 0.77	O	258 157	21
R812rfs/WT		0.49	0.38	0.43 0.45		0.78	0.88 0.92	A	186 154	1
P812rfs/WT		0.48	0.53	0.29 0.33		1.10	0.60 0.68	O	>300 >300	12
P812rfsR854Q	0.12	0.28	0.28	0.22 0.31	0.25 0.89	0.96 0.43	0.92 1.12	O	290 223	2N
P812rfsR854Q	0.23	0.49	0.51	0.54 0.52	0.39 0.96	1.04 0.47	1.10 1.06	nt	nt	2N
P812rfsR854Q	0.22	0.61	0.44	0.61 0.63	0.36 1.02	0.72 0.32	1.01 1.04	nt	nt	2N
R854Q/WT		0.79	0.68	0.61 0.62	0.74 0.94	0.86	0.77 0.79	O	126 nt	Carrier 2N
R854Q/WT	0.71	0.80	0.81	0.84 0.80	0.70 0.81	1.01 0.89	1.05 1.00	O	139 nt	Carrier 2N
R854Q/WT	0.58	0.58	0.66	0.62 0.68	0.52 0.89	1.14 1.00	1.07 1.18	nt	nt	Carrier 2N
R854Q/WT	0.61	0.71	0.57	0.75 0.66	0.57 0.81	0.80 0.86	1.06 0.93	nt	nt	Carrier 2N
R854QY1584C	0.35	0.30	0.28	0.26 0.31		0.93 1.17	0.87 1.03	A	277 nt	1
R854QY1584C	0.42	0.46	0.49	0.54.6 0.53		1.07 0.91	1.17 1.15	O	277 nt	1

as VWD 2C with decreased VWF: pp values and prolonged PFA-CT (Table 3).

VWD 2N due to VWF D' domain mutations

The heterozygous/WT D' mutation P812rfs/WT mutation in nine were diagnosed as VWD type 1 in seven and type 2 in two using the classical VWF assays and diagnosed as type 1 in eight and type 2 in one using the VWF GPIbR and VWF:GPIbM assays (Table 4). The heterozygous P812Rfs/WT mutation is featured by decreased VWF: Ag values (0.31-0.53 U/dL), normal VWFpp/Ag ratios (0.64-1.19) and moderate to pronounced prolonged PFA-CT. In contrast, four cases of heterozygous R854Q/WT carriers of FVIII binding defect mutations had normal VWF values diagnosed as normal with normal PFA-CT. Interestingly double heterozygosity of R854W/Y1584C was associated with symptomatic type 1 VWD in two patients respectively with decreased VWF:RCo (0,28- 0.49 U/dL) and prolonged PFA-CT (Table 4).

The VWF: PP values are decreased in 9 cases heterozygous/WT for P812Rfs/WT (range 0,25-0.55) and in 3 VWD 2N cases double

heterozygous for P812rfs/R854W (range 0.25-0.39) as compared to heterozygous R854W/WT mutation (range -.52-0.70).

VWD type 1, 1C, 2E and 2E clearance type (2E C) due to VWF D3 domain mutations

Using a complete set of six sensitive VWF assays (VWF: Ag, VWF: RCo, VWF: CB, VWF: GPIbM, VWF: GPIbR, VWFpp and medium resolution VWF multimeric analysis) VWD patients with the mutation W1144G/WT in 6 patients and Y1146C/WT in two patients are diagnosed as pronounced type dominant VWD 1 E or 2 E according to multimeric pattern associated with increased clearance (C) as documented by increased VWFpp/Ag ratios above 2.1 (Table 5). The performances of the VWF: RCo and VWF: CB versus VWF: GPIbM and VWF: GPIbR in typing VWD 1 or 2 did not essentially differ from each other regarding to the classification type 1 or 2 according to ISTH and ECLM classifications (Table 1).

All VWD patients with the S979N/WT mutation were typed as 2E with normal VWFpp/VWF: Ag ratios (0.65-2.04) indicating the absence of a clearance (C) defect (Table 5). The R924G/WT mutation

Table 5: Results in VWD patients due to mutations in the D3 domain.

Mutation	VWF:Ag	VWF:RCo	VWF:CB	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	VWFpp/	ECLM	GPIbM
D3	U/dL	U/dL	U/dL	U/dL	U/dL	vs CB/Ag	GPIbR/Ag	Ag ratio	diagnosis	GPIbR
						ratios	ratios			
R1205H	0.10	0.06	0.29	0.05	0.09	0.60 2.90	0.50 0.90	7.20	1C	1C
R1205H	0.15	0.10	0.26	0.05	0.09	0.67 1.76	0.35 0.59	4.87	1C	1C
W1144G	0.76	0.62	0.72	0.88	0.83	0.82 0.95	1.16 1.10	0.86	1E	1E
W1144G	0.24	0.16	0.20	0.17	0.19	0.67 0.82	0.70 0.79	2.25	1/2 EC	1EC
W1144G	0.13	0.07	0.06	0.06		0.59 0.46	0.49	3.19	2 EC	2EC
W1144G	0.19	0.14	0.12	0.10	0.18	0.74 0.61	0.54 0.94	3.20	1/2 EC	2/1 EC
W1144G	0.22	0.04	0.14	0.15	0.18	0.18 0.63	0.69 0.80	2.57	2EC	2/1 EC
W1144G	0.32	0.12	0.17	0.15	0.22	0.37 0.54	0.47 0.67	2.79	2EC	2 EC
Y1146C	0.18	0.09	0.07	0.09	0.12	0.50 0.39	0.51 0.67	2.78	2EC	2 EC
Y1146C	0.14	0.11	0.09	0.13	0.16	0.78 0.93	0.94 1.13	4.43	1EC	1 EC
S2179F D4	0.12	0.06	0.09	0.10	0.11	0.50 0.74	0.84 0.95	3.92	1mC	1mC
Mutation	VWF:Ag	VWF:RCo	VWF:CB	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	VWFpp	ECLM	GPIbM
	U/dL	U/dL	U/dL	U/dL	U/dL	vs CB/Ag	GPIbR/Ag	/Ag rat	diagnosis	GPIbR
S979N	0.52	0.34	0.37	0.22	0.28	0.63 0.66	0.41 0.53	2.00	2E	2E
S979N	0.44	0.23	0.28	0.18	0.24	0.38 0.86	0.40 0.56	2.04	2E	2E
S979N	0.24	0.15	0.16	0.17	0.14	0.29 0.32	0.73 0.60	1.09	2E	1E 2E
S979N	0.26	0.10	0.22	0.10	0.16	0.65 0.71	0.61 0.77	1.20	2E	2E 1E
S979N	0.35	0.10	0.11	0.14	0.16	0.52 0.64	0.40 0.47	1.41	2E	2E
S979N	0.37	0.08	0.26	0.18	0.39	0.22 0.70	0.48 1.04	0.65	2E	2E 1E
S979N	0.37	0.05	0.17	0.19	0.18	0.46 1.38	0.52 0.49	1.38	2E	2E
									BS/ECLM	ABO PFA-CT EPI vs ADP
R924Q	0.73	0.72	0.50	0.68	0.67	0.99 0.68	0.94 0.93	0.89	BS Zero/ N	nt nt nt
R924Q	0.57	0.50	0.45	0.48	0.51	0.88 0.80	0.83 0.83	1.35	BS2/ Low 1	O 150 nt
R924Q	0.41	0.41	0.26	0.37	0.33	1.00 0.64	0.89 0.80	2.08	BS2/ Low 1	O 150 nt
R924Q	0.89	0.90	0.71	0.91	0.77	1.01 0.80	1.01 0.87	1.00	BS Zero/ N	A 246 130
R924Q	0.84	0.88	0.82	0.92	0.79	0.98 1.01	1.10 0.94	1.01	BS Zero/ N	Nt 179 132
D4 Domain										
P2063S/WT	0.57	0.53	0.53 0.51	0.59 1.03	0.93 0.79	1.15 1.13	1.19	O	146 nt	11m

in 5 asymptomatic cases with low or normal Bleeding Score (BS) had variable VWF values between 0.26 to 0.89 U/dL but normal VWFpp/Ag and VWF:RCo/Ag ratios and normal or near normal PFA-CT. This observation is in line with the UK Arg924Gln mutation study.

As shown in Table 4, out of 51 cases with Low VWF or normal VWF but no causative VWF mutation detectable or no evidence for VWD related to PFA-CT and blood group O vs non-O four cases (8%) changed from phenotype 2 to 1 using the VWF: GPIbM/Ag or VWF: GPIbR/Ag ratio compared to the classical VWF: RCo/Ag ratio.

Discussion

Yin et al. documented that 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

[14]. Clinical and laboratory characteristics of double heterozygous mutations G39R/D141N, K157E/C1165R in the D1 domain in the study of Yin et al. [14] are featured by recessive severe type 1 VWD (Rec 1) secretion defect and FVIII binding defect. Mutations in the D1 domain do not split off VWFpp from proVWF and retain proVWF in the Endoplasmatic Reticulum (ER), which is associated with severe secretion and intracellular multimerization 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 is distinct from VWD type 3, 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 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 lack of of multimerization of the C379G mutant. The missense rVWF D141N, K157E and C379G mutants

Table 6: Result in 51 cases with no causative VWF mutation detectable or no evidence for VWD related to PFA-CT and blood group O vs non-O.

Diagnosis		VWF:Ag	VWF:RCo	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	GPIbR/Ag	Bleeding	PFA-CT	PFA-CT
Clinical	Lab	U/dL	U/dl	U/dL	U/dL	ratio	ratio	ratio	Score ABO	EPI	ADP
No VWD	N	1.07	0.90	0.69	0.66	0.84	0.64	0.62	4 nt	131	nt
Or1	1	0.30	0.25	0.34	0.26	0.83	1.12	0.85	2 O	nt	nt
Or1	1	0.34	0.43	0.41	0.39	1.26	1.19	1.15	3 O	239	136
No VWD	N	1.18	1.19		1.22	1.01	1.14	1.03	zero A	140	nt
No VWD	N	1.77	1.35	nt	1.55	0.76	0.84	0.88	zero A	145	nt
Or1	1	0.47	0.36	0.33	0.33	0.77	0.70	0.71	10 O	188	145
Or1	1	0.59	0.41	0.22	0.26	0.69	0.38	0.45	9 O	>300	136
1	1	0.73	0.52	0.67	0.65	0.71	0.92	0.89	zero	145	nt
Or1	1	0.72	0.43	0.25	0.24	0.60	0.35	0.34	8 O	177	188
1	1	0.47	0.37	0.38	0.38	0.79	0.80	0.81	2 A	>300	158
1	1	0.58	0.59	0.53	0.49	1.02	0.91	0.84	zero A	nt	nt
No VWD	N	1.15	0.99	1.13	1.16	0.77	0.97	1.01	zero A	189	89
1	1	0.54	0.52	0.50	0.52	0.96	0.93	0.96	zero A	172	113
1	1	0.53	0.55	0.53	0.50	1.04	1.01	0.94	2 A	106	nt
No VWD	N	0.78	0.88	0.74	0.71	1.13	0.95	0.91	1 O	100	nt
No VWD	N	0.62	0.79	0.58	0.62	1.27	0.94	1.00	1 A	nt	nt
No VWD	N	0.78	0.88	0.72	0.80	1.13	0.92	1.03	zero B	186	122
OrN		0.70	0.62	0.59	0.63	0.89	0.84	0.90	9 O	163	119
Or1	1	0.60	0.38	0.52	0.47	0.63	0.86	0.78	10 O	161	116
OrN		0.75	0.65	0.70	0.72	0.87	0.93	0.96	2 O	109	nt
1	1	0.58	0.46	0.49	0.46	0.79	0.84	0.79	15 O	190	179
1	1	0.45	0.33	0.38	0.32	0.73	0.84	0.72	11 O	215	131
Or1	1	0.71	0.54	0.58	0.55	0.76	0.82	0.78	11 O	199	140
Or1	1	0.77	0.52		0.98	0.68	1.41	1.28	6 O	166	140
1	1	1.16	0.62		1.13	0.53	0.89	0.98	2 B	125	nt
No VWD	N	1.27	0.98		1.39	0.77	1.26	1.09	1 B	98	nt
Or1	1	0.56	0.43	0.45	0.43	0.77	0.81	0.77	4 O	155	nt
1	1	0.58	0.30	0.48	0.46	0.52	0.83	0.80	2 A	146	nt
1	1	0.50	0.51	0.41	0.46	1.02	0.82	0.93	2 A	179	nt
1	1	0.57	0.62	0.54	0.53	1.09	0.95	0.94	2 A	104	nt
1	1	0.75	0.58	0.63	0.68	0.77	0.84	0.91	zero A	137	
Or1	1	0.46	0.43	0.41	0.45	0.93	0.90	0.98	6 O	151	nt
Or1	1	0.62	0.49	0.48	0.41	0.79	0.77	0.66	4 O	124	
Or1	1	0.53	0.60	0.58	0.52	1.13	1.10	0.96	zero O	179	127
Or1	1	0.31	0.31	0.27	0.31	1.00	0.86	1.01	1 O	229	138
1	1	0.49	0.40	0.45	0.38	0.82	0.91	0.77	3 A	205	nt
No VWD	N	1.03	1.15		1.08	1.12	1.07	1.05	zero A	205	
No VWD	N	0.94	0.88		1.01	0.94	1.09	1.07	zero A	168	101
1	1	0.54	0.55	0.49	0.55	1.02	0.91	1.01	7 A	163	137
Or1	1	0.45	0.41	0.33	0.33	0.91	0.73	0.73	zero A	186	141
Or1	1	0.72	0.53	0.61	0.69	0.74	0.84	0.95	1 O	140	nt
No VWD	N	0.93	0.93		1.18	1.00	1.18	1.26	7 O	139	nt
No VWD	N	0.84	0.75		0.88	0.89	1.39	1.05	3 O	139	nt

No VWD	N	1.03	1.10	0.76	0.89	1.07	0.74	0.87	zero	140	83
No VWD	N	1.03	0.97	0.91	1.04	0.95	0.89	1.02	zero	139	nt
Or1	1	0.63	0.51	0.52	0.57	0.81	0.82	0.90	1 O	169	117
Or1	1	0.70	0.57	0.62	0.56	0.81	0.87	0.80	4 O	139	117
No VWD	N	1.04	0.99		1.09	0.95	1.07	1.05	3 O	108	nt
Or1	1	0.59	0.59	0.52	0.54	1.00	0.88	0.92	7 O	>301	144
No VWD	N	0.69	0.69	0.64	0.68	1.00	0.92	0.98	6 A	143	nt
No VWD	N	0.88	0.64	0.67	0.82	0.73	0.76	0.93	6 B	160	nt

showed a less severe but pronounced secretion and multimerization defect with a bold pro-VWF protomer as compared to WT rvWF. In the study of Yin et al G39R/D141N D1-D1 mutation is associated with VWD severe type 1.HA due 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. The K157E/C1165R D1/D3 mutation is associated with severe type 1/HA due to severe secretion defect and multimerization defect. The mutation G39R/D141N and K157E/C1165R are associated with severe type 1/HA mimicking type 3 VWD [14]. 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 [14]. 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.

The Platelet Function Analyzer Closur Times (PFA-CT) in the Brno study are slightly prolonged in VWD type 1 Low VWF (0,30 and 0,50), and to a less extend or normal in Low VWF between 0.40 to 0.70 U/dL in 51 cases with the absence of a causative mutation in the VWF gene. The PFA-CT are strongly prolonged (>300 sec) in recessive VWD type 1 and 2C, and in dominant 2E and in pronounced type 1. The cut off level 0.70 was used for VWF: RCo/Ag, VWF: GPIbM/Ag and VWF: GPIbR ratios to define VWD type 1 versus type 2 VWD [15]. In the present study we compared the VWF: GPIbM and VWF: GPIbR assays against ECLM criteria as the gold standard (Table 1) and assessed the role of FVIII: C/VWF: Ag ratio and the VWFpp/VWF: Ag ratio in all VWD type 1, 2N and 2 E due to mutations in the D1, D2, D'and D3 domains of the VWF gene (Table 3). In the Brno study we used a complete set of Von Willebrand Factor (VWF) assays for the diagnosis of Von Willebrand Disease (VWD) according to ECLM criteria. The VWF: RCo/Ag, VWF: GPIbM/Ag and VWF: GPIbR ratios are normal (above 0.7) in VWD type 1 due to heterozygous/WT mutations in the D1 domain consistent with type 2 (Table 2), and mild VWD in LowVWF without a detectable underlying molecular defect (Table 6). The VWF: RCo/Ag, GPIbR/Ag and GPIbM/Ag ratios are variable around the cut off level of 0.70 in VWD type 2 due to multimerization defect in the D3 domain and therefore diagnosed as either type 1E or type 2E. This study clearly demonstes that the combined use of FVIII: C, VWF; Ag, VWF: PP, VWF: GPIbM or VWF: GPIbR and VWF mulrimeric analysis are mandatory to make a correct diagnosis of VWD type 1, 1C, 1E, 2E or 2 EC caused by mutations in the D3 domain (Table 5). The heterozygous/WT S2179F in the D4 is diagnosed as featured

by VWD type 1 Secretion and Clearance (SCD) similar as decribed. The heterozygous P2063S/WT in the D4 domain is diagnosed as mild VWD type 1m.

In a separate study we found that dominant VWD 2A and VWD 2B due to mutations in the A2 and A1 domain, the VWF: GPIbM/Ag and VWF: GPIbR/Ag ratios are pronounced decreased as compared to VWF: RCo/Ag and VWF: CB/Ag ratios due to the proteolytic loss of large and intermediate VWF multimers (manuscript in press). VWD 2M due to los of function mutation in the A3 domain is featured by decreased VWF: Rco/Ag ratio and normal VWF: CB/Ag ratio, but the VWF: GPIbR/Ag ratio (range 0.14-28) and the VWF: RCo/Ag ratio (range 0.10-0.33) were similar low but the VWF: GPIbM/Ag ratio somewhat higher (range 0.32 to 0.36) indicating the need to retain the VWF: CB assay to make a correct diagnosis of VWD 2M. The introduction of the rapid VWF: GPIbM or VWF: GPIbR assays as compared to the classical VWF: RCo assay will change VWD type 2 into type 1 in about 10 to 12%. VWD type 1 due to a heterozygous mutation in the D1 domain is featured by a persistence of proVWF as the cause of secretion/multimerization and FVIII binding defect mimicking VWD type 3, decreased valus for VWFpp, VWFpp/Ag ratios (0.51 to 0.99 with one exception) together with moderate to pronounced prolonged PFA-CT.

Borderline VWD

The result in 51 cases with no causative VWF mutation detectable or no evidence for VWD related to PFA-CT and blood group O vs non-O in Table 6 were tentatively diagnosed as VWD type 1 in 17, Blood group O related VWF deiciency in 20 and No VWD in 17 cases. The laboratory diagnosiswere mild VWD type 1 (LowVWF [16]) in 33 and Normal (N) in 17 cases. The PFA-CT closure times were normal or slighly prolonged in the majority of LowVWF cases and three cases had strongly prolonged PFA-CT for Epinephrine and much shorter values for PFA-CT.

Authors Contributions

JJM, PS, and KM initiated the study and wrote the manuscript on the direct compares two rapid VWF activity assays VWF: GPIbM and VWF: GPIbR in the Brno cohort of VWD patients against a complete set of FVIII: C and VWF parameters. PS, and JJM collected all clinical data and plasma and DNA samples. IV and AG performed the laboratory and molecular analysis. KM and GM measured the normal range of the two rapid VWF activity assays. ZB, UB, JB and MP served as scientific advisor.

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