Journal of Hematology & Thromboembolic Diseases

Michiels et al., J Hematol Thrombo Dis 2019, 6:5
DOI: 10.4172/2329-8790.1000299

Research Article Open Access

Determination of Two Rapid Von Willebrand Factor (VWF) Activity assays VWF: Gpibm and VWF: Gpibr in Well Defined Von Willebrand Disease Patients Using a Complete Set of Classical and Sensitive VWF Assays

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Received date: November 06, 2018; **Accepted date:** January 16, 2019; **Published date:** January 23, 2019

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Abstract

The VWF: RCo/VWF: Ag, VWF: GPIbM/VWF: Ag and VWF: GPIbR/VWF: Ag ratios are normal (above 0.7) in all variants of VWD type 1 and lowVWF and decreased (below 0.7) in VWD type 2 A, 2B and 2M. The VWF: RCo/VWF: Ag, GPIbR/VWF: Ag and GPIbM/VWF: Ag ratios are variable around the cut off level of 0.70 in VWD type 2A (II E) due to a multimerization defect in the D3 domain and therefore diagnosed as either type 1E or type 2E. The mutation W1144G/WT and Y1146C/WT result in dominant VWD type 1E or 2E associated with a secretion and clearance defect in the mutation S979N/WT result in dominant VWD 2 E with the absence of a clearance defect. Heterozygous R924Q/WT and homozygous R924Q/E924Q result in reduction of VWF and FVIII consistent with VWD type in particular when associated with blood group O. In dominant VWD 2A (IIA) due to G1597R mutations in the A2, the VWF: GPIbM/VWF: Ag and VWF: GPIbR/VWF: Ag ratios are markedly decreased to a similar degree as VWF: RCo/VWF: Ag and VWF: CB/VWF: Ag ratios due to the proteolysis loss of large and intermediate VWF multimers. In VWD 2B the VWF: GPIbM/VWF: AG ratios were more markedly decreased (below 0.40, range 0.14-0.37) compared to decreased VWF: RCo/VWF: Ag and VWF: CB/VWF: Ag ratios (range 0.17-0.68) due to the proteolytic loss of large VWF multimers. For the R1306W, R1308C and R1341W VWD 2B mutations (N=7) the activity/antigen ratios ranged from 0.15 to 0.88 for VWF: RCo, 0.44 to 0.80 for VWF: CB and markedly lower ratios from 0.15 to 0.48 for VWF: GPIbM and 0.09 to 0.33 for vWF: GPIbR. VWD 2M mutation R1359K due loss of RIPA function mutation in the A1 domain is featured by normal VWF: CB/VWF: AG ratio (range 0.80-1.05) with decreased VWF: RCo/VWF: Ag ratio (range 0.10-0.38) and similarly decreased VWF: GPIbR/VWF: Ag ratio (range 0.14-28) but the VWF: GPIbM/VWF: Ag ratio was somewhat higher (range 0.32 to 0.36) indicating the need to retain the VWF: CB and RIPA assay to differentiate between VWD 2M and VWD 2B. Mild VWD 2M due to mutations G1415D, P1266L/V1281I and E1292D/WT (N=7) have decreased VWF: RCo/Ag ratios but normal VWF: CB/Ag, VWF: GPIbM/Ag and VWF: GPIbR/Ag ratios in the majority of them. Addition of VWF propeptide (VWF: PP) assay, VWF multimer analysis, mutation detection and responses of VWF parameters to DDAVP will significantly better characterize the phenotype of each individual VWD patient. The Platelet Function Analyzer Closure Times (PFA-CT) are moderately prolonged between the upper limit of normal to 300 seconds in mild VWD type 1 patients due to heterozygous/WT mutations, including mild VWD due to mutations located in the A1 (P1266L) and A2 (Y1584C) domains. The PFA-CT are strongly prolonged (>300 sec) in VWD 2A, 2B, 2C, 2D and 2M due to mutations in the A1, A2, D2 and CK domains respectively and in recessive severe type 1 VWD due to double heterozygous null/missense mutations in the A2/D1 and A2/D domains.

Keywords: Mutation; Analyzer; Heterozygous; Platelet; Phenotype

Introduction

The International Society of Thrombosis and Haemostasis (ISTH) classification of Von Willebrand Diseases (VWD) was first designed in 1994 and subsequently revised in 2000. The 1994/2000 ISTH classification is based on the combined use of the Von Willebrand Factor (VWF) assays of VWF antigen (VWF: Ag), VWF ristocetin cofactor (VWF: RCo), Ristocetin Induced Platelet Aggregation (RIPA) and VWF multimeric analysis in a low SDS resolution gel [1-3]. The ISTH classifications defined VWD type 2A by loss of large multimers thereby lumping dominant 2A, 2B, 2E and recessive 2C together, defined VWD 2B by spontaneous RIPA and introduced a subgroup of 2M showing normal VWF multimers in a low resolution gel [1-4]. Mild type 1 VWD type 1 LowVWF patients are characterized by variable penetrance of bleeding, increased prevalence of blood group O, VWF: Ag and VWF: RCo values between 0.30 and 0.60 U/dL, normal VWF Act/VWF: Ag ratios and normal VWF multimers [5,6]. The Rotterdam 2002 modification of the ISTH criteria distinguishes VWD type 3, recessive severe type 1, dominant type 1 with values between 0.05-0.30 U/dL), and mild LowVWF with values between

0.30-0.60 U/dL [6]. RIPA is decreased VWD 2M due to loss of function mutation in the A1 domain [4-7]. RIPA is normal in mild and moderate but decreased in pronunced or severe VWD 2A, 2C and 2D due to pronounced VWF: RCo deficiency secondary to proteolysis in 2A, due to a secretion/multimerization defect in 2C, and the result of a dimerization defect in the CK domain in 2D [8-12]. RIPA is normal in dominant VWD 1 Secretion Defect (SD) and clearance defects (Table 1)[8-16]. Michiels & Van Vliet [6] introduced in 2002 the use of VWF collagen type I-III binding assay (VWF: CB-I-III), in combination with VWF: RCo, RIPA, sensitive VWF multimer analysis and DDAVP challenge test to much better separate variants of VWD type 1 from VWD recessive 2C and dominant 2A (normal or decreased RIPA) [8-16], and 2B with increased RIPA from VWD 2M featured by decreased to zero RIPA, decreased VWF: RCo/Ag ratio, normal VWF: CB/Ag ratio (Table 1)

Type	Classification
Type 1: VWF: Ag< 0.30 U/mL normal VWF: CB/VWF: Ag and VWF: RCo/VWF: Ag ratio >0.70	D1
LowVWF type 1: VWF: Ag levels >0.30-0.60 U/mL. Normal VWF: CB/VWF: Ag and VWF: RCo/VWF: Ag ratio >0.70	-
Type 3: VWF: Ag and FVIII: C undetectable or very VWF due double null VWF gene mutation.	-
Severe type 1 hemophilia A phenotype due to mutations in the D1 domain (manuscript in preparation)	-
Severe type 1 VWD VWF:Ag and VWF: RCo detectable, increased FVIII: C/VWF: Ag ratio due to secretion defect.	-
Recessive von Willebrand Disease: VWD (ISTH and ECLM in blue)	-
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	-
Recessive 2N (2N) FVIII:c/VWF:Ag ratio <0.5. FVIII-VWF binding defect	D-D3
Recessive 2C (IIC) FVIII:C.VWF:Ag increased, secretion mulimerization defect	D2
Recessive severe 1sm, dominant 1 m or 1 sm with normal (m) or smeary (sm) MM	D4 C1-6
Dominant von Willebrand Disease VWD: ECLM and ISTH in brackets	A1
2E: (IIE) type 1/2, loss of large multimers, no triplets and increased clearance	D3
2A: (IIA) Loss of large MM due to increased VWF proteolysis, RIPA N or decreased	A2
2M: (2M) RIPA and VWF:RCo/Ag ratio decreased, VWF:CB/Ag ratio	normal
2B: (IIB) Increased RIPA (0.8 mg/mL) and thrombocytopenia with VWD type 2	A1
2CB Collagen binding defect, VWF:RCo/VWF:Ag normal and CB/VWF:Ag ratio < 0.7	A3
2D: (IID) Dimerization defect, loss of large MM, intervening bands and absence of triplets	CK

Table 1: International Society of Thrombosis Haemostasis (ISTH) criteria and European Clinical Laboratory and Molecular (2018 ECLM) Classification of Von Willebrand Disease (VWD) related to functional von Willebrand factor (VWF) domain location.

LowVWF mild VWD type 1 patients are frequently type 3 or severe type 1 carriers. The combination of C1584Y mutation and blood group O is frequent within the LowVWF cohort of mid VWD patients [9]. In this report we directly compare the performances of the rapid INNOVANCE (VWF: GPIbM) VWF activity assay in the absence of ristocetin *vs* the VWF: RCo HemosIL (VWF: GPIbR) in the presence of ristocetin against the classical VWF: RCo assay in cross sectional studies of patients classified according to the updated ISTH and ECLM criteria [9-16]. As the rapid VWF: GPIbR (VWF: RCo HemosIl) assay is quite similar as the classical VWF: RCo the introduction of the VWF: GPIbR to replace the VWF: RCo assay will not essentially change the concept and strenghts of the ISTH classification [17]. The rapid VWF: GPIbR assay will detect the clinically insignificant D1472H and P1467S Single Nucleotide Polymorphism (SNP) in a similar way to the combersome and insensitive classical VWF: RCo assay. Because of the absence of ristocetinin and using gain of function mutants of platelet GPIb ligand, the VWF: GPIbM (INNOVANCE) assay will not detect the D1472H and P1467S SNP as 'dysfunctional' [17,18]. We here predict similar results of both VWF: GPIbR and VWF: GPIbM assays in all variant VWD type 1, LowVWF and 2N with no VWF functional defects but expect significant differences between the VWF: GPIbM (INNOVANCE) *vs* VWF: GPIbR (HemosIL) results in VWD 2B and 2M caused by gain or loss of RIPA function mutations in the A1 respectively. We here address the question whether the VWF collagen binding (VWF: CB) assay is still needed for the differential diagnosis between VWD type 1m or 1sm and VWD 2M and the differentiation between VWD 2M *vs* VWD 2A and 2B in the setting of the ISTH and ECLM classification of VWD using a complete set of VWF assays.

Methods

The Innovance VWF: Ac assay (VWF: GPIbM) on a Sysmex CS series analysers (Sysmex UK, Milton Keynes, UK) utilizes a recombinant GPIbα fragment containing two gain-of-function mutations G233V and M239V in the platelet Glycoprotein Ib (GPIb) [19-22]. ligand, which spontaneously bind to the GPIb receptor on the A1 domain of plasma VWF in the absence of ristocetin (Figure 1)

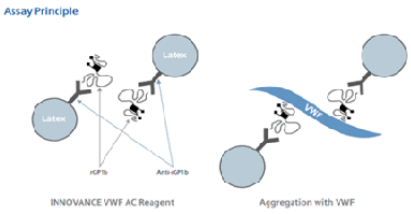

Figure 1: Patients with suspected Von Willebrand Disease (VWD) and a prolonged Platelet Function Analyser (PFA) Closure Time (CT) are candidates for screening for VWD with one of the Von Willebrand Factor (VWF) function assays.

The rapid VWF activity innovance (VWF: GPIbM) assay consist of Polystyrene microparticles covered with an anti-GPIb monoclonal antibody. The recombinant GPIb (rGPIb) which contains the gain of function mutations G223 and M239V is provided in a separate reagent. As soon as the particle reagent, the rGPIb reagent are mixed, the rGPIb binds to antiGPIb latex particles and the A1 domain of VWF in plasma sample subsequently spontaneously binds to the bound rGPIb, which is measured as decrease of light transmission in a fully automated agglutination assay [21,22].

The latex particles are coated with mutant GPIb coated on the antibodies and addition of plasma to the coated latex particles will cause agglutination and subsequent decrease in light transmission which is directly proportional to the VWF: GPIb-binding activity of VWF in the plasma [23].The VWF: RCo rapid activity assay VWF: GPIbR (HemosIL) [23] is based on reagent containing fixed, lyophilized particles coated with GPIb fragment on their surface which bind active VWF present in the plasma through its GPIb receptor in the presence of ristocetin (Figure 2).

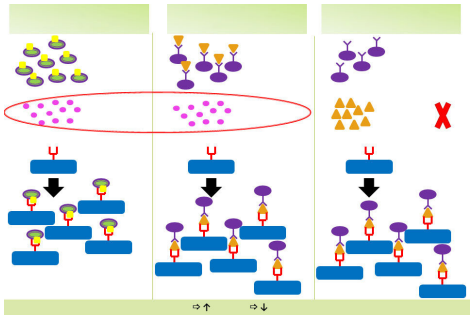

Figure 2: Assay principles of different von Willebrand Factor GPIb-binding activity kits.

According to Vangenechten Left: BC-VWF:RCo, VWF:RCo (Siemens Healthcare Diagnostics) uses formalin-fixed platelets with intact GPIb which will bind VWF through his GPIb receptor in the presence of ristocetin. Middle: HemosIL-VWF:RCo, VWF:GPIbR (Werfen) involves binding of plasma VWF to recombinant GPIb-antibody fragments attached to latex microparticles *via* monoclonal antibodies in the presence of ristocetin. Right: Innovance-VWF Ac, VWF:GPIbM (Siemens Healthcare Diagnostics) uses microparticles coated with an antibody against GPIb which bind recombinant GPIb-antibody containing two gain-of-function mutations (G223V, M239V) where no ristocetin is required for agglutination. Using all reagents platelets or GPIb-coated latex microparticles will spontaneously agglutinate is in proportion to the GPIb-binding activity of VWF in the sample and is determined by measuring the decrease of light transmission caused by this agglutination. GPIb fragments coated onto latex particles in the HemosIL GPIbR assay is used in the same way as for GPIbM assay. In the HemosIL GPIbR assay VWF-GPIb particle agglutination occurs, which is proportional to the capacity of plasma VWF to bind platelets in the presence of ristocetin, which is determined by measuring the decrease of light transmission caused by this agglutination [23]. The classical VWF: RCo is based on reagent containing formalin-fixed platelets which bind functional VWF in plasma in the presence of ristocetin in the aggregometer through binding of platelet GPIb ligand to the GPIb receptor in the A1 domain of plasma VWF. In the present study the VWF: GPIbM, VWF: GPIbR and the classical VWF: RCo activity assays were measured as described in detail [17-23]. At the time of measuring the VWF activity in clearly defined VWD patients VWF antigen (VWF: Ag) was determined with HemosIL VWF: Ag immunoassay on an ACL TOP 500 (Werfen UK). Platelet Function Analyserclosure times were measured as described by Van Vliet et al [24].

The German SIEMENS study used the commercialized INNOVANCE VWF: Ac assay (VWF: GPIbM) on a Sysmex CS2000i analyser (Sysmex UK, Milton Keynes, UK) in a large German population of VWD patients classified according to ISTH criteria in the MCMDM-1VWD study [22-25]. The Dutch Nijmegen VWF: activity assay study used the commercialized INNOVANCE VWF: Ac assay (VWF: GPIbM) [26]. The French study compared the commercialized assays VWF: AC INNOVANCE=VWF: GPIbM and VWF: RCo Acustar=VWF: GPIbR [27]. VWF: RCo in the German SIEMENS and Czech-Belgian studies was measured by using the BC-VWF: RCo (SIEMENS Healthcare Diagnostics), on STA-R Evolution (Diagnostica Stago) [27-33]. In the Brno/Antwerp/London the VWF antigen(VWF;Ag) determination using VWF: GPIbM and VWF:

VWF: GPIbR (HemosIL) and VWF: Ag immunoassay (HemosIL) on the ACL TOP 500 was used to generate reference values in 201 controls related to Blood group O *vs* Non-O [28-33]. Blood group O *vs* non-O specific cut-offs, mean, median and Reference Intervals (RI) for the VWF: GPIbM activity assay and VWF: Antigen (VWF: Ag) in a large group of 201 healthy donors [29] (Table 2A).

Donor population	Mean	VWF:GPIbM	VWF:GPIbM
N M/NY	Median	Marburg: M	New York: NY
0	RI	0.85	0.85
129/119	Mean	0.8	0.8
1	Median	0.46-1.45	0.44-1.48
Non-O	RI	1.12	1.16
134/122	Mean	1.21	1.18
0.48-1.73	Median	0.61-1.79	0.81-1.76
O/non-O	RI	1.01	1.01
263/241	0	0.46-1.62	0.99

Table 2A: The Germany study comparing normal values and Reference Interval (RI) of VWF: GPIbM assay (INNOVANCE) in Marburg (M) *vs* New York (NY) USA in large numbers of healthy donors related to blood group O *vs* Non-O].

Results and Discussion

Normal values of rapid VWF activity and antigen assays in healthy blood donors

The reference ranges for the GPIbM assay according to blood group O *vs* non-O in the German SIEMENS INNOVANCE study are shown in [22]. The lowest normal values of VWF: GPIbM are much lower in O *vs* Non-O healthy donors (0.21 and 0.35 U/dL), wheras the PFA-CT normalises at VWF: CB levels of 0.70 U/dL and above (Van Vliet et al.) [24]. The results of the VWF: GPIbM and VWF: GPIbR assays in the Antwerp-London study are shown in (Table 2B).

Donor population		VWF:GPIbM	VWF:GP1bR	VWF:Ag
Blood group		IU/dL	IU/dL	IU/dL
O	Mean	0.71	0.77	0.87
N=95	Median	0.67	0.72	0.83
-	RI	0.32-1.05	0.45-1.23	0.53-1.27
Non-O	Mean	1.01	1.02	1.13
N - 84	Median	1	0.98	1.11
--	RI	0.37-1.64	0.50-1.54	0.65-1.86
O/non-O	Mean	0.85	0.89	0.99
N=201	Median	0.8	0.87	0.94
-	RI	0.38-1.65	0.47-1.53	0.53-1.70

Table 2B: The London normal values of VWF:GPIbM and VWF:GPIbR assays in a large group of 183 healthy donors according to ABO blood group O (N=95) and Non-O (N=84) and O/Non-O (N=201).

The lower levels Of VWF: GPIbM and VWF: GPIbR are 0.32 and 0.45 U/dL in Blood group O and 0.37 and 0.50 U/dL in Non-O blood group respectively. The Reference Intervals (RI) are much lower in the VWF: GPIbM compared to VWF: GPIbR (difference 0.13 U/dL)[23]. The Reference Interval (RI) of VWF: Ag assay are much higher as compared to VWF: GPIbM assay and somewhat less for VWF: GPIbR assay. The lowest normal values of VWF: GPIbM are much lower in O *vs* Non-O healthy donors (0.21 and 0.35 U/dL) in the German SIEMENS INNOVANCE stuy. This observation is of huge importance since the fact that the PFA-CT normalises at VWF: CB levels of 0.70 U/dL and above [24]. The lower levels of VWF: GPIbM and VWF: GPIbR in the London study are 0.32 and 0.45 U/dL in Blood group O and 0.37 and 0.50 U/dL in Non-O blood group respectively. The upper and lower Reference Interval (RI) limits are much lower in the VWF: GPIbM compared to VWF: GPIbR (difference 0.13 U/dL). The upper and lower Reference Interval (RI) limits of VWF: Ag assay are much higher as compared to VWF: GPIbM assay and somewhat less for VWF: GPIbR assay.

Results in ISTH defined VWD patients in the German, Dutch Nijmegen and French studies

Comparison of the VWF: GPIbM versus classical VWF:RCo assays in the German study :The commercialized VWF: Act INNOVANCE assay in the German SIEMENS study was performed in a large cohort of 153 VWD patients classified according to the ISTH in the MCMDM-1VWD European study [25]. The VWF: Ag, VWF: RCO and VWF: GPIbM were measured in 79 type 1, 32 type 2A lumping IIA and IIE, 24 type 2B (increased RIPA), 9 type 2Msm (in fact type 1) 6 type 2N (FVIII: C/VWF: Ag ratio <0.5) but only in 3 cases 2M (Figure 3).

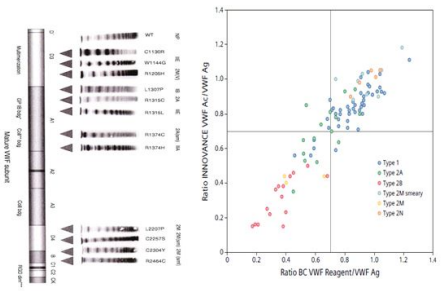

Figure 3: According to the sensitive VWF multimeric analysis alone in the MCDMD-1.

VWD 2E patients with multimerization molecular defects in the D3 domain were diagnosed as IIE or 2M (V) and VWD 2M patients with loss of RIPA function defects in the A1 domain were diagnosed as IB, 2A, 2A(sm), and IIa, whereas VWD type 1 patients with molecular

defects in the D4, B and C1 domains werediagnosed as 2M or 2M(sm). For translation into ISTH and ECLM classification of VWD

The VWD patients used in the Patzke German SIEMENS study stem from the MCMDM-1VWD study and were classified according to the German ISTH-Hamburg hybrid classification and reclassified in this report into the ISTH-UK and ECLM classifications (Table 3).

Mutant	VWF:Ag	VWF:RCo	RCo/Ag	Large MM	Domain	ISTH-Hamburg	ISTH:UK VWF	ECLM classification
	U/dL	U/dL	ratio	Low resol		RCo/Ag ratio	RCo/CB/Ag	
C1130R/WT	0.17	0.1	0.59	49%	D3	2A (IIE)	2 E	2E
C1130R/WT	0.12	0.07	0.58	49%	D3	2A (IIE)	2 E	2E
C1130R/WT	0.22	0.13	0.59	67%	D3	2A (IIE)	2 E	2E
C1130G/WT	0.1	0.1	1	57%	D3	2A (IIE)	1 E	1E
C1130F/WT	0.08	0.04	0.5	73%	D3	2A (IIE)	2 E	2E
C1130F/WT	0.08	0.03	0.38	33%	D3	2A (IIE)	2 E	2E
C1130F/R2263P	0.13	0.11	0.85	77%	D3 D4	2A (IIE)	1 E	1E
Y1146C/S1378F	0.14	0.13	0.93	94%	D3 A1	2A (IIE)	1 E	1E
R924Q/R1315L	0.42	0.13	0.31	46%	D3 A1	2A (IIE)	2 E	2E
W1144G/WT	0.31	0.12	0.39	72%	D3	2A (IIE)	2 E	2E
R1342C	0.2	0.03	0.15	96%	A1	2A	2M	2M
V1485fs/Y1584C	0.09	0.03	0.33	140%	null A2	1	Recessive 1	Recessive 1
V1822G/WT	0.19	0.26	1.37	148%	A3	1	1	1m
Q2520P/WT	0.19	0.27	1.42	71%	C4	1	1	1m
G1415D/WT	0.14	0.03	0.21	76%	A1	1	2M	2M
G1415D/WT	0.14	0.14	1	70%	A1	1	2M	2M
Exon 21 skip	0.44	0.36	0.82	115%		1	1	1m
Exon 21 skip	0.21	0.07	0.33	130%		1	2U	2U
R1374C/WT	0.2	0.03	0.15	94%	A1	2A	2M	2M
R1374C/WT	0.1	0.07	0.7	83%	A1	2A	2M	2M
R1374C/WT	0.15	0.04	0.27	86%	A1	2A	2M	2M
R1374C/WT	0.1	0.03	0.3	71%	A1	2A	2M	2M
R1374C/P2145S	0.1	0.03	0.3	71%	A1 D4	2A	2M	2M
R1315C/WT	0.25	0.1	0.4	52%	A1	2A	2M	2M
R1315C/WT	0.07	0.03	0.43	58%	A1	2A	2M	2M
D1277/L1278delinsE	0.18	0.03	0.17	70%	A1 null	2A		
SS1285P/WT	0.16	0.03	0.19	75%	A1	1 (1B)	2M	2M
I1416N/WT	0.19	0.03	0.16	67%	A1	1 (1B)	2M	2M
P1266L/R1315H	0.27	0.11	0.41	91%	A1 A1	1 (1B)	2M	2M
L1307P/WT	0.16	0.07	0.44	56%	A1	2A (IIA)	2M	2M
R1374H/WT	0.29	0.1	0.34	37%	A1	2A (IIA)	2M	2M

R1374H/WT	0.74	0.16	0.22	29%	A1	2A (IIA)	2M	2M
S2469P/R1779X	0.03	0.03	1	64%	C2	severe 1	severe 1	Severe 1
R2464C/WT	0.25	0.25	1	104%	C2	2M sm	1	1sm
R2464C/WT	0.33	0.3	0.91	122%	C2	2M sm	1	1sm
R2464/Y1584C	0.19	0.25	1.32	133%	C2 A2	2M sm	1	1sm
C2362F/WT	0.35	0.34	0.97	116%	C2	2M sm	1	1sm
C2304Y/WT	0.2	0.19	0.95	95%	C1	2M sm	1	1sm
G2441C/WT	0.22	0.2	0.91	108%	C3	2M sm	1	1sm
C2477Y/WT	0.38	0.33	0.87	124%	C3	2M sm	1	1sm
C2477S/WT	0.28	0.4	1.43	85%	C3	2M sm	1	1sm
C2257S/WT	0.29	0.42	1.45	97%	D4	2M sm	1	1sm
R1315C/WT	0.2	0.09	0.45	71%	A1	2M sm	2M	2Msm
R1315C/WT	0.1	nt	nt	81%	A1	2M sm	2M	2Msm
L2207P/WT	0.26	0.19	0.73	97%	D4	2M	1	1m
R1205H/WT	0.07	0.1	1.43	70%	D3	2M (Vic)	1 C	1 C
R1205H/WT	0.1	0.04	0.4	131%	D3	2M (Vic)	1 C	1 C
R1205H/WT	0.05	0.03	0.6	138%	D3	2M (Vic)	1 C	1 C
R1205H/WT	0.8	1.08	1.35	150%	D3	2M (Vic)	1 C	1 C
R1205H/M740I	0.14	0.06	0.43	151%	D3 D2	2M (Vic)	1 C	1 C
R1205H/M740I	0.08	0.06	0.75	144%	D3 D2	2M (Vic)	1 C	1 C
R1205H/M740I	0.06	0.03	0.5	129%	D3 D2	2M (Vic)	1 C	1 C

Table 3: VWD type 1 patients in the MCMDM-1 VWD study diagnosed according to ISTH Hamburg (ISTH-Hamburg) classification.

Using ISTH combined with multimer analysis by Budde [26] and its translation into the ISTH-UK using the ISTH criteria on top of VWF: RCo/VWF:Ag and VWF:CB/VWF:Ag ratios and subsequent translation into the ECLM classification using a complete set of sensitive VWF assays, VWF multimeric analysis and molecular defect domain location Translation of the 54 of 150 (36%) VWD patients in the MCMDM-1VWD study who had abnormal multimers and VWF: RCo/VWF: Ag ratio and/or VWF: RCo/VWF: Ag ratio below 0.7 (Type 2A) or above 0.7 (Type 1) according to the ISTH-Hamburg, ISTH-UK and ECLM classifications of VWD patients This group of patients in fact consisted of four main distinct groups: VWD type 1 E or 2 E due to mutations in the D3 domain; VWD 2 M due to loss of function mutation in the A1 domain; VWD type 1 due to mutations in the D4-C1-6 domains and VWD Vicenza type 1 C. The mutants C1130R/WT, C1130G/WT, C1130G/R2263P, Y1146C/S1378F R924Q/R1315L and W1144G were diagnosed as 2A (IIE) 5 of they had VWF: RCo/Ag ratio below 0.7 (type 2 E) and 2 above 0.7 (type 1 E) The A1 loss of function mutation L1307P (IB), R1315C (2A), and R1315H (II E) were diagnosed as 2A, but are in fact typical cases of VWD 2 M in the ECLM classification. The number of VWD 2M patients is very small in the Patzke et al. INNOVANCE study [25]. Three cases of VWD type 1 had activity/antigen ratios below 0.70 with the VWF:GPIbM and VWF:RCo assays. Out of 32 patients with VWD 2A, seven had normal antigen/activity ratios in both VWF activity assays (VWD type 1), three were discrepant, and six were below 0.7 in both assays (VWD Type 2). The broad range of VWF: Ag and all VWF functional parameters in VWD 2A from very low to normal in Table 4 is due to lumping VWD IIA and IIE in ISTH defined VWD 2A. All type 2B and 2M cases were diagnosed as type 2 [25]. All nine cases of socalled '2M smeary' according to Budde based on VWF multimer analysis alone were reclassified and diagnosed as VWD type 1sm according to ECLM criteria [25]. The overall conclusion is that the performances of the VWF: GPIbM and VWF: RCo in terms of activity ratios are identical in VWD type 1, 2B, 2M, and type 2Msnf=type 1, but that the VWF: GPIbM/VWF:Ag ratio in VWD 2B is significantly lower as compared to the VWF: RCo/VWF: Ag ratio (Table 4).

VWD type	**Type 1**	**2A**	**2B**	**2M**	**1sm**	**2N**
	ISTH-SCC	**IIA plus IIE**	**2B**	**2M**	**1**	**2N**

Number of patients	79	32	24	3	9	6
VWF:Ag %	51	44	52	50	72	87
range	Dec-84	9-119	28-107	18-81	22-201	51-139
VWF:RCo %	39	25	18	21	72	74
range	Oct-71	Oct-95	Oct-73	Dec-32	16-172	38-117
VWF:GPIbM %	38	25	14	21	72	79
range	Apr-72	4-111	Apr-47	Aug-35	22-176	44-125
VWF:RCo/VWF:Ag	0.84	0.6	0.36	0.48	0.99	0.89
range	0.46-1.35	0.16-1.20	0.17-0.68	0.39-0.66	0.74-1.24	0.53-1.04
VWF:GPIbM/VWF:Ag	0.8	0.56	0.26	0.43	1	0.93
range	0.39-1.119	0.13-1.15	0.12-0.50	0.40-0.44	0.88-1.18	0.61-1.05

Table 4: Direct comparison of the classical VWF: RCo assay and the rapid VWF GPIbM INNOVANCE activity assay in the German study of Patzke et al.[22] in VWD patients reclassified according to the ECLM classification.

The values of VWF:Ag and all VWF functional parameters in VWD 2A lumping VWD IIA and IIE show a broad range from very low to normal due to lumping VWD IIA and IIE in ISTH defined VWD 2A. The VWF:GPIbM values and VWF:GPIbM/VWF:Ag ratios in VWD 2B are lower than the corresponding VWF:RCO values and ratios against VWF:Ag due to the loss of large and some of the intermediate VWF multimers in VWD 2B. The GPIbM and VWG:RCo values and their ratios against VWF:Ag are equally decreased to a less extend in VWD 2M indicating the need to retain RIPA and VWF:CB-I-III assays in the diagnostic work-up of VWD patients to correctly differentiate between VWD 2B and 2M.

Four VWD type 1 patients had decreased VWF: GPIbM/VWF/Ag ratio and decreased BC VWF reagent/VWF: Ag ratio and diagnosed as type 2 according to ISTH-Hamburg. All type 2B and type 2M have indeed VWD type 2 in both functional assays. ISTH-Hamburg defined type 2M smeary in Figure 3 showing normal VWF;RCo/VWF: Ag ratios in are reclassified as type 1 VWD in the ISTH-UK and ECLM classifications are indeed VWD type 1 in INNOVANCE and VWF: RCo functional assays. All type 2N in the Figure 3 are type 1 VWD in the INNOVANCE and VWF:RCo assays. VWD type 2A (IIA and II E) consists of two groups: five cases are type 2 in both functional assays (very likely 2A); seven cases are type 1 in both assays (very likely IIE); two cases are type 1 in the VWF: GPIbM innovance assay and one case is type 1 in the VWF: RCo BC VWF Reagent assay. These result discrepancies in VWD type 2A are due to a mixture of VWD 2A (IIA *vs* IIE) due to mutation in the A2 domain and of VWD 2E due to mutations in the D3 domain

Conclusion

Comparison of VWF: GPIbM INNOVANCE and classical RICOF (VWF: RCo) in the Dutch Nijmegen study: The Laboratory of Hematology Thrombosis and Hemostasis Unit, University Medical Center (UMC), Nijmegen compared the ristocetin independent gain of function GP1bM (INNOVANCE) activity assay with the VWF ristocetin assay [26] VWF: RICOF (=VWF: RCo) [27] (Figure 4).

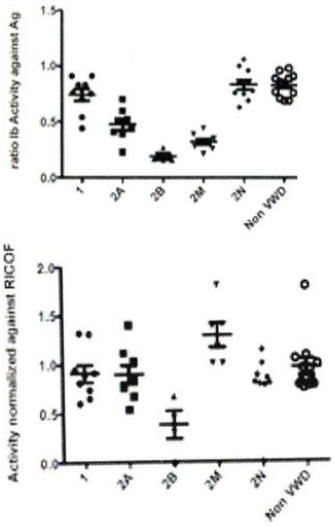

Figure 4: Results of the Dutch Nijmegen study. Please note that VWF: RCOF=VWF:RCo and Ib Activity=INNOVANCE=VWF:GPIbM.

Left: The ratios GP1bM/VWF:Ag were around 1.0 in Non-VWD and in 2N VWD patients. The GP1bM/VWF: AG ratios in nine VWD type 1patients.

Right: The GP1bM/VWF:RCo ratios were normal in non-VWD and 2N and showed a broad range from 0.60 to 1.5 in VWD

It was above 0.70 in 7 of 9 and below 0.70 in 2 patients. The GP1bM/VWF:Ag ratios in nine VWD 2A patients were around 0.5 and below 0.70 except in one case. The GP1bM/VWF:Ag ratios in six VWD 2B were significantly lower with values below 0.5 and mean values around 0.25. The ratios GP1bM/VWF:Ag ratios were normal around 1.0 (range 0.7-1.4) in non-VWD and 2N VWD patients. The GPIbM/VWF:Ag ratios in nine VWD 2M were significantly lower 0.5 and

range from 0.3 to 0.7. type 1 and VWD 2A but decreased and below 0.60 in VWD 2B and increased above 0.70 in VWD 2M indicating that the combined use of VWF:Ag, VWF:RCo and VWF:CB are needed to distinguish VWD 2M with the presence of large VWF multimers from both VWD 2A and 2B lacking the large and some of the intermediate VWF multimers.

The VWF: RCOF (VWF: RCo) is a cumbersome assay and affected by clinically insignificant polymorphisms present in the ristocetin binding site of VWF resulting in *in vitro* deceased VWF activity. In the Dutch Nijmegen study type 1 and type 2 VWD patients were classified according to ISTH criteria confirmed by genotype. The overall slope between VWF: RCOF (VWF: RCo) *vs* VWF Ib=GP1bM (INNOVANCE) in the Dutch study was 1.26+0.09 indicating that the VWF Ib=GP1bM (INNOVANCE) assay showed overall lower VWF levels compared to VWF: RCOF (2 outlier values). The Dutch Nijmegen comparative study on VWF GPIb (INNOVANCE) *vs* RICOF (VWF: RCo) comparison included 44 genetically confirmed VWD patients: type 1 N=9, type 2A N=9, type 2B N=6, type 2M N=9, type 2 2N N=6, type 1/2N N=4, type 3 N=1 Acquired VWF Syndrome (AVWS) N=1 and 12 non VWD patients directly comparing GP1bM INNOVANCE versus VWF: RCOF. The overall slope between VWF: RCOF (VWF: RCo) *vs* GP1b (INNOVANCE) was 1.26=0.09 indicating that the GP1bM (INNOVANCE) showed overall lower VWF levels compared to VWF: RCOF (VWF: RCo) and 23 samples (23%) were at least 20% higher in the GP1b (INNOVANCE) assay as compared to VWF: RCOF (data not shown). As illustrated in Figure 4, the GP1bM/VWF: Ag ratios were around 1.0 in Non-VWD and in 2N VWD patients. The ratios GP1bM/VWF: AG was below 0.70 in 2 and above 0.70 in 7 of 9 VWD type 1 patients. The GP1bM/VWF: Ag ratios were around 0.40 in nine VWD 2B patients, but significantly lower in VWD 2B around 0.25 with no overlap with VWD 2A except in one. The INNOVANCE/VWF: RCOF ratios were normal in non-VWD and 2N and showed a broad scattering in VWD type 1 and type 2A idicating equal sensitivity to diagnose VWD 2A. The INNOVANCE/VWF: RCOF ratios are increased between 1.0 and 1.8 in VWD 2M and very low ratios around 0.50 in clasical VWD 2B. The important observation from the Dutch Nijmgen study of ISTH and genetically defined VWD patients is that GP1bM (INNOVANCE) values are significantly lower compared to the VWF: RCo (VWF: RCOF) assays in VWD 2B patients lacking the large and some of the intermediate VWF multimers, but the VWF: GPIbM (INNOVANCE) values are significantly higher as compared to VWF: RCo values in VWD 2M patients, who usually show the presence of large VWF multimers. The VWF: GP1bM/VWF: Ag and VWF: RCo/VWF: Ag ratios were normal in non-VWD and 2N, above 0.70 in in the majority of VWD type 1 patients and below 0.70 in the majority of VWD type 2A patients. the VWF: GPIbM/VWF: Ag ratio was the lowest in VWD 2B below 0.40 and decreased around 0.40 in VWD 2M indicating that the combined use of VWF: Ag, VWF: RCo, RIPA and VWF: CB are needed to distinguish VWD 2M from VWD 2B. VWD 2M patients have normal VWF: CB-I-III function and decreased RIPA function due to loss of function mutation in the A1 domain. Data on VWF: CB and VWF: CB/VWF: Ag ratios are not available in this Dutch Mijmegen study. The VWF: GPIbM/VWF: RCo ratio is around 0.50 in VWD type 2B similar as seen in the German INNOVANCE study and the French study (Figure 5).

	Type 1 n = 22	Type 2A n = 15	Type 2B n = 13	Type 2M n = 16	Type 2U n = 3	Type 3 n = 6	Moderate deficiency n = 47
VWF:Ag	26.4 ± 7.5	36.7 ± 23.2	44.7 ± 15.6	26.1 ± 12.5	21.7 ± 13.1	< 1	50.6 ± 10.5
VWF:RCo	21.8 ± 6.5	18.3 ± 13.7	24.3 ± 17.1	12.2 ± 8.1	17.3 ± 13.1	< 5	48.8 ± 10.8
VWF:RCo-Acu	22.5 ± 7.7	17.2 ± 11.1	21.8 ± 14.3	12.9 ± 7.5	16.3 ± 11.2	< 0.5	48.2 ± 11.8
VWF:Ac	23.5 ± 7.4	16.6 ± 9.0	18.6 ± 10.4	14.0 ± 10.0	16.0 ± 12.5	< 5	47.2 ± 10.4

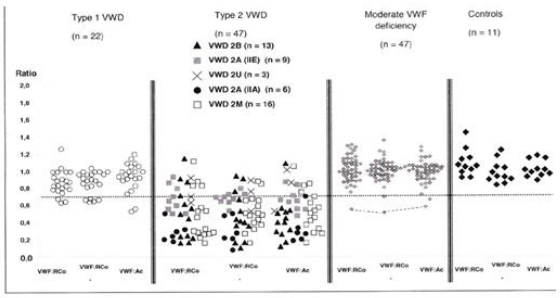

Figure 5: Results of the French study of ISTH defined VWD type 1 patients had VWF levels below 0.30 U/L and Low VWF patients above 0.30 U/L.

The VWF/VWF: Ag ratio in 22 Type 1 VWD patients was below 0.70 for VWF; RCo in 5, for VWF: GPIbR in 2 and for VWF; GPIbM in 3. All LowVWF patients had normal VWF Act/Ag ratios for all three VWF activity assays except 1 bordeline and 1 case below 0.70.Type 2 VWD patients in the French study all VWD patients were classified according to the ISTH data base 2013 and confirmed by genotype [27]. The values in the table of VWF: RCo, VWF: GPIbR and VWF: GPIbM are comparative in VWD 2A and 2M, whereas in VWD 2B the values for VWF: GPIbM were lower than for VWF: RCo with values for VWF: GPIbR in between. In type 2 VWD the VWF Act/Ag ratio of all three VWF activity assays was below(<) 0.70 in 6 of 6 IIA; 6 of 9 IIE; 9 of 13 2B; 12 of 16 2M; and 1 of 32 U patients with the following exception. The Act/Ag ratio was above 0.70 in 3 of 13 2B cases for VWF: RCo and VWF: GPIbR assays and in 1 of 13 2B cases for VWF: GPIbM. The Act/Ag ratio was above 0.60 in 5, 8 and 6 of 9 VWD IIE patients for VWF: RCo, vWF: GPIbR and VWF: GPIbM assays respectively. The Act/Ag ratio was below 0.60 for all three VWF activity assays in all 6 IIA VWD patients with one exception for the VWF: VWF: GPIbM assay.

Indicating that the VWF:GPIbM assay is more sensitive to the loss of large and some of the intermediate VWF multimers. Michiels & Vangenechten found significant lower levels for lower VWF: GPIbM/VWF: Ag ratios as compared to deceased VWF: CB/VWF: Ag ratios in 5 affected VWD 2B members of one family with the 2B mutation R1306W (personal observations Michiels and Vangenechten, (Table 5).

Mutation	VWF:Ag	VWF:RCo	VWF:CB	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	VWF/pp	ECLM	GPIbM
	U/dL	U/dL	U/dL	U/dL	U/dL	*vs* CB/Ag	GPIbR/Ag	Ag ratio	diagnosis	GPIbR
R1205H	0.1	0.06	0.29	0.05	0.09	0.60 2.90	0.50 0.90	7.2	1 C	1 C
R1205H	0.15	0.1	0.26	0.05	0.09	0.67 1.76	0.35 0.59	4.87	1 C	1 C

W1144G	0.76	0.62	0.72		0.88	0.83	0.82 0.95	1.16 1.10		0.86	1 E	1E
W1144G	0.24	0.16	0.2		0.17	0.19	0.67 0.82	0.70 0.79		2.25	1/2 E C	1 E C
W1144G	0.13	0.07	0.06		0.06		0.59 0.46	0.49		3.19	2 E C	2 E C
W1144G	0.19	0.14	0.12		0.1	0.18	0.74 0.61	0.54 0.94		3.2	1/2 E C	2/1 E C
W1144G	0.22	0.04	0.14		0.15	0.18	0.18 0.63	0.69 0.80		2.57	2 E C	2/1 E C
W1144G	0.32	0.12	0.17		0.15	0.22	0.37 0.54	0.47 0.67		2.79	2 E C	2 E C
Y1146C	0.18	0.09	0.07		0.09	0.12	0.50 0.39	0.51 0.67		2.78	2 E C	2 E C
Y1146C	0.14	0.11	0.09		0.13	0.16	0.78 0.93	0.94 1.13		4.43	1 E C	1 E C
S2179F	0.12	0.06	0.09		0.1	0.11	0.50 0.74	0.84 0.95		3.92	1m C	1m C
Mutation	VWF:Ag	VWF:RCo	VWF:CB	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	0	ECLM	GPIbM		
	U/dL	U/dL	U/dL	U/dL	U/dL	*vs* CB/Ag	GPIbR/Ag	0	diagnosis	GPIbR		
S979N	0.52	0.34	0.37	0	0.28	0.63 0.66	0.41 0.53	0	2 E	2E		
S979N	0.44	0.23	0.28	0	0.24	0.38 0.86	0.40 0.56	0	2 E	2 E		
S979N	0.24	0.15	0.16	0	0.14	0.29 0.32	0.73 0.60	0	2 E	1 E 2 E		
S979N	0.26	0.1	0.22	0	0.16	0.65 0.71	0.61 0.77	0	2 E	2 E 1 E		
S979N	0.35	0.1	0.11	0	0.16	0.52 0.64	0.40 0.47	0	2 E	2 E		
S979N	0.37	0.08	0.26	0	0.39	0.22 0.70	0.48 1.04	0	2 E	2 E 1 E		
S979N	0.37	0.05	0.17	0	0.18	0.46 1.38	0.52 0.49	0	2 E	2 E		
R924Q	0.73	0.72	0.5	1	0.67	0.99 0.68	0.94 0.93	0	BS Zero/ N	nt	nt	nt
R924Q	0.57	0.5	0.45	0	0.51	0.88 0.80	0.83 0.83	0	BS2/ Low 1	O	150	nt
R924Q	0.41	0.41	0.26	0	0.33	1.00 0.64	0.89 0.80	0	BS2/ Low 1	O	150	nt
R924Q	0.89	0.9	0.71	1	0.77	1.01 0.80	1.01 0.87	0	BS Zero/ N	A	246	130
R924Q	0.84	0.88	0.82	1	0.79	0.98 1.01	1.10 0.94	0	BS Zero/ N	Nt	179	132

Abbreviations: VWF: Von Willebrand Factor, Ag: Antigen, RCo: Ristocetine Cofactor, CB: Collagen Binding, GPIbR: Glycoprotein Ib Ristocetine, GPIbM: Glycoprotein Ib Monoclonal Antibody, PP: Propeptide, BT: Bleeding Time, ABO bloodgroup, PFA: Platelet Function Analyzer, EPI: Epinephrine,ADP: Adenoside Diphosphate.

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

Comparison of VWF: GPIbM *vs* VWF: GPIbR assays in the French cross sectional VWD study: De Maistre evaluated and compared the performance of two newly commercialized assays VWF: Ac INNOVANCE=VWF: GPIbM and VWF: RCo Acustar=VWF: GPIbR in a cross sectional study of 123 pathological samples of VWD patients classified according to the ISTH classification in Figure 5 [27]. The mean values and standard deviations of VWF: Ag, VWF: RCo, VWF: GPIbR and VWF: GPIbM measurements in 22 Type 1, 15 type 2A (lumping IIA and IIE), 13 type 2B and three type 2U and in 47 Low VWF patients in the elegant French study of De Maistre et al. are informative and characteristic findings for each of the VWD subtypes (Figure 6).

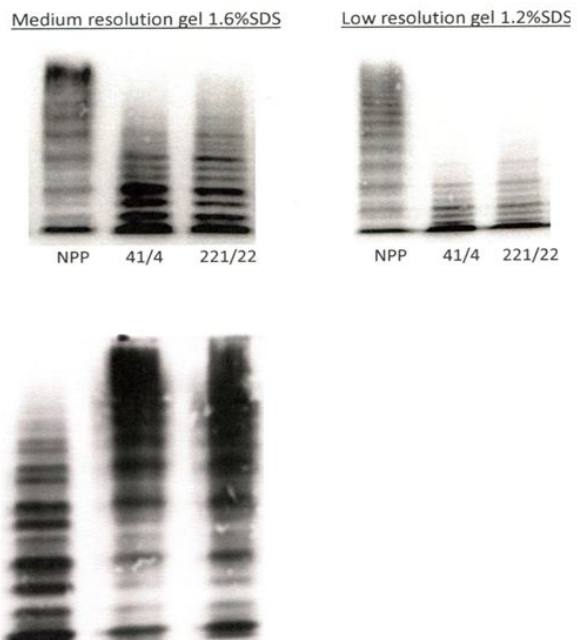

Figure 6: Left: Von Willebrandfactor (VWF) multimeric analysis in a medium resolution SDS gel showing the absence of large and some of the intermediate VWF multimers. **Right:** VWF multimer analysis in a medium and low resolution SDS gel showing the absence of large and intermediate VWF multimers and pronounced triplet structure of remaining VWF bands from one case of the Brno family with dominant VWD type 2A caused by the G1579R mutation.

The VWF: RCo/VWF: Ag ratios are completely normal in VWD type 1 and Low VWF and somewhat lower but still normal in VWD 2U. The VWF: RCo/VWF: Ag ratios are somewhat higher as compared the VWF: GPIbR/VWF: Ag and VWF: GPIbM/VWF: Ag ratios in VWD 2A (lumping IIA and IIE) and 2B, but VWF: GPIbM/VWF: Ag ratio exhibited the most pronounced decreased in VWD 2B as compared to 2M and 2A (lumping IIA and IIE). The VWF activity/antigen ratios using the three methods were comparable in VWD type 1 (n=22), Low VWF (n=47) and controls (n=11) (Figure 5) [27]. Separation of VWD 2A (n=15) into IIA (n=9) and IIE (n=6) clearly show very low ratios of 0.20 to 0.50 for VWD IIA in three functional VWF assays VWF: RCo, VWF: GPIbR and VWF: GPIbM, but the VWF activity/antigen ratios ranged from about 0.50 to 0.90 in VWD IIE in the three functional assays VWF: RCo, VWF: GPIbR and VWF: GPIbM. The results in Figure 5 show comparable results for the activity/antigen ratios in the classical VWF: RCo, in the rapid VWF: GPIbR and the VWF: GPIbM assays for type 1, VWD type IIA, II E 2U and 2M, LowVWF, and controls except VWF: GPIbM for type 2B. The VWF: GPIbM showed markedly lower activity/antigen ratios since 12/13 VWD 2B patients had ratios below 0.7 as compared 9 of 12 in the VWF: GPIbR and 8 of 12 in the classical VWF: RCo assay.

The results of the French comparative analysis of two rapid VWF activity assays are completely in line with similar finding in the German, Dutch Nijmegen and the Czech/Belgian/UK studies. Comparison of the IMMUNONANCE VWF: GPIbM/VWF: Ag ratio versus the classical VWF: RCo/VWF: Ag ratio in 38 VWD patients in the UK/Italian study of Lawrie et al revealed that out of 38 VWD patients 4 cases (10.5%) changed from type 2 into type 1 and one case from type 2 into type 1 with the use of the VWF: GPIbM/VWF: Ag ratio [28]. In the Brno study, Smejkal and Michiels found that out of 108 VWD patients 12 cases (11%) changed from type 2 into type 1 with the replacement of the classical VWF: RCo assay by the VWF: GPIbM assay (data on file Dr Smejkal 2017)(Table 6).

VWD 2B	patient	FVIII:C	VWF:Ag	VWF:GP1bM	VWF:CB	VWFpp	FVIII:Ag ratio	VWF:IbM/Ag ratio	VWF:CB/ Ag ratio	VWF:pp/Ag ratio
R1306W/WT	Born date	%	%	%	%	%	%	%	%	%
Case 1	#########	47	47	11,5	21	74	1,00	0,24	0,45	1.57
Case 2	#########	42	47	6,5	9	85	0,89	0,14	0,19	1.81
Case WT/WT	08-06-1958	108	114	99,8	104	65	0,95	0,88	0,91	0.57
Case 3	#########	47	33	6,3	12	99	1,42	0,19	0,36	3
Case 4	#########	114	68	23,8	32	125	1,68	0,35	0,47	1.83
Case 5	#########	46	38	5,4	22	67	1,21	0,14	0,58	1.76

Table 6: Results of FVIII:C and WF parameters in a Dutch family with dominant VWD 2B caused by the R1306W mutation (Personal observations by Michiels and Vangenechten).

The VWF: GPIbM/VWF: Ag ratios in VWD 2B mutation R1306W are evidently lower than the VWF: RCo/VWF: Ag ratios. The VWF: pp/VWF: Ag ratio ranged from 1.57 to 3.00 as the result of increased proteolysis of mutant R1306W VWF similar as observed in VWD 2A mutant V1499E in a large Dutch family and in the VWD 2A mutants G1579R and W1609R.

Results in eclm defined vwd patients from the brno vwd study

VWD patients with the mutation W1144G/WT in 6 patients and Y1146C/WT in two patients are diagnosed as pronounced type dominant VWD 1E or 2E according to multimeric pattern associated with Increased Clearance (IC) as documented by increased VWFpp/Ag ratios above 2.1. 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. All VWD patients with the S979N/WT were typed as 2 E with normal VWFpp/Ag ratios (0.65-20.4) indicating the absence of a Clearance (C) defect. The R924G/WT mutation in 5 asymptomatic cases with low or normal Bleeding Score (BS) had variable VWF values between 0.26-0.89 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 of Hickson et al. [29] showing that the phenotypes in five simple R924Q/WT cases and in one homozygous R924Q/R924Q case had normal VWF multimers, and FVIII: B with variable median bleeding score3, FVIII: C 0.77 U/dL, VWF: RCo 0.64 U/dL, and VWF: Ag 0.64 U/dL. The R924Q mutation has no influence of the VWF: RCo/VWF: Ag ratio and *in vitro* expression studies of recombinan R924Q as a single genetic variant did not result in altered VWF expression]. The results of phenotypic manifestations of six Index Cases (IC) and in Affected Family Member (AFM) and Unaffected Family Members (UFM) in the study of Hickson et al. [29] in Table 5A were the following: heterozygous R924Q/WT and homozygous R924Q/R924Q result in VWD type 1 and heterozygous R924Q/C1927/WT in VWD type 1 or 2M smeary as compared to normal VWF parameters in 1533 1G>T/WT. Double heterozygous R924/C1927R/1533 1G>T resulted in severe type 1 secretion defect, double heterozygous R924Q/R854Q in VWD 2N with FVIII: B of 0.09 U/L as compared to the carrier state R854Q/WT with FVIII: B of 0.65 U/L. Double heterozygous R924Q/R1205H result in more severe VWD Vincenza phenotype as compared to R1205H/WT Vicenza VWD. Heterozygous R924Q/R1315L/WT is associated with mild VWD type IIE (2E) and double heterozygous R924Q/R1315L/Y1584C resulted in pronounced VWD type IIE (2E).

A1 domain 2B and 2M: The values of VWF: GPIbM and VWF: GPIbR as compared to VWF: RCo and VWF: CB and its ratios in R1306W, R1308C and R1341W mutated cases of VWD 2B are significantly lower (Table 7A).

Mutation	VWF:Ag	VWF:RCo	VWF:CB	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	VWFpp/	ECLM	GPIbM/Ag ratio
	U/dL	U/dL	U/dL	U/dL	U/dL	*vs* CB/Ag	GPIbR/Ag	Ag ratio	diagnosis	GPIbR/Ag ratio
R1306W	0.54	0.38	0.43	0.2	0.08	0.70 0.80	0.37 0.14	2.36	2B C	2B C
R1306W	0.43	0.19	0.26	0.12	0.08	0.44 0.60	0.29 0.30	3.08	2B C	2B C
R1306W	0.27	0.04	0.12	0.05	nt	0.15 0.44	0.15 nt	2.44	2B C	2B C
R1308C	0.26	0.12	0.09	0.08	0.03	0.46 0.36	0.30 0.09	2.54	2B C	2B C
R1308C	0.29	0.06	0.11	0.06	0.02	0.21 0.37	0.22 0.06	2.69	2B C	2B C
R1341W	0.42	0.37	0.27	0.2	0.14	0.88 0.64	0.48 0.34	1.49	2B	2B
R1341W	0.62	0.23	0.25	0.21	0.26	0.37 0.40	0.35 0.42	0.85	2B	2B
Range	-	0.06-0.38	0.11-0.43	0.05-0.21	0.03-0.14	-	-	-	-	-

Table 7A: Results from the Brno cohort in VWD 2B due to gain of function mutation in the A1 domain.

The values of the VWF: GPIbM are lowe than the values of VWF: CB in the Dutch family with dominant VWD 2B showing the absence of large VWF multimers . The higher sensitivity of the two rapid VWF: GPIbM and VWF: GPIbR assays as compared to the classical VWF: RCo assay in VWD 2B is very likely related to the absence of large and some of the intermediate VWF multimers in VWD 2B patients. E1359K mutated VWD 2M patients showed decreased VWF: RCo/VWF: Ag ratios and normal VWF: CB/VWF: Ag ratios, and the VWF: GPIbR and VWF: GPIbM values and the ratios for VWF: GPIbR/VWF: Ag were similar low (0.14-0.28) as the VWF: RCo/VWF: Ag ratios. The ratios for VWF: GPIbM/VWF: Ag was somewhat higher (0.32-0.36) than for VWF: RCo/VWF: Ag (0.10-0.33) indicating the need to retain the VWF: CB (I-III) assay to make a correct diagnosis of VWD 2M. Double heterozygous P1266L/V1278I mutation in two patients and heterozygous E1292D/WT mutation in three patients in the A1 domain were diagnosed as VWD 2M associated with a Secretion Defect (SD) or some increase of Clearance (C) (Table 7B).

Mutatie	VWF:Ag	VWF:RCo	VWF:CB	GPIbM	GPIbR	RCo/Ag	GPIbM/Ag	VWFpp/	ECLM	GPIbM/R ratio
	U/dL	U/dL	U/dL	U/dL	U/dL	*vs* CB/Ag	GPIbR/Ag	Ag ratio	diagnosis	on top of CB/Ag ratio
E1359K	1.36	0.3	1.27	0.46	0.25	0.22 0.93	0.34 0.18	0.93	2M	2M
E1359K	0.54	0.18	0.57	0.2	0.08	0.33 1.05	0.36 0.14	1.37	2M	2M
E1359K	0.73	0.07	0.77	0.25	0.19	0.10 1.05	0.34 0.26	1.25	2M	2M

E1359K	0.5	0.08	0.45	0.16	0.07	0.16 0.89	0.32 0.14	0.72	2M	2M
E1359K	0.89	0.08	0.53	0.31	0.25	0.16 0.59	0.34 0.28	1.24	2M	2M
G1415D	0.08	0.03	0.1	0.15	0.17	0.38 1.22	1.90 2.16	2.33	2M C	2M C
G1415D	0.19	0.06	0.15	0.16	0.31	0.32 0.80	0.85 1.62	1.81	2M	2M

Table 7B: Results from the Brno cohort in VWD 2M due to loss of function in the A1 domain.

In VWD 2B, the ratios of VWF: RCo, VWF: CB, VWF: GPIbM and VWF: GPIbR versus VWF: Ag is all decreased due to loss of large and some of the intermediate VWF multimers (Figure 7A). In addition clearance of VWF as measured by increased VWFpp/VWF: Ag ratio is increased in the VWD 2B mutants R1306W and R1308C, but not in VWD 2B mutant R1341W (Tables 5 and 6). In classical VWD 2B the VWF: GPIbM and VWF: GPIbR values and ratios of VWF: GPIbM/VWF: Ag and VWF: GPIbR/VWF: Ag are lower than the corresponding VWF: RCo and VWF: CB values. VWD 2M patients have decreased RIPA, normal VWF: CB/VWF: Ag ratios and decreased ratios for VWF: RCo/VWF: Ag, VWF: GPIbM and VWF: GPIbR indicating to retain RIPA and VWF: CB-I-III for the laboratory differentiation between VWD 2M and VWD 2B. Mild cases of VWD 2M with decreased VWF: RCo/Ag ratios have near normal or normal valus for VWF: CB, VWF: GPIbM and VWF: GPIbR (Table 8).

Mutation	**VWF:Ag**	**VWF:RCo**	**GPIbM/R**	**VWF:pp**	**RCo/Ag**	**GPIbM/R**	**VIII/C**	**ABO**	**PFA-CT**	**ECLM** *vs*
A1 Domain	U/dL	U/dl	U/dL	U/dL ratio	CB/Ag ratio	ratios	Ag ratio		EPI ADP	GPIbM/R
P1266L/V1278I	0.25	0.13	0.25 0.23	0.43 1.70	0.52 0.84	0.99 0.91	2.36	A	>300 237	2M SD 1M
P1266L/V1278I	0.45	0.33	nt	0.46 1.02	0.52 0.84	nt	1.36	O	215 131	2M SD nt
E1292D/WT	0.26	0.26	0.26 0.86	0.31 1.18	1.00 1.00	0.86 1.00	2.38	B	211 165	1M SD 1M
E1292D/WT	0.46	0.16	0.26 0.30	0.79 1.72	0.35 0.66	0.57 0.66	1.11	O	>300 297	2M 2M
E1292D/WT	0.35	0.13	0.25 0.27	1.12 2.00	0.37 0.83	0.71 0.71	1.26	B	nt nt	2M 1M

Table 8: Results in mild VWD 2M or 1M due to mutations in the A1 domain.

A2 domain: The results o in 16 cases of G1579R mutated VWD type 2A from 5 families the rapid VWF: GPIbM (0.04-0.12 U/dL) *vs* VWF: GPIbR (0.03-0.20 U/dL) assays are similar low and do not differ (Table 9). The results of the two rapid VWF activity assays VWF: GPIbM and VWF: GPIbR are significantly lower than the decreased values for the classical assay VWF: RCo (0.04-0.28 U/dL) and VWF: CB 0.11-0.30 U/dL) in 16 cases of G1579R mutated VWD type 2A from 5 families. Similarly, much lower VWF: GPIbM/VWF: Ag ratios and VWF: GPIbR/VWF: Ag ratios (0.03 to 0.27 U/dL) as compared to VWF: RCo/VWF: Ag and VWF: CB/VWF: Ag ratios (0.06-0.53 U/dL) were recorded in sixteen G1579R mutated VWD 2A patients and in one G1609R/WT mutated VWD 2A patient. The higher sensitivity of the two rapid VWF: GPIbM and VWF: GPIbR assays as compared to VWF: RCo assay in G1579R mutated VWD 2A is very likely related to the absence of large and some of the intermediate VWF multimers (Figure 6). The VWF: pp/VWF: Ag ratios were slightly increased with values between 1.11 and 2.15 in 16 cases of G1579R mutated VWD 2A. As the consequence of slightly shortened VWF: Ag half-life times due to increased proteolysis of VWF. Similar findings of slightly increased VWFpp/VWF: Ag ratios has been reported in 10 affected patients in a large Dutch family with dominant VWD 2A Gouda due to the V1499E mutation [30,31]. Seven evaluable patients heterozygous for the Y1584C/WT mutation were diagnosed as VWD 1m (normal VWF multimers). Double heterozygosity the Y1584C in the A2 domain with a mutation in the D1 domain Y1584C/G39r in two cases and with a mutation in the D' domain Y1584C/P812rfs in one case resulted in severe type 1 VWD secretion defect (SD with increased FVIII/VWF: Ag ratio) with strongly prolonged PFA-CT above 300 sec (Table 9).

Mutation	**VWF: Ag**	**VWF: RCo**	**VWF: CB**	**GPIbM**	**GPIbR**	**RCo/Ag**	**GPIbM/Ag**	**VWF/pp**	**ECLM/**	
G1579R	U/dL	U/dL	U/dL	U/dL	U/dL	*vs* CB/Ag	GPIbR/Ag	Ag ratio	GPIbM R	
Range	0.41-0.95	0.04-0.28	0.11-0.37	0.04-0.12	0.03-0.20	0.06-0.53	0.03-0.27	1.11-2.15	2A	
G1609R	0.46	0.09	0.14	0.04	0.04	0.20 0.31	0.13 0.23	1.3	2A	

Results from the Brno cohort of VWD type 1 due to mutations in the A2 domain										
Mutation	**VWF: Ag**	**VWF: RCo**	**GPIbM/R**	**VWF: pp**	**RCo/Ag**	**GPIbM/R**	**VIII:/C**	**ABO**	**PFA-CT**	**ECLM** *vs*
A2 Domain	U/dL	U/dl	U/dL	U/dL ratio	CB/Ag r	ratios	Ag ratio		EPI ADP	GPIbM/R
Y1584C/WT	0.3	0.23	0.31 0.33	0.57 1.91	0.77 1.10	1.03 1.11	0.77	O	232 206	1m 1m
Y1584C/WT	0.42	0.36	0.28 0.33	0.70 0.71	0.86 0.71	0.66 0.78	1	O	242 192	1m 1m
Y1584C/WT	0.36	0.23	0.28 0.30	0.59 1.63	0.64 0,67	0.79 0.83	1.22	O	265 249	2/1m 1m
Y1584C/WT	0.61	0.55	0.53 0.59	0.54 0.88	0.90 0.77	0.87 0.96	1.2	A	205 148	1m 1m
Y1584C/WT	0.72	0.48	0.54 0.68	0.61 0.85	0.67 0.68	0.74 0.94	1.1	A	>300 174	2/1m 1m
Y1584C/WT	0.53	0.46	0.41 0,41	0.53 2.16	0.87 0.92	0.76 0.76	0.53	O	192 155	1m 1m
Y1584C/WT	0.76	0.49	0.61 0.61	0.68 0.90	0.64 0.92	0.80 0.80	0.82	A	145 nt	2/1m 1m
Y1584C/G39R	0.12	0.09	0.13 0.16	0.21 1.77	0.75 1.03	1.07 1.31	1.63	A	>300 >300	Severe 1SD
Y1584C/G39R	0.07	0.11	0.18 0.29	0.24 3.00	1.38 1.49	2.19 3.60	2.09	A	>300 >300	Severe 1SD
Y1584C/P812rfs	0.08	0.02	nt 0.25	0.12 1,54	0.25 1.54	nt 3.10	3.45	O	>300 116	Severe 1SD

Table 9: Results from the Brno cohort of VWD 2A patients with the G1579R mutation in the A2 domain in 16 affected members from 5 families and in 1 case with the G1609R in the A2 domain.

The values and ratios of VWF: RCo, VWF: CB, VWF: GPIbM andVWF: GPIbR against VWF: Ag is all very low in the VWF A2 domain mutants G1579R and G1609R in the Brno study. VWD type 1m heterozygous Y1584/WT mutant in the A2 domain have prolonged PFA-CT ranging from the upper limit of normal to 300 seconds. Three of seven cases of Y1584C/WT mutants show discrepant findings of type 1 in the rapid VWF activity assays as compared to type 2 VWD in the VWF: RCo assay. Double heterozygous Y1584/G39R and A2Y1584C/P812rfs missense/null mutations are associated with severe VWD type 1 Secretion Defect (SD) and strongly prolonged PFA-CT.

Conclusion

The broad range of VWF: Ag and all VWF functional parameters in VWD 2A from very low to normal in Table 4 is due to lumping VWD IIA and IIE in ECLM defined VWD 2A. The VWF: GPIbM values and ratios of VWF: GPIbM/VWF: Ag in VWD 2B are lower than the corresponding VWF: RCo values. The activity ratios for VWF: RCo and VWG: GPIbM are equally decreased in VWD 2M showing normal VWF: CB ratios indicate the need to retain RIPA and VWF: CB-I-III to correctly differentiate between VWD 2B and 2M.

The values and ratios of VWF: RCo, VWF: CB, VWF: GPIbM and VWF: GPIbR against VWF: Ag is all very low in the VWF A2 domain mutant G1579R and G1609R in the Brno study [32]. VWD type 1m heterozygous Y1584/WT mutant in the A2 domain have prolonged PFA-CT ranging from the upper limit of normal to 300 seconds. Three of seven cases of Y1584C/WT mutants changed from type 2 with decreased VWF: RCo/VWF: Ag ratios into type 1 with normal ratios for VWF: GPIbM/VWF: Ag and VWF: GPIbR/VWF: Ag. Double heterozygous Y1584/G39R and Y1584C/P812rfs missense/null mutations are associated with severe VWD type 1 secretion defect [32].

A3 and D4 domains: Sevens patients double heterozygous for D1691E/WT or T1728S/WT and one case heterozygous for P2063S/WT had normal ratios for VWF: RCo/VWF: Ag, VWF: GPIbR/VWF: Ag and were diagnosed as VWD type 1m (Table 10). Three out of seven patients double heterozygousforD1691E/WT or T1728S/WT had decreased VWF: CB/VWF: Ag but normal VWF: CRo/VWF: Ag ratio. The values and ratios of VWF: RCo, VWF: CB, VWF: GPIbM andVWF: GPIbR against VWF: Ag due to mutations in the A3 domain are in line with the diagnosis of VWD type 1 or type 2 within the same mutation in the VWF: RCo, VWG: GPIbM and VWF: GPIbR assays but all are type 1 in the VWF;CB-I-III assay consistent with VWD type 1m with normal VWFpp/Ag and FVII: C/VWF: Ag ratios indicating the absence of a secretion or clearance defect.

The role of Platelet Function Analyzer Closure Times (PFA-CT) in VWD patients: Nummi et al. measured PFA-CT with Epinephrin (EPI) and Adenosinediphosphate (ADP) cartridges in a one center cohort of 54 ISTH defined VWD patients: LowVWF in 10, type 1 in 7, type 2A

in 14, Type 2B in 9, type 2N in 1, type 3 in 13 and normal VWF in no VWD 19 (Figure 7) [33]. The VWF: RCo and VWF: Ag levels were between 0.14 -0.33 U/dL and 0.08-0.25 IU/dL in pronounced VWD type 1 and between 0.43-0.64 U/dL and 0.36-0.64 in LowVWF respectively. PFA-CT EPI and ADP were normal (less than 150 seconds in healthy controls and in the group of no VWD with normal VWF levels. The PFA CT EPI was prolonged with values between 150-250 seconds in 6 of 10 and in 2 of 10 Low VWF patients. The PFA CT EPI and ADP were strongly prolonged above 250 seconds in VWD 2A, 2B, 2M and type 3. The PFA CT EPI and ADP prologations in VWD type 1 are inbetween the values seen in low VWF *vs* VWD type 2A, 2B and 2M. The PFA-CT were strongly prolonged (>300 sec) in the Brno study in VWD patient with recessive type 1 and 2C, and in dominant 2A, 2B, 2M (Smejkal data on file 2017). The PFA-CT results in the Brno study are slightly prolonged with values between the upper range of normal to 300 seconds in VWD type 1 Low VWF (0.30 and 0.60) due to mutations in the VWF gene (Tables 5, 7-9). The PFA CT was normal or slighly prolonged in LowVWF with VWF values between 0.40 to 0.70 U/dL in 51 cases with the absence of a causative mutation in the VWF gene (manuscript in preparation). The PFA-CT in heterozygous C1584C/WT VWD type 1 patients was slightly prolonged with values between the upper limit of normal to 300 seconds (Table 10).

Mutation	VWF: Ag	VWF: RCo	GPIbM/R	VWF: pp	RCo/Ag	GPIbM/R	VIII/ C	ABO	PFA-CT	ECLM *vs* EPI
A3 Domain	U/dL	U/dl	U/dL	U/dL ratio	CB/Ag ratio	ratio	Ag ratio		EPI ADP	GPIbM / R
D1691E/WT	0.31	0.32	0.44 0.43	0.55 1.77	1.03 1.39	1.43 1.38	0.41	O	195 167	1m
D1691E/WT	0.63	0.65	0.52 0,64	0.65 0.80	0.78 0.80	1.43 1,38	0.59	O	nt nt	1m 1
D1691E/WT	0.59	0.39	0.52 0.63	0.70 1.19	0.66 1.09	0.64 0.80	0.68	O	nt nt	2 or 1m 2 or 1
T1728S/WT	0.98	0.63	0.88 0.90	1.05 1.08	0.64 0.82	0.89 0.92	1.29	O	214 164	2 or 1m 1
T1728S/WT	0.56	0.31	0.38 0.37	0.57 1.02	0.55 0.73	0.68 0.66	1.14	O	>300 140	2 or 1m 2
V1706I/WT	0.55	0.47	0.45 0.42	0.51 0.93	0.85 1.11	0.82 0.77	1.11	A	153 nt	1m 1
V1706I/WT	0.61	0.69	0.62 0.59	0.57 0.93	1.13 1.20	1.02 0.96	1.36	O	137 nt	1m 1
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	1m 1

Table 10: Results from the Brno cohort of mild VWD due to mutations in the A3 and D4 domains

The values and ratios of VWF: RCo, VWF: CB, VWF: GPIbM andVWF: GPIbR against VWF: Ag due to mutations in the D3 domain are in line with the diagnosis VWD type 1 or type 2 within the same mutation in the VWF: RCo, VWG: GPIbM and VWF: GPIbR assays but all are type 1 in the VWF;CB-I-III assay consistent with VWD type 1m with normal VWF: pp/VWF: Ag and FVIII: C/VWF: Ag ratios indicating the absence of a secretion and clearance defect.

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