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ORIGINAL ARTICLE

A comparative study in patients with type 2 von Willebrand disease using 4 different platelet-dependent von Willebrand factor assays

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Abstract

Background: Several assays are now available to evaluate platelet-dependent von Willebrand factor (VWF) activity.

Objective: To report the results obtained using 4 different assays in patients with von Willebrand disease (VWD) carrying variants mainly in the A1 domain, which is critical for VWF binding to glycoprotein Ib (GPIb) and ristocetin.

Methods: We evaluated 4 different assays, 2 gain-of-function mutant GPIb binding (VWF:GPIbM) and 2 ristocetin cofactor (VWF:RCo) assays, in 76 patients with type 2 VWD. Patients and healthy controls were tested using VWF:GPIbM enzyme-linked immunosorbent assay (ELISA), VWF:GPIbM automated, VWF:RCo aggregometric, and VWF:RCo automated assays.

Results: There was a good correlation (Pearson's r>0.82) and agreement (Bland-Altman plots assessment) between the 4 assays, although several outliers existed among the type 2B without high-molecular-weight multimers (HMWM). The VWF activity/VWF:antigen ratios, calculated for each assay, were used to establish the percentage of a correct diagnosis of type 2 (ratio<0.60) in these patients: VWF:RCo aggregometric, 2A(100%), 2M(78%), 2M/2A(100%), 2B(68%); VWF:RCo automated, 2A(88%), 2M(89%), 2M/2A(100%), 2B(63%); VWF:GPIbM ELISA, 2A(96%), 2M(67%), 2M/2A(67%), 2B(0%); VWF:GPIbM automated, 2A(73%), 2M(44%), 2M/2A(75%), 2B(84%). In type 2B patients with HMWM, all assays gave a ratio ≥0.60.

Conclusion: The VWF:GPIbM-automated assay is the most effective to diagnose as type 2 the 2B variants, whereas the VWF:RCo assays are the most effective in detecting 2M and 2M/2A variants. The VWF:GPIbM ELISA greatly overestimates the activity of the type 2B patients lacking HMWM. In this study, the use of a VWF activity/VWF:antigen ratio cut-off of 0.70 halved the number of misdiagnosed patients.

Paola Colpani and Luciano Baronciani contributed equally to this study.

KEYWORDS
von Willebrand factor, von Willebrand disease, Ristocetin-Willebrand factor, glycoprotein Ib alpha, enzyme-linked immunosorbent assay, gain-of-function mutation

Essentials

- The von Willebrand factor (VWF) activity assay is crucial in von Willebrand disease (VWD) diagnosis.
- The 4 different VWF activity assays correlate well but may give inconsistent results.
- Discrepancies between the 2 commercial assays are mainly found in type 2B and 2M VWD.
- The choice of VWF activity assays affects the diagnosis of VWD type 2.

1 | INTRODUCTION

Von Willebrand disease (VWD) is the most common inherited bleeding disorder with a 1% prevalence in the global population and equal distribution among males and females [1]. VWD is due to quantitative or qualitative defects of the adhesive multimeric plasma glycoprotein (GP) von Willebrand factor (VWF) [2]. According to the *International Society on Thrombosis and Haemostasis* guidelines, VWD is classified into 3 main types: types 1 and 3 are characterized by the partial or complete deficiency of VWF, whereas type 2, which includes 4 distinct subtypes (2A, 2B, 2M, and 2N), is due to qualitative VWF defects. VWD type 2A is characterized by decreased VWF-dependent platelet adhesion caused by a deficiency of high-molecular-weight multimers (HMWM), whereas type 2B includes VWF variants with an increased affinity for the platelet receptor glycoprotein Ib (GPIb) with or without a deficiency of HMWM. Type 2M is due to decreased VWF-dependent platelet adhesion despite an intact VWF multimeric pattern, and VWD type 2N includes VWF variants with decreased binding affinity to factor (F)VIII [3,4]. The diagnosis of VWD is established on personal and family history of mucocutaneous bleeding and abnormal results of laboratory tests that measure plasma VWF and FVIII [5]. However, an accurate diagnosis is often difficult because of a variable bleeding tendency and heterogeneous laboratory phenotypes among different VWD types. For the initial laboratory investigation, the measurements of plasma FVIII coagulant activity (FVIII:C), VWF antigen (VWF:Ag), and platelet-dependent VWF activity, historically evaluated as ristocetin cofactor activity (VWF:RCo), are performed [6,7]. The VWF:RCo assay measures the ability of VWF to agglutinate platelets in the presence of ristocetin. The original method used formalin-fixed platelets and ristocetin by aggregometry [8]. This assay has been improved with the use of automated instruments and the availability of commercial reagents containing lyophilized platelets and ristocetin [9]. Nevertheless, several new methods have emerged in the last few decades as an alternative to VWF:RCo to overcome some limitations, mainly due to the need for platelets and ristocetin [10]. Some assays still use ristocetin but have replaced platelets with beads coated with wild-type recombinant (r) GPIb molecules (VWF:GPIbR) using immunoturbidimetric or chemiluminescent methods [11]. Other assays avoid both ristocetin and platelets, using latex beads coated with a monoclonal anti-VWF-A1 domain antibody (VWF:Ab) [12] or gain-of-function rGPIb molecules on enzyme-linked immunosorbent assay (ELISA) plates [13] or with latex beads coated with anti-GPIb antibodies (VWF:GPIbM) [14].

In this study, we chose to compare a manual VWF:RCo aggregometric assay, a commercially available VWF:RCo automated assay, a VWF:GPIbM in-house ELISA, and a commercially available VWF:GPIbM-automated assay. We evaluated 2 in-house methods based on the same principle along with the only 2 commercially available assays that use either platelets and ristocetin or gain-of-function rGPIb molecules. This strategy should help to better evaluate the possible advantages or disadvantages of the 2 different ploys used to measure *in vitro* platelet-dependent VWF activity. For the same purpose, we have mainly investigated patients with VWD-carrying variants in the A1 domain (2B, 2M, and 2M/2A), because this domain plays a key role in VWF binding to the GPIb platelet receptor as well as ristocetin.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

We evaluated 76 patients with VWD diagnosed at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center in Milan and 50 healthy controls. The healthy controls, 25 males and 25 females with a median age of 48 years (IQR, 42-53 years), had no history of hemorrhagic episodes and did not use antiplatelet or anticoagulant medications for at least 10 days before blood collection. Their blood samples were voluntarily obtained at the Transfusion Center of Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca' Granda Ospedale Maggiore Policlinico. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, and all enrolled cases signed informed consent to participate. All 76 patients with VWD and the 50 healthy controls were Caucasians. The patients who were a convenience sample selected based on confirmed VWD diagnoses and *VWF* mutations were enrolled from 2016 to 2017 and analyzed within 6 months of collection. Blood samples were collected from all cases in 3.2% buffered citrate solution (1:9

anticoagulant to whole blood) (VACUETTE Blood Collection Tubes; Greiner Bio-One S.R.L.). Platelet-poor plasma prepared by blood centrifugation at 1500 *g* for 15 minutes at room temperature was stored at -80 °C and thawed within 5 minutes at 37 °C before performing the tests. The patients with VWD, 30 males and 46 females with a median age of 48 years, (IQR, 29-59 years) were diagnosed in agreement with the guidelines of the International Society on Thrombosis and Haemostasis-Scientific and Standardization Committee guidelines and the National Heart, Lung, and Blood Institute [3,4]. At the time of establishing a diagnosis for these patients, the accepted cut-off for platelet-dependent VWF activity/VWF:Ag ratio (VWF activity/VWF:Ag ratio) was 0.60. This cut-off helps to detect VWD type 2 by identifying disproportionately reduced VWF function caused by multimer abnormalities or mutations within specific functional domains of VWF compared with VWF:Ag [15]. Current guidelines for VWD diagnosis [16] suggest a cut-off value of 0.70, but we chose in this study to maintain the cut-off at 0.60, i.e., the ratio used at the time of diagnosis. Patients were classified as type 2A (n = 26), type 2B without HMWM (n = 19), type 2B with HMWM (2B HMWM; n = 10), type 2M (n = 9), and type 2M/2A (n = 12). The biochemical and molecular historical data of the patients are reported in the Supplementary Table 1. At the time of diagnosis, the VWF activity was measured by using an adapted VWF:RCo assay employing the BC von Willebrand Reagent (Siemens) [17]. Patients' diagnoses were confirmed using the reported genotypic-phenotypic association of the variants on online databases [18,19]. Of the 29 variants evaluated, only 3 were reported to be associated with different VWD types (p.Arg1315Leu, p.Arg1374Cys, and p.Arg1374His). Each of these variants was mainly associated with either type 2M [20, 21, 22], 2A [23, 24, 25], or even as type 2A/2M (p.Arg1374Cys) [25]. In our center, these variants were classified as type 2M/2A [26]. Although the latter definition (2M/2A) is not included in the official VWD classification [3], we have extensively described the characteristics of these variants in a previous study [26]. In brief, patient diagnosis was based on markedly reduced VWF activity/VWF:Ag ratios with only mildly reduced VWF:CB/VWF:Ag ratios and a modest loss of HMWM due to a mutation in the A1 domain (Supplementary Table 1).

2.2 | Laboratory measurements

VWF:Ag was determined using the VWF Ag reagent (Siemens Healthcare Diagnostics) with an automated analyzer CS-2400 (Sysmex). We used 4 different methods to measure platelet-dependent VWF activity; the details regarding these 4 assays are summarized in Supplementary Table 2. For the VWF:RCo assay, ristocetin was used to induce the interaction between the VWF A1 domain and platelets *in vitro* through the GPIb-IX-V receptor complex using 2 different methods, i.e., an aggregometric and an automated assay. The first method employed a two-channel aggregometer with in-house formalin-fixed platelets and ristocetin at a final concentration of 1 mg mL^{-1}, (limit of detection [LOD]: 6 IU dL^{-1}) [27]. The slope of the agglutination curve was calculated automatically by the AGGRO/LINK Software (Chrono-Log) in the NCCLS Format for Windows, rev. 4.75. The second method used the commercial BC VWF Reagent (Siemens Healthcare Diagnostics) based upon the use of lyophilized platelets and ristocetin with the CS-2400 analyzer (LOD: 12.5 IU dL^{-1}). Both the VWF:GPIbM assays are based upon the gain-of-function mutant rGPIb molecules that spontaneously interact with the VWF A1 domain and used 2 methods, i.e., an ELISA and an automated assay. The in-house ELISA was performed with immobilized gain-of-function rGPIb molecules, as previously described [28]. In brief, the monoclonal anti-GPIb antibody 2D4 was bound to an ELISA plate. The day after the gain-of-function rGPIb was added and incubated overnight, plasma samples and calibrator were added and incubated at room temperature for 2 hours. Then, bound VWF was detected with an anti-VWF horseradish peroxidase–labeled polyclonal antibody (P0226; Dako; LOD: 1 IU dL^{-1}). We also used the automated method INNOVANCE VWF Ac reagent (Siemens Healthcare Diagnostics) on the CS-2400 analyzer, following strictly the manufacturer's protocol. In brief, gain-of-function rGPIb molecules are added to patient plasma and spontaneously bound to VWF. Then, polystyrene particles coated with an anti-GPIb antibody are added. Binding between polystyrene beads and rGPIb-VWF complexes results in a decrease of light transmission that is directly proportional to the VWF-GPIb binding activity in plasma (LOD: 4.2 IU dL^{-1}). All tests were calibrated against the World Health Organization 6th International Standard (National Institute for Biological Standards and Control, NIBSC: 07/316).

2.3 | Statistical analysis

Continuous variables were expressed as medians and IQR, and categorical variables were expressed as counts and percentages. Median VWF activity/VWF:Ag ratios obtained performing the 4 different activity tests were compared for each group of patients with VWD using the non-parametric Kruskal-Wallis test. Dunn post-hoc analysis has been performed whenever necessary. *P* values of <.05 were considered statistically significant. The correlation between the 4 assays was calculated using the Pearson's method. Bland-Altman plots were used to graphically assess the agreement between methods by plotting the differences between the values of the 2 compared methods against the means of the values. For these analyses, test results below the LOD of each assay were arbitrarily set at half the value (ie, 3 IU dL^{-1} for VWF:RCo aggregometric, 6.25 IU dL^{-1} for VWF:RCo automated, and 2.1 IU dL^{-1} for VWF:GPIbM automated) to ensure a correct definition and comparison between the different assays. The results were reported as mean difference and 95% CI. Analysis was performed using the statistical software R, (release 3.2.3; R Foundation for Statistical Computing) and Graph Pad PRISM version 7.3.

3 | RESULTS

The results obtained for the 76 patients with type 2 VWD are summarized, along with patients' genetic variants, in Table 1. We calculated the median and IQR of the VWF activity/VWF:Ag ratio for the 4 assays in

TABLE 1 Platelet-dependent VWF activity/VWF:Ag ratios.

VWD type	VWF:Ag (*IU dL^{-1}*)	VWF:RCo aggregometric/ VWF:Ag ratio (A)	VWF:RCo automated/ VWF:Ag ratio (B)	VWF:GPIbM ELISA/VWF:Ag ratio (C)	VWF:GPIbM automated/ VWF:Ag ratio (D)	Kruskal-Wallis *P* value	Amino acid change
2A (n. 26)	29 (15-53)	0.23 (0.11-0.32)	0.22 (0.13-0.42)	0.26 (0.19-0.43)	0.31 (0.20-0.59)	A-D: $P = 0.0321$	p.Asp366Leufs*16/p.Asn528Ser (Pt. n=1); p.Ser1506Leu/WT (Pt. n=5); p.Ser1517Arg/WT (Pt. n=3); p.Arg1597Trp/WT (Pt. n=2); p.Ile1628Asn/WT (Pt. n=1); p.Ile1628Thr/WT (Pt. n=2); p.Gly1629Arg/WT (Pt. n=5); p.Gly1631Asp/WT (Pt. n=5); p.Leu1657Pro/WT (Pt. n=1); p.Val1665Glu/WT (Pt. n=1)
2M (n. 9)	18 (16-21)	0.22 (0.18-0.49)	0.35 (0.30-0.39)	0.43 (0.37-0.65)	0.60 (0.43-0.63)	All comparisons $P > 0.05$	p.Asp1277_Leu1278delinsGlu/WT (Pt. n=2); p.Asp1283His/WT (Pt. n=1); p.Arg1315Cys/WT (Pt. n=3); p.Tyr1321Cys/WT (Pt. n=3)
2M/2A (n. 12)	25 (21-32)	0.14 (0.11-0.25)	0.29 (0.22-0.32)	0.53 (0.42-0.62)	0.39 (0.28-0.51)	B-C: $P = 0.0477$ A-D: $P = 0.0098$ A-C: $P < 0.0001$	p.Arg924Gln-p.Arg1315Leu/WT (Pt. n=1); p.Arg1374Cys/WT (Pt. n=1); p.Arg1374His/WT (Pt. n=10)
2B (n. 19)	35 (25-52)	0.50 (0.44-0.61)	0.55 (0.43-0.66)	1.17 (1.08-1.26)	0.34 (0.26-0.43)	C-A: $P < 0.0001$ C-B: $P = 0.0002$ C-D: $P < 0.0001$	p.Gly1172Val/p.Arg1308Cys (Pt. n=1); p.His1268Asp/WT (Pt. n=1); p.Arg1306Trp/WT (Pt. n=8); p.Arg1308Cys/WT (Pt. n=2); p.Val1316Met/WT (Pt. n=4); p.Pro1337Leu/WT (Pt. n=1); p.Arg1341Gln/WT (Pt. n=2)
2B HMWM (n. 10)	49 (41-75)	1.04 (0.86-1.45)	1.09 (0.95-1.26)	1.18 (1.03-1.35)	0.95 (0.90-1.05)	All comparisons $P > 0.05$	[#]p.Pro1266Leu/WT (Pt. n=2); [#]p.Pro1266Leu/WT (Pt. n=2); [#]p.Pro1266Leu/WT (Pt. n=4); p.Arg1308Leu/WT (Pt. n=1); p.Ile1372Ser/WT (Pt. n=1)
Healthy subjects (n. 50)	93 (74-115)	1.00 (0.83-1.04)	0.96 (0.86-1.05)	1.17 (1.03-1.26)	0.99 (0.92-1.05)	-	-

VWD, von Willebrand disease; VWF, von Willebrand factor; GPIb, platelet receptor glycoprotein Ib; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; VWF:GPIbM, VWF gain-of-function mutant GPIb binding; HMWM, high molecular -weight multimers; Pt, patient; WT, wild-type. The ratios are reported as median and interquartile ranges for each VWD type. $P < 0.05$ was considered statistically significant. [#]Three different gene conversions were detected in type 2B HMWM, with p.Pro1266Leu being the major variant (see Supplementary Table 1).

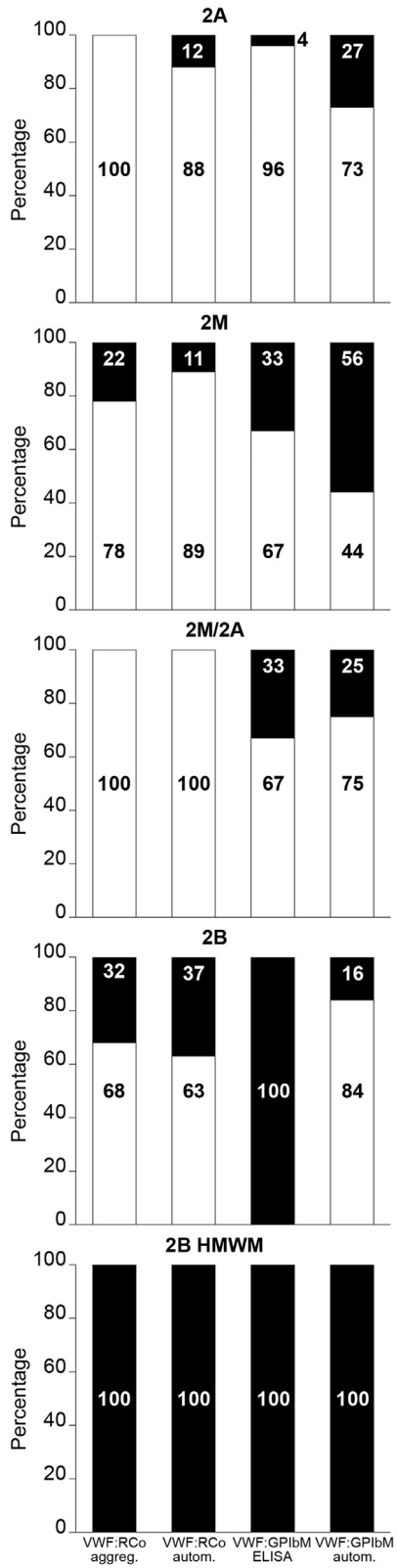

FIGURE 1 Percentage of correct diagnosis/misdiagnosis for each assay considering a cut-off value of 0.60. The white column represents the percentage of correct diagnoses, whereas the black column represents the percentage of misdiagnoses. VWF:RCo, VWF ristocetin cofactor activity; VWF:GPIbM, VWF gain-of-function mutant GPIb binding; HMWM, high-molecular-weight multimers.

the 5 VWD types. Figure 1 shows the percentage of correct type 2 diagnosis (white columns) using the VWF activity/VWF:Ag ratios for each assay, and considering a cut-off of 0.60. Type 2A and 2M/2A patients showed median ratios <0.60 with all the 4 assays, but a higher proportion of patients was misdiagnosed using the VWF:GPIbM assays (black columns). In the type 2M, the median ratios were <0.60 with all assays, with the exception of the VWF:GPIbM-automated assay (median ratio: 0.60). Using the latter, 5 of 9 patients with type 2M were misdiagnosed as type 1 (Table 2). In patients with type 2B lacking HMWM, all assays showed a median ratio <0.60, with the exception of the VWF:GPIbM ELISA (median ratio: 1.17). The latter assay was misdiagnosed as type 1 all these patients with type 2B. On the contrary, the VWF:GPIbM-automated assay was the most accurate to identify as type 2 in this group of patients. As expected, the type 2B HMWM showed a median ratio >0.60 for all assays. Pertaining to the 76 patients with VWD, the correlation between the 4 assays, calculated using Passing-Bablok regression (Figure 2), yielded a high correlation between the 2 VWF:RCo assays (Pearson's r = 0.93; Figure 2D) and between the VWF:GPIbM-automated assay and each VWF:RCo method (Pearson's r 0.93 and 0.95, respectively; Figure 2E and F). There was also a high correlation between the VWF:GPIbM ELISA and the other assays with Pearson coefficients ranging between 0.81 and 0.90 (Figure 2A, B and C). The Bland-Altman plots were employed to evaluate the differences between the 4 assays (Figure 3). The analysis of the VWF:GPIbM ELISA and VWF:GPIbM-automated assay showed 5 outliers and a mean bias of 10.41 IU dL^{-1} (95% CI: 42.07 to -21.26; Figure 3A). The distribution of the differences between the latter assays in each VWD group is shown in Figure 4. When the VWF:GPIbM ELISA was compared with the 2 VWF:RCo assays, 8 outliers were identified, with a mean bias of 9.84 IU dL^{-1} (95% CI: 41.06 to -21.4) and 9.05 IU dL^{-1} (95% CI: 32.8 to -14.7) for the VWF:RCo aggregometric and the automated assays, respectively (Figure 3B and C). The last 3 Bland-Altman analysis revealed the smallest mean bias (<1 IU dL^{-1}): 0.03 IU dL^{-1} (95% CI: 21.36 to -21.30; Figure 3D) for the 2 VWF:RCo assays (with one outlier) and 0.81 IU dL^{-1} (95% CI: 23.53 to -25.16; Figure 3E) for the VWF:GPIbM automated vs VWF:RCo aggregometric or -0.70 IU dL^{-1} (95% CI: 15 to -16.04; Figure 3F) vs VWF:RCo automated (with a total of 6 outliers). All outliers were identified in patients with type 2B lacking HMWM, with one exception, a patient with type 2B HMWM (Supplementary Table 3). This patient gave an unexplained higher value with the VWF:RCo aggregometric assay than the other assays, which was confirmed in 2 different sessions.

The 50 healthy subjects had VWF activity/VWF:Ag median ratios higher than 0.60 using the 4 different assays. The statistical analyses, employing the Passing-Bablok regression, yielded a Pearson correlation coefficient between 0.79 and 0.95. Regarding the Bland-Altman plots, a good agreement was observed notwithstanding the presence of 12 outliers (Supplementary Figures S1 and S2).

4 | DISCUSSION

In this study, we report data obtained by using 4 assay methods to measure platelet-dependent VWF activity: two VWF:RCo (an

TABLE 2 Platelet-dependent VWF activity/VWF:Ag ratios and amino acid change in patients with type 2M.

ID Patient	VWF:Ag (IU dL^{-1})	VWF:RCo aggregometric/ VWF:Ag ratio	VWF:RCo automated/ VWF:Ag ratio	VWF:GPIbM ELISA/ VWF:Ag ratio	VWF:GPIbM automated/ VWF:Ag ratio	Amino acid change
052	19	0.69	0.33	0.37	0.47	p.Asp1277_Leu1278delinsGlu/WT
063	14	0.22	0.45	0.36	0.99	p.Asp1277_Leu1278delinsGlu/WT
070	21	0.75	0.29	0.57	0.43	p.Asp1283His/WT
08	14	0.22	1.00	0.65	0.60	p.Arg1315Cys/WT
09	21	0.15	0.30	0.24	0.28	p.Arg1315Cys/WT
034	23	0.40	0.28	0.71	0.63	p.Arg1315Cys/WT
036	17	0.18	0.37	0.42	0.38	p.Tyr1321Cys/WT
076	16	0.49	0.39	0.43	0.63	p.Tyr1321Cys/WT
077	18	0.17	0.35	0.72	1.07	p.Tyr1321Cys/WT

VWD, von Willebrand disease; VWF, von Willebrand factor; GPIb, platelet receptor glycoprotein Ib; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; VWF:GPIbM, VWF gain-of-function mutant GPIb binding; WT, wild-type. Patients carrying the same type 2M variant are related.

aggregometric and an automated one) and 2 VWF:GPIbM assays (an ELISA and an automated assay). All the 5 groups of patients with VWD investigated showed reduced VWF:Ag median values, with patients with type 2M showing the lowest VWF levels. The type 2A group was the most heterogeneous because of the 11 different genetic variants, whereas almost all patients with type 2M/2A had the same variant (p.Arg1374His) [26]. The VWF activity/VWF:Ag ratio, which is crucial to distinguish VWD type 1 from most type 2 variants, was evaluated using the 4 different assays. Regarding the 26 patients with VWD type 2A, 11 (42%) showed a ratio ≥0.60 with at least one of the 4 assays. Seven of these 11 patients (27%) showed ratios ≥0.60 using the VWF:GPIbM-automated assay. Should a cut-off of 0.70 be used according to the new guidelines [16], 5 patients (19%) would be wrongly classified as type 1. Regarding the 19 patients with type 2B lacking HMWM, the median ratio was <0.60 with all assays with the exception of the VWF:GPIbM ELISA. Although the diagnosis of type 2B is based on an enhanced ristocetin-induced platelet agglutination [29], the identification of these variants as type 2 is often based on a low VWF activity/VWF:Ag ratio. Accordingly, the VWF:GPIbM-automated assay appears to be the most reliable, with 16 of 19 patients with a ratio <0.60, followed by the VWF:RCo aggregometric (13 of 19) and the VWF:RCo automated assays (12 of 19). On the other hand, the VWF:GPIbM ELISA appears to be unreliable for these type 2B cases, yielding a ratio much higher than 0.60. In the 10 patients with type 2B with HMWM, all assays showed a median ratio higher than 0.60, consistently with the results obtained in the first type 1 New York study (p.Pro1266Leu) [30]. In all the 12 patients with VWD type 2M/2A, the VWF activity/VWF:Ag ratios were lower than 0.60 with both VWF:RCo assays. On the contrary, employing the results of the 2 VWF:GPIbM assays, 7 patients would be classified as type 1, but only 2 of them would be misdiagnosed using the cut-off of 0.70. Pertaining to the 9 patients with VWD type 2M, the VWF:RCo assays appeared to be the most reliable and VWF:GPIbM assays the least reliable. In this small group of patients, the use of the VWF:GPIbM-automated assay would misdiagnose 5 of them as type 1. However, also using the VWF:RCo assays, 3 of these patients had a VWF activity/VWF:Ag ratio higher than 0.60. The inconsistencies of the results obtained in patients with the same type 2M variants may be partially explained by the markedly reduced VWF:Ag values of these patients. Nevertheless, the 2 VWF:GPIbM assays showed the highest degree of concordance among patients with type 2M, with the highest VWF activity/VWF:Ag ratios. If the cut-off of 0.70 was applied using the VWF:GPIbM-automated assay, only 2 patients with VWD type 2M would be misdiagnosed as a type 1. In the 50 healthy subjects evaluated, except for 5 individuals, a VWF activity/VWF:Ag ratio higher than 0.70 was found using all the 4 different assays. These results support the use of a cut-off ratio of 0.70, instead of 0.60, to diagnose patients with VWD type 2. Indeed, in 5 healthy subjects, ratios lower than 0.70 (between 0.66 and 0.69) were obtained, but only using the in-house VWF:RCo assay.

A general concordance was observed for the 4 different assays, although the Bland-Altman plots showed 9 outliers of whom 8 were among type 2B cases lacking HMWM. The VWF:GPIbM ELISA showed remarkably higher values in these patients in comparison with those measured with the other assays, as previously reported [28,31]. Six outliers were identified when the VWF:GPIbM-automated assay was compared to the 2 VWF:RCo assays, the values obtained for outliers using the VWF:GPIbM-automated assay were lower than those obtained with the VWF:RCo assays. Using the 2 automated assays, this trend was seen for the great majority of 2B patients, with or without HMWM. The largest discrepancy observed with the VWF:GPIbM assays for 2B patients is likely due to the lack of HMWM in some of these patients. This aspect is apparently irrelevant using the ELISA, but it seems to affect the automated assay as the agglutination of the beads cannot be mediated by 2B variants lacking HMWM. The different results obtained with the VWF:GPIbM automated and VWF:RCo assays in most 2B variants have already been reported by Boender et al. [32], suggesting that the 2 ploys used in these methods

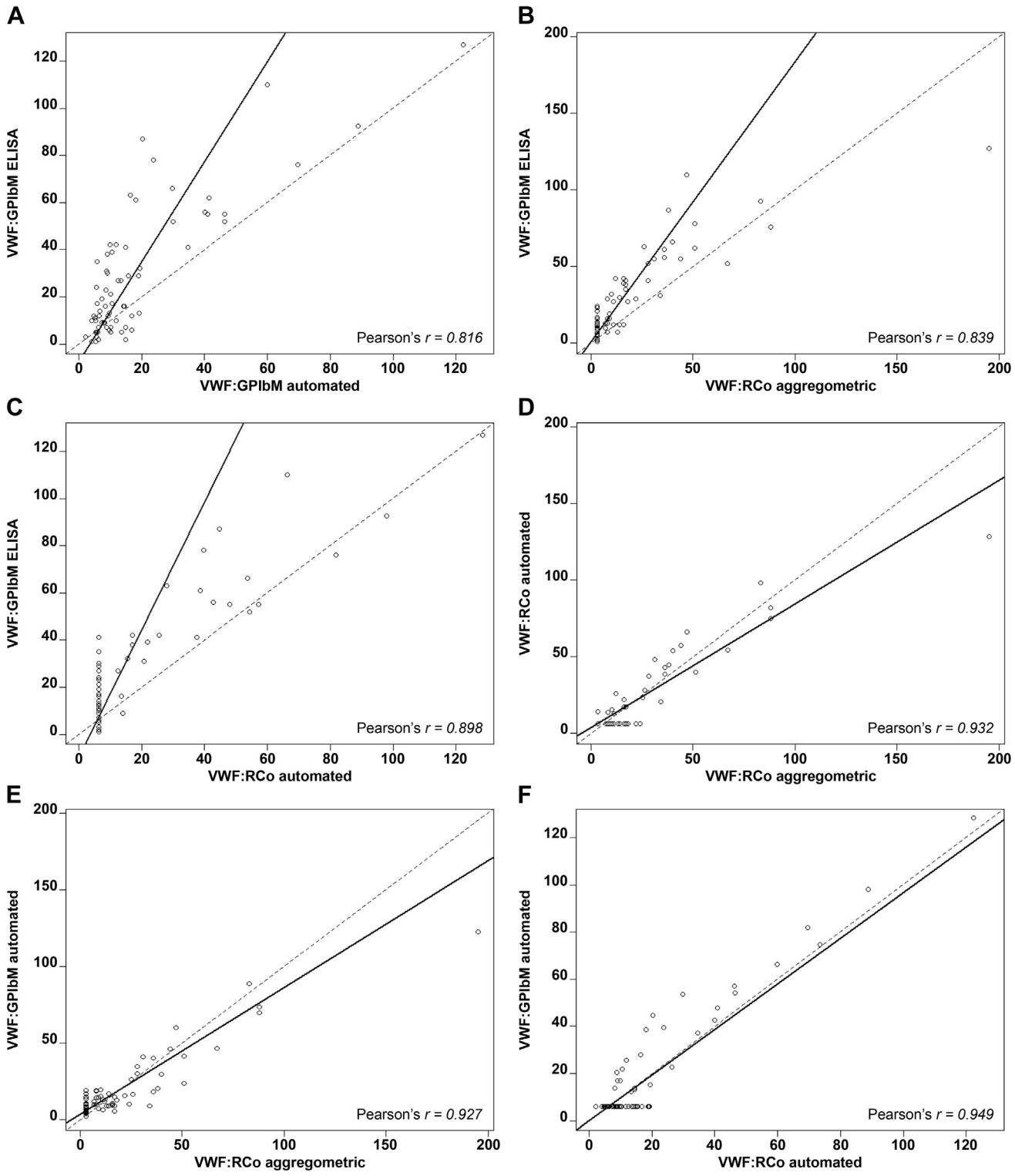

FIGURE 2 Comparison between the different assays to evaluate platelet-dependent VWF activity in type 2 patients. Correlation between assays was determined using the Pearson correlation coefficient. The slope was calculating using Passing-Bablok regression analysis. Solid line depicts the Passing-Bablok regression line. Dashed line depicts the line of identity. VWF, von Willebrand factor; VWF:Ag, VWF antigen; GPIb, platelet receptor glycoprotein Ib; VWF:RCo, VWF ristocetin cofactor activity; VWF:GPIbM, VWF gain-of-function mutant GPIb binding.

behave differently with these variants. Considering that in the ristocetin-induced platelet agglutination assay, the type 2B VWF agglutinated platelets at low ristocetin concentrations [29], we speculate that the use of ristocetin in these variants might grant an increased capacity to agglutinate platelets also for the VWF:RCo assay.

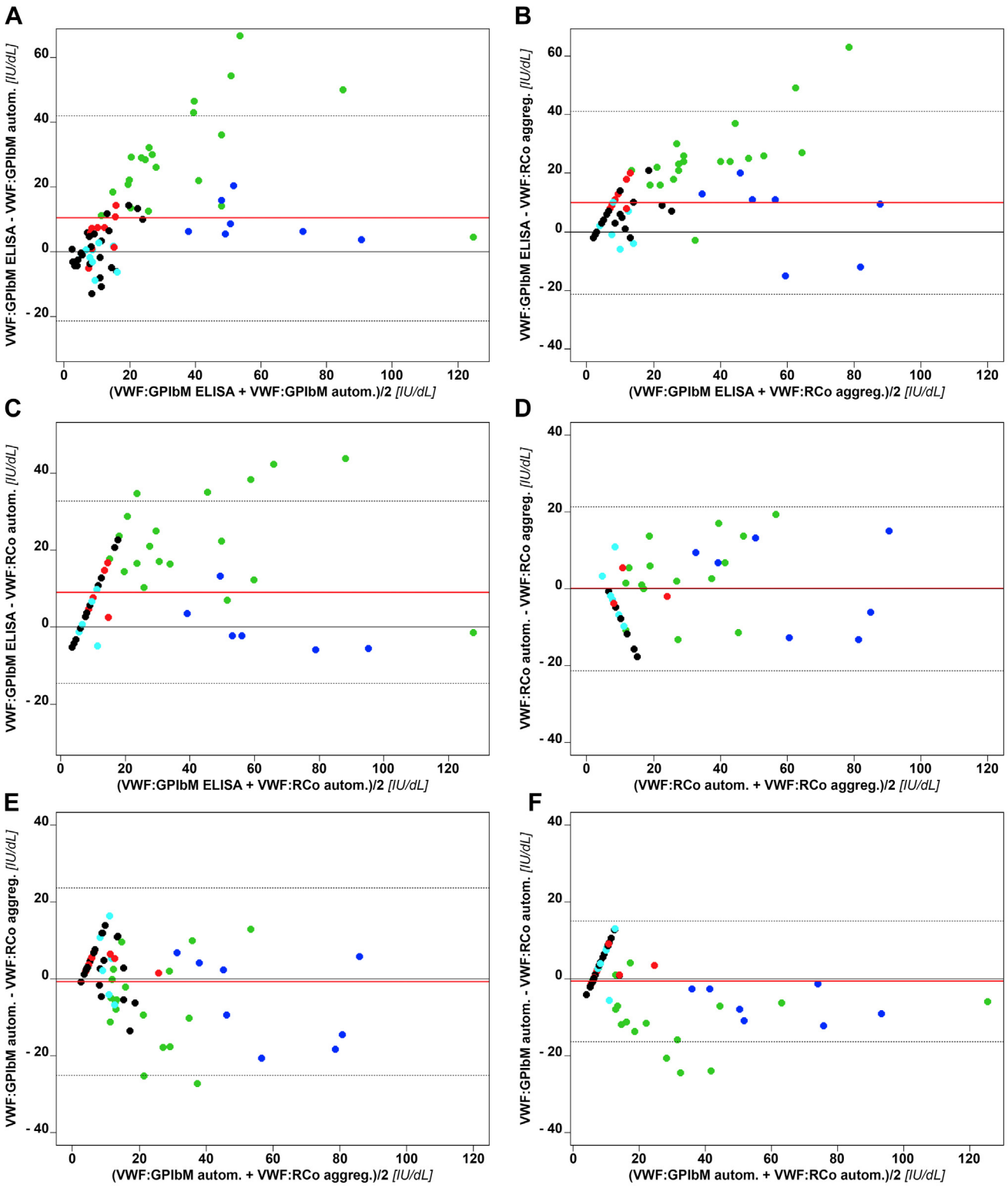

FIGURE 3 Bland-Altman plots show the differences in the assays against the means of the results. The red line, line of bias; black line, line of identity; dashed line, 95% limits of agreement between test results. Green dots, type 2B without HMWM; dark blue dots, type 2B with HMWM; light blue dots, type 2M; black dots, type 2A and red dots, type 2M/2A. An outlier was remarkably high with the VWF:RCo aggregometric assay (Supplementary Table 3) and was not reported in panels B, D and E to allow a clearer distribution of the other samples. VWF, von Willebrand factor; VWF:RCo, VWF ristocetin cofactor activity; GPIb, platelet receptor glycoprotein Ib; HMWM, high-molecular-weight multimers; VWF:GPIbM, VWF gain-of-function mutant GPIb binding.

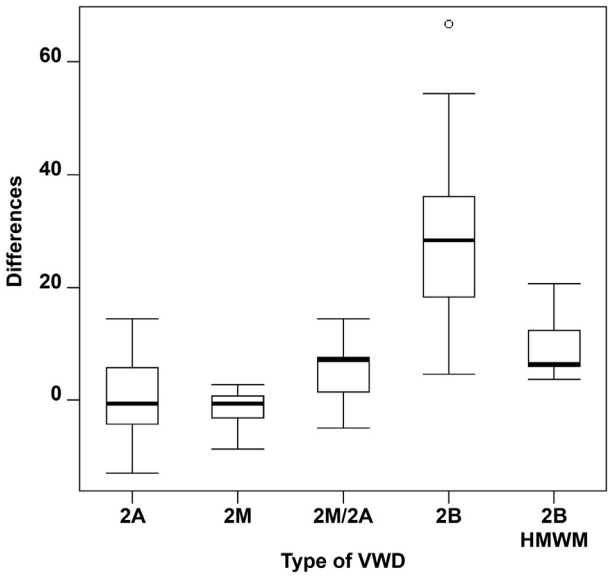

FIGURE 4 The figure represents the boxplot of distribution of difference between VWF:GPIbM ELISA and VWF:GPIbM automated in VWD types. Each boxplot represents the IQR with median value (horizontal line). The vertical bars represent 2 SD, open circle indicate outlier. VWD, von Willebrand disease; VWF, von Willebrand factor; GPIb, platelet receptor glycoprotein Ib; HMWM, high-molecular-weight multimers; VWF:GPIbM, VWF gain-of-function mutant GPIb binding.

Our study has some limitations. The first limitation consists of a small sample size. In addition, the 5 VWD groups are not equally represented, with a slightly excess of patients with type 2B and 2M/2A. The former group was enrolled to further evaluate the previously reported differences between the in-house VWF:GPIbM ELISA and the commercial VWF:GPIbM assay [31]. The latter group was selected to investigate patients more accurately with variants in the A1 domain. The second limitation is that 2 of the 4 methods analyzed are not commercially available. Nevertheless, we have considered useful to compare the 2 commercial methods with our 2 in-house assays, as they are based on the same methodological approach. Finally, we only used a single reagent lot in each assay to analyze the samples, and we did not examine the variations lot-to-lot. Therefore, the results obtained might partly reflect the specific reagent lot used.

In conclusion, this study confirms that the VWF:GPIbM ELISA shows higher results in patients with VWD type 2B lacking HMWM than the other assays. The VWF:GPIbM-automated assay is the most accurate to diagnose VWD as type 2 in those patients with VWD type 2B lacking HMWM, whereas the VWF:RCo assays are the most effective to diagnose VWD as type 2 in patients with type 2M and 2M/2A variants. The use of a cut-off value of 0.70 for the VWF activity/VWF:Ag ratio to differentiate patients with VWD type 2 from those with type 1 appears to prevent misdiagnosis in patients with type 2.

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ETHICS STATEMENT

The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico.

AUTHOR CONTRIBUTIONS

P.C. and L.B. wrote the manuscript. P.C, F.S., and G.C. performed sample biochemical characterization, M.T.P carried out part of the experiments, M.B. performed the statistical analyses and E.B. enrolled the patients. F.P. critically reviewed the manuscript. All authors approved the final version of the manuscript.

RELATIONSHIP DISCLOSURE

F.P. reports participation at educational meetings and advisory board of Grifols, Sanofi, Sobi, Takeda, Roche, and Biomarin. The other authors state that they have no conflicts of interest.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2023.100139

Update

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ERRATUM

Erratum to 'A comparative study in type 2 von Willebrand disease patients using four different platelet-dependent von Willebrand factor assays.' [Research and Practice in Thrombosis and Haemostasis Volume 7, Issue 3, March 2023, 100139]

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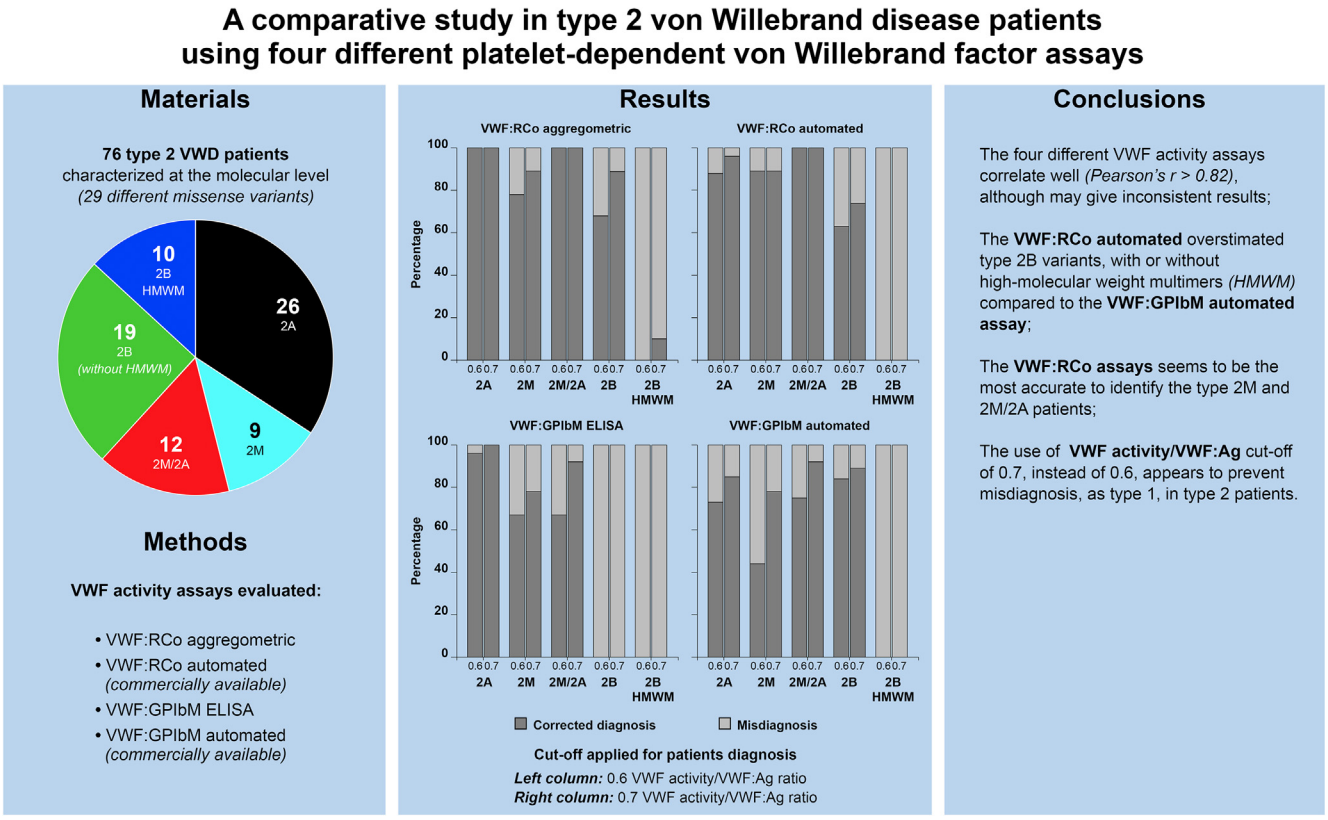

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