[haematologica]
2004;89:77-85

A sensitive ristocetin co-factor activity assay with recombinant glycoprotein Ibα for the diagnosis of patients with low von Willebrand factor levels

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A B S T R A C T

Background and Objectives. The assay of ristocetin co-factor activity of von Willebrand factor (VWF:RCo) is used in the screening of patients with suspected von Willebrand's disease (VWD), the most frequent inherited bleeding disorder. A correct diagnosis of VWD relies on platelet agglutination tests that have a low accuracy within and between assays. A more accurate VWF:RCo assay would improve VWD diagnosis and classification.

Design and Methods. We describe here an ELISA method in which a recombinant fragment of the α-subunit of platelet glycoprotein Ib-IX-V complex (rGPIbα) is bound to an anti-GPIbα monoclonal antibody immobilized onto microtiter plate wells and which captures plasma VWF in the presence of ristocetin. The results obtained with this ELISA assay were compared blindly with values calculated from the agglutination test in normal subjects (n=60) and in patients with type 1 (n=8), type 2A (n=16), type 2B (n=13), type 2M (n=17) or type 2M Vicenza (n=8) VWD characterized by low VWF levels.

Results. The two assays gave similar results in both normal subjects and VWD patients (r=0.93), but the ELISA test had a higher sensitivity (0.1 versus 6.25 U/dL). The repeatability and reproducibility of the ELISA had coefficients of variation of 9% and 10%, respectively, as compared to 14% and 15% for the agglutination test.

Interpretation and Conclusions. This ELISA assay can be useful in the identification and classification of VWD patients in that it may provide a more accurate distinction between type 2 disease with abnormal VWF function and type 1 disease with a low plasma concentration of VWF.

Key words: von Willebrand factor, von Willebrand's disease, ristocetin co-factor activity, von Willebrand factor activity, diagnosis and classification.

http://www.haematologica.org/journal/2004/1/77/

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Von Willebrand's disease (VWD), the most frequent inherited bleeding disorder, is caused by a deficiency and/or abnormality of von Willebrand factor (VWF).[1,2] The revised classification of VWD identifies two major categories, characterized by quantitative (types 1 and 3) or qualitative (type 2) VWF defects.[3] Within the first group, type 1 VWD is characterized by a partial deficiency of VWF in plasma and/or platelets with concomitantly reduced VWF activity and antigen but presence of all the high molecular weight forms observed in normal individuals, whereas in type 3 VWD there is a total absence or only trace amounts of VWF in plasma and platelets.[1-3] Type 2 VWD has been classified into four distinct subtypes that reflect different pathophysiologic mechanisms.[3] Type 2A VWD is characterized by the absence of high molecular weight VWF multimers in plasma and decreased efficiency of the functions dependent on VWF binding to the platelet glycoprotein (GP) Ib-IX-V complex. Type 2B VWD is also characterized by a loss of the largest plasma VWF multimers, but in this case as a result of an increased affinity for platelet GP Ib-IX-V complex. Type 2M VWD exhibits decreased VWF-dependent platelet functions in the presence of apparently normal VWF multimers, suggesting that is caused by mutations affecting VWF activity but not its multimeric structure and assembly. Lastly, type 2N (Normandy) VWD is characterized by a functional defect in the N-terminal region of the molecule where the binding site for coagulation factor VIII resides, with no change in the distribution of plasma VWF multimers.[3]

The ristocetin co-factor activity (VWF:RCo) assay has been widely used in the diagnosis of VWD because it reproduces

in vitro the ability of VWF to interact with one of its platelet receptor, the glycoprotein (GP) Ib-IX-V complex.[1,2] The original method for this assay was described in 1974 by MacFarlane and is based on the property of the antibiotic ristocetin to agglutinate formalin-fixed normal platelets in the presence of VWF.[4] As the most common test of VWF activity, the VWF:RCo assay, together with VWF:Ag, is traditionally considered the first step in the diagnosis of VWD types.[1,2] The use of the VWF:RCo/Ag ratio has been recommended to discriminate type 1 from type 2A, 2B and 2M VWD, as reported in the guidelines for the diagnosis and treatment of VWD in Italy. In fact, VWF:RCo values in patients with type 1 VWD and normal subjects are equivalent to those of VWF:Ag, and the resulting VWF:RCo/Ag ratio is above the normal lower limit. In contrast, levels of VWF:RCo lower than VWF:Ag, with a VWF:RCo/Ag ratio below the normal lower limit, are indicative of type 2A, 2B and 2M VWD.[5]

Since the original description, new *ex vivo* methods have been proposed to measure *VWF activity*. Several *in house* and commercial tests have been developed to assay VWF:RCo, and attempts have also been made to develop a ristocetin-independent ELISA[6] but these assays have not gained general acceptance.[7] The availability of an ELISA assay for the collagen binding activity of VWF (VWF:CB), with a sensitivity as low as 0.1 U/dL, has prompted several laboratories to use this method instead of the VWF:RCo assay, on the assumption that both tests are sensitive to the presence of the VWF multimers of largest size and can be used to discriminate between patients with normal or abnormal VWF multimeric structure.[8,9] However, assaying VWF:CB cannot in principle substitute for the measurement of VWF:RCo because only the latter directly reflects the interaction mediated by the platelet glycoprotein Ib-IX-V receptor that is essential for VWF function in hemostasis and thrombosis.[10,11]

More recently, a reliable and reproducible ELISA method to measure VWF:RCo using a recombinant fragment of the α-subunit of the GP Ib (rGPIbα), the VWF binding subunit of the GP Ib-IX-V complex, has been developed, but this test has not yet been used extensively in clinical practice and is not commercially available.[12] Here we describe a similar ELISA method in which plasma VWF binds to a different recombinant fragment of the α-subunit of the GP Ib (rGPIbα). Our results demonstrate that the sensitivity of this assay exceeds that of any currently available method for the measurement of VWF:RCo. The method can be used to discriminate type 2 VWD from previously defined type 1 VWD patients, especially when VWF:Ag levels are low, and should facilitate the diagnosis, classification and management of VWD.

Design and Methods

Plasma from VWD patients and normal controls

Blood from 60 healthy volunteers and 62 patients with VWD attending the Angelo Bianchi Bonomi Hemophilia Thrombosis Center of Milan was drawn into 1/10th volume of 3.13% sodium citrate. Patients and normal individuals gave their informed consent according to the rules of the local Institutional Review Board. Platelet-poor plasma (PPP) was prepared by centrifugation at 1,500 × g for 15 min. Plasma was stored in small aliquots at –70° C prior to testing. The VWD patients were selected from among the 484 followed at the Center according to their previous diagnosis of type 1 with low VWF:Ag levels (n=8), 2A (n=16), 2B (n=13), 2M (n=17) or 2M "Vicenza" (n=8) VWD. Criteria for these diagnoses were derived from the recommendations of the Scientific Standardization Committee on VWF of the International Society of Thrombosis and Haemostasis[3] and they were translated into a diagnostic flow chart, as recently reported in the guidelines for diagnosis and treatment of VWD in Italy.[5] In order to prove the potency of the assays to measure VWF activity, we also selected 9 patients with very low (<15 U/dL) VWF:Ag levels and defined genetic abnormalities (*see below*).

Assays for VWD diagnosis already in use: VWF:Ag-ELISA

Microtiter plates (96 wells) were coated overnight at 4°C with 125 mL/well of polyclonal anti-VWF-antibody (Dako): 1 to 1000 in 50 mM carbonate buffer, pH 9.6. After washing four times with PBS-0.01% Tween 20, the plates were blocked for 1 hour at room temperature (RT) with 250 mL/well of a 5% BSA-TBS solution. After additional washing, standard plasma pool dilutions (from 1:50 to 1:3200) and diluted test samples, all in 0.1% BSA-TBS solution, were added in duplicate to the wells and incubated for 2 hours at RT. The wells were then washed and a rabbit anti-human-VWF antibody labeled with horse radish peroxidase (HRP, 0.5 μg/mL in 0.1% BSA-TBS) was added and incubated for 1 hour at RT (Dako, Glostrup, Denmark). After a final washing, the bound antibody was quantified with a colorimetric reaction by adding the HRP substrate 1,2 *ortho*-phenylendiamine dihydrochloride (OPD) at the concentration of 400 μg/mL. The reaction was stopped with 4 mol/L H_2SO_4 and the absorbance read at 492 nm. VWF:Ag was calculated by extrapolation from the log calibration curve. The sensitivity of this *in house* VWF:Ag assay was 0.1 U/dL. The average normal value of VWF:Ag obtained in 100 normal individuals with O and non-O blood groups is 112±36 U/dL, with a range of 50-218 U/dL.

VWF:RCo by agglutination

The *in house* assay for VWF:RCo determined by platelet agglutination was derived from the original one described by MacFarlane. In this assay human formalin-fixed platelets derived from blood bank donors were used as a source of GPIbα. The test was performed in a two-channel aggregometer connected to a recorder (Chronolog). Fifty microliters of dilutions of normal or patients' plasma were added to 200 μL of formalin-fixed platelets (250×10^9/L) prepared in a siliconized glass tube containing a stirring bar. After 15-20 seconds used to calibrate the aggregometer and to record the baseline curve, 5 μL of ristocetin solution (1 mg/mL final concentration) were added into the stirring cuvette, and the maximal agglutination was determined. A standard curve with 5 dilutions of the normal plasma pool was created at the beginning and at the end of the daily assay and at least three dilutions of test plasma were used in duplicate to measure VWF:RCo. The standard curve of the normal pool was obtained by plotting, on a semilog scale, the maximum rate of agglutination, measured from the maximum slope of each agglutination trace, as a function of plasma dilution. The 1:2 dilution of the normal pool was considered as 100 U/dL and significant agglutination was usually seen with a 1:32 dilution, corresponding to 6.25 U/dL. The average normal value of VWF:RCo measured by agglutination obtained in 100 normal individuals with 0 and non-0 blood groups was 110±35 U/dL, with a range of 46-202 U/dL. The VWF:RCo/Ag ratio in this control group was 0.99±0.23, with a range of 0.63-1.75.

VWF:CB-ELISA

Microtiter plates (96 wells) were coated overnight at RT with 110 μL/well of collagen solution (25 μg/mL; 95% type I and 5% type III) previously diluted in Horm buffer (HORM-NYCOMED 7806141). After washing four times with 0.02 M TBS-0.1% Tween, the wells were blocked by over-coating with a 3% BSA-TBS solution for 1 hour at RT. After additional washing, 100 μL/well of diluted plasma samples were added. At least 7 normal pool dilutions (from 1:50 to 1:3200) were used to prepare the standard curve and 3 test sample dilutions were assayed. After 2 hours of shaking incubation at RT, the wells were washed and 100 mL of diluted HRP-conjugated anti-VWF antibody were added to each well, as described for the VWF:Ag ELISA. After an additional 1 hour incubation at RT, the wells were washed and the bound antibody was quantified with a colorimetric reaction, as described for the VWF:Ag ELISA. VWF:CB was calculated by extrapolation from a bi-log calibration curve. The sensitivity of this *in house* VWF:CB assay was 0.1 U/dL. The average value of VWF:CB obtained in 100 normal individuals with 0 and non-0 blood groups was 115±46 U/dL, with a range of 50-194 U/dL. The VWF:CB/Ag ratio in this control group was 1.03±0.26, with a range of 0.59-1.87.

New VWF:RCo ELISA test: monoclonal antibodies

The anti-GPIbα monoclonal antibody, LJ-P3, has been previously described.[13] This antibody recognizes a conformation-specific epitope present in the amino terminal domain of GPIbα (residues 1-290) and does not inhibit VWF binding to GPIbα. The reactivity of LJ-P3 with a recombinant fragment of GPIbα has been previously described.[14]

Culture of insect cells expressing recombinant GPIbα

The amino terminal domain of GPIbα was produced by stably transfected *Drosophila melanogaster* S2-cells (Invitrogen, Carlsbad, CA, USA), as described elsewhere.[15] The fragment comprises residues 1-290 of the mature protein and contains a mutation of the unpaired Cys65 residue to Ala, introduced to prevent the slow formation of dimers observed when the protein was kept in solution for prolonged periods. The atomic structure of this recombinant fragment has recently been determined by X-ray crystallography.[15] For these studies, the culture medium of transfected insect cells was used as the source of GPIbα fragment (dilutions 1:10-1:100).

Method for the VWF:RCo ELISA test

The principles of the method are described in Figure 1. An anti-GPIbα monoclonal antibody (LJ-P3) was bound to an ELISA plate to capture rGPIbα from the culture medium of transfected insect cells. The rGPIbα fragment bound to LJ-P3, in turn, captured plasma VWF in the presence of ristocetin; then, bound VWF was detected by a polyclonal anti-VWF antibody conjugated with HRP. The VWF:RCo was derived from the amount of VWF bound in the well and determined through a colorimetric reaction. In detail, microtiter plates (96 wells) were coated overnight at 4°C with 100 μL of monoclonal antibody LJ-P3 (5 μg/mL in PBS). After washing four times with PBS-0.01%-Tween 20, 100 μL of a solution containing 2-8 mg/L rGPIbα were added. After overnight incubation at 4°C, the plates were washed and blocked with 5% BSA (250 μL/well) for 1 hour at RT. After additional washing, 50 μL of standard plasma pool dilutions in PBS-0.1%-BSA-0.01% Tween 20 (from 1:100 to 1:6400) and diluted test samples were added to the wells. Plasma pool dilutions were assayed in triplicate and test samples in duplicate. At this point, 50 μL/well of 1.6 mg/mL ristocetin solution (Sigma) in PBS were added, and the

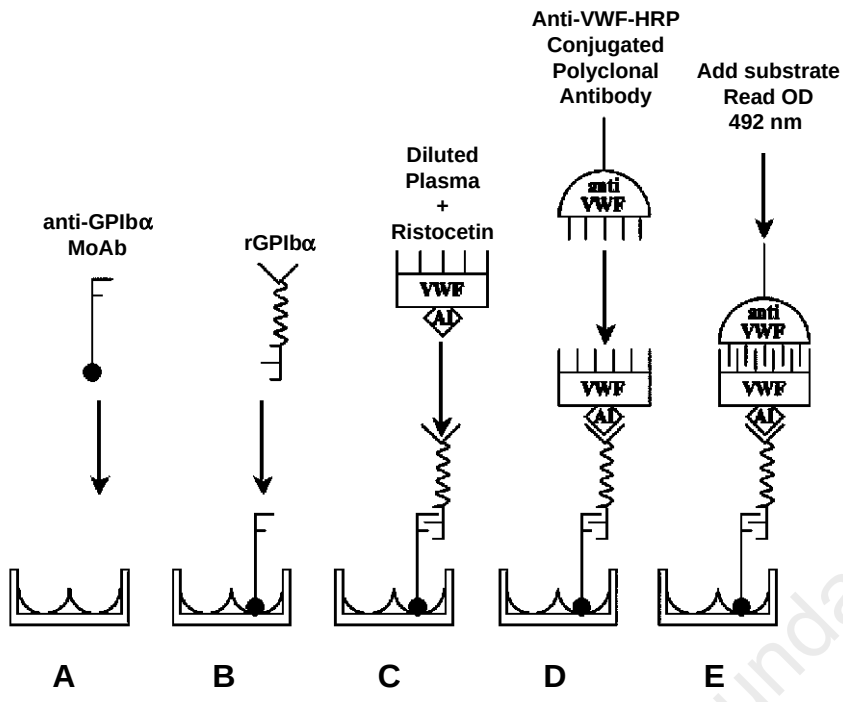

Figure 1. Schematic representation of the VWF:RCo assay by ELISA using rGPIbα. An anti-GPIbα monoclonal antibody (MoAb) is bound to an ELISA plate (A) and captures rGPIbα (residues His^{1}-Arg^{290}) present in the culture medium of transfected insect cells (B). The rGPIbα fragment bound to LJ-P3, in turn, captures plasma VWF in the presence of ristocetin (C) and the captured VWF is detected by a polyclonal anti-VWF antibody conjugated with horse radish peroxidase (D). The VWF:RCo is calculated by measuring the OD at 492 nm.

mixtures were incubated for 2 h at RT. Following this step and after washing, a rabbit anti-human-VWF antibody with conjugated HRP (Dako, Glostrup, Denmark) was added at a concentration of 0.5 mg/mL in PBS-0.1%-BSA-0.01% Tween 20 and incubated for 1 hour at RT. After additional washing, the amount of bound VWF was determined by a colorimetric reaction as described for the assay of VWF:Ag by ELISA (*see above*).

Validation of the VWF:RCo ELISA test in comparison with VWF:RCo by agglutination

The VWF:RCo ELISA was compared with the *in house* VWF:RCo performed by platelet agglutination (*see above*). The repeatability of the two assays was determined by assaying 6 replicates of 5 dilutions of a normal plasma pool in one run, and the reproducibility by assaying 6 replicates of the same plasma samples over 3 days, with the test performed by two technicians. The sensitivity was expressed as the minimal value measurable in the series of diluted standards.

Statistical analysis

Laboratory data were expressed as means with ranges. The t-test was used to compare the results obtained with the assay of VWF:RCo by agglutination and ELISA; p values < 0.05 were accepted as statistially significant.

Results

Ristocetin-induced binding of plasma VWF to rGPIbα

The binding of plasma VWF to the captured rGPIbα in the presence of various concentrations of ristocetin was tested in the assay. An optimal binding curve was obtained at a ristocetin concentration of 0.8 mg/mL with no significant differences observed at 1 mg/mL (Figure 2). Therefore, a standard concentration of 0.8 mg/mL was used to peform the VWF:RCo ELISA. The specificity of the binding of VWF to rGPIbα in the presence of ristocetin was determined by using the medium of non-transfected cells as a negative control (*data not shown*).

Assessment of VWF:RCo by ELISA

The new ELISA method was evaluated in our laboratory in a blind comparison with the previously available VWF:RCo assay. The VWF:RCo levels in a series of dilutions (1:100 to 1:3200) of a plasma pool were measured in the ELISA. By plotting optical density versus VWF:RCo activity we obtained a curve in a semi-log scale (Figure 3). The repeatability of the VWF:RCo assays by platelet agglutination and ELISA had a mean coefficient of variation (CV) of 14% and 9%, respectively; the reproducibility had a mean CV of 15% and 10%, respectively. In contrast, the sensitivity of ELISA

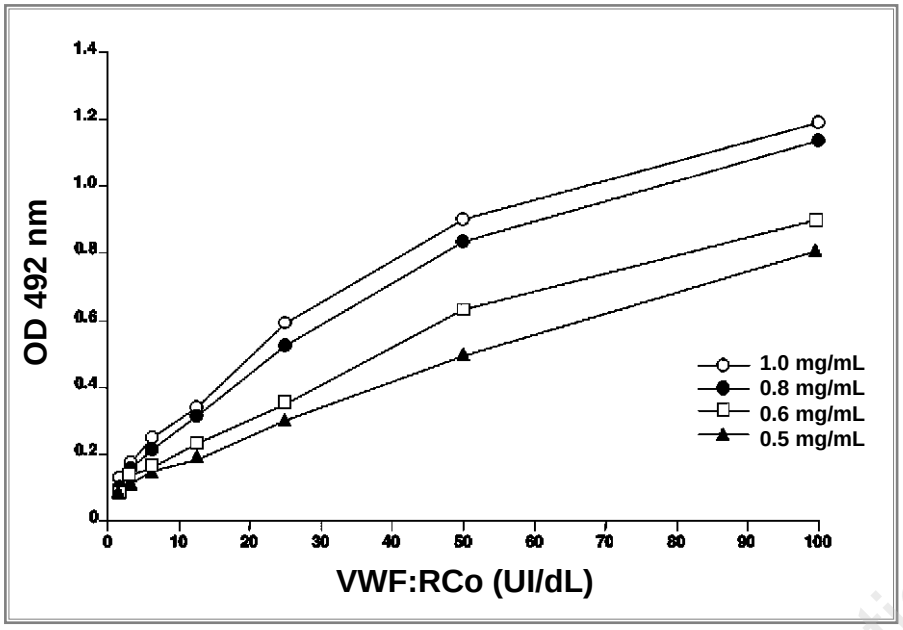

Figure 2. Ristocetin-induced binding of plasma VWF to rGPIbα. Binding of plasma VWF (dilutions from 1:32 to 1:512) to rGPIbα was studied in the presence of increasing concentrations of ristocetin: 0.5 mg/mL (closed triangles), 0.6 mg/mL (open squares), 0.8 mg/mL (closed circles) and 1 mg/mL (open circles). There was not a significant difference between the results for the 0.8 and 1 mg/mL final ristocetin concentrations.

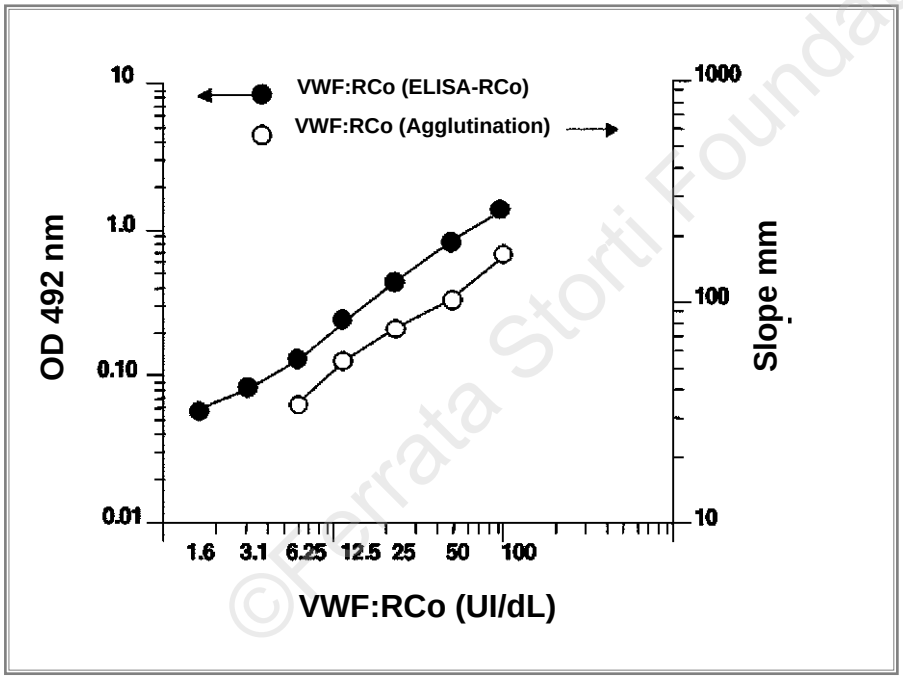

Figure 3. Sensitivity of the two VWF:RCo assays as tested by standard curves obtained with decreasing dilutions of a normal plasma pool. The log of the VWF:RCo concentrations (U/dL) in the plasma dilutions is shown on the abscissa; the log of the OD at 492 nm for the ELISA (left) or the maximum slope in the agglutination curve, for the platelet agglutination test, is shown on the ordinate. Note that the sensitivity of the ELISA test is greater than that of the agglutination test.

was much higher than that of the *in house* agglutination method: 0.1 versus 6.25 U/dL (Figure 3).

Measurements of VWF:RCo in plasma from normal individuals and from VWD patients

The mean values and ranges of VWF:RCo levels measured with the two assays in both normal individuals and in different VWD patients are reported in Table 1 and a comparison of the corresponding absolute values is presented as a scatter plot in Figure 4. In terms of absolute levels of VWF:RCo, the two assays gave similar results in both normal subjects and VWD patients (r=0.93). The VWF:RCo/Ag ratios were also calculated using the values from both assays. When the VWF:RCo level measured by agglutination was < 6 U/dL, we used the arbitrary value of 5 U/dL to perform statistical analyses and to calculate the VWF:RCo/Ag ratio. The ratios obtained with the two VWF:RCo assays were not statistically different in normal individuals and in patients with types 2A, 2B and 2M VWD, the slight differences found being probably related to the CVs of these assays. Only the two groups of patients with type 2M *Vicenza* and those with previously diagnosed type 1 *platelet low* VWD had significantly different ratio values: 0.7 and 0.4 using the

Table 1. Mean values (ranges) of VWF:RCo levels in normal individuals and in patients with different types of VWD.

Individuals tested (n)	*VWF:RCo (U/dL) Agglutination*	*VWF:RCo-(U/dL) ELISA*	*VWF:RCo/Ag Agglutination*	*VWF:RCo/Ag ELISA*
Normal subjects	106	96	0.90	0.80
(n=60)	(50-166)	(46-168)	(0.7-1.4)	(0.7-1.2)
Type 1 low	15	12	1.4	0.7(*)
(n=8)	(<6-37)	(0.5-29)	(0.7-2.5)	(0.3-1.2)
Type 2A	9	9	0.3	0.2
(n=16)	(<6-36)	(1-53)	(0.1-0.4)	(0.1-0.6)
Type 2B	21	17	0.5	0.4
(n=13)	(11-54)	(4-44)	(0.3-0.9)	(0.1-0.8)
Type 2M	11	9	0.3	0.2
(n=17)	<6-38)	(4—37)	(0.1-0.6)	(0.1-0.6)
Type "Vicenza"	< 6	3	0.7	0.4(*)
(n=8)	(<6)	(0.9-6)	(0.5 -1.0)	(0.2 - 0.7)

To calculate the mean values of VWF:RCo and the VWF:RCo/Ag ratios, VWF:RCo levels below the limit of sensitivity of the agglutination assay (<6 IU/dL) were arbitrarily assigned the values of 5 U/dL (*) $p < 0.05$.

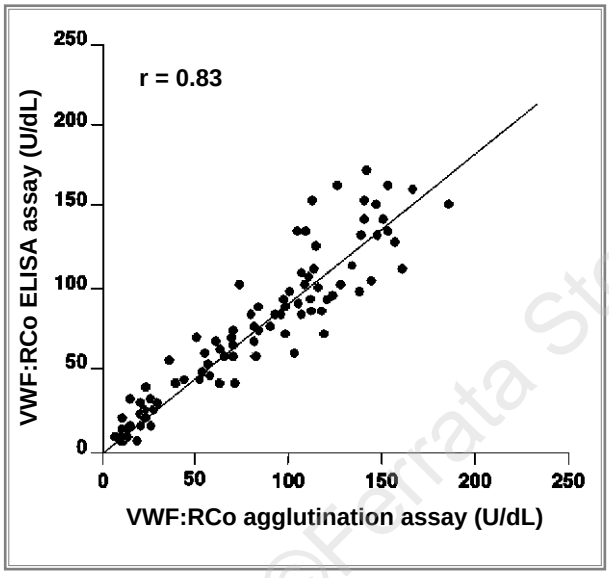

Figure 4. Scatter plot analysis comparing the plasma values of VWF:RCo (U/dL) in normal individuals and patients with VWD obtained with the agglutination (x axis) or the ELISA (y axis) assays. There is a good correlation between the two methods (r=0.93).

results of the ELISA test, versus 1.4 and 0.7 using the platelet agglutination assay (Table 1).

To explore further the potency of both VWF:RCo assays in making a correct VWD diagnosis we selected 9 patients with VWF:Ag < 15 U/dL and known mutations in the VWF gene. The data from all the available assays performed in these VWD patients are summarized in Table 2. Patients #1, 2, and 3 were originally diagnosed as having type 1 *platelet low* VWD because of low VWF levels in both plasma and platelets, a normal multimeric pattern of VWF, and reduced ristocetin-induced platelet agglutination (RIPA). Testing VWF:RCo by agglutination these patients exhibited VWF:RCo/Ag ratios of 1.5, 1.00 and 0.75, respectively, consistent with the corresponding VWF:CB/Ag ratios of 0.90, 0.83, 0.75, which suggested a diagnosis of type 1 VWD. Using the more sensitive ELISA, the VWF:RCo/Ag ratios were 0.05, 0.06, 0.10 suggesting type 2 defects. All these VWD patients had mutations in specific VWF exons (Table 2).

Patients #4, 5, and 6 were members of three different families with the well-characterized type 2M *Vicenza* VWD showing *supranormal* multimers in their plasma and the same VWF gene defect (R1205H of the entire pre-pro-VWF subunit). Using the VWF:RCo agglutination test, these patients had a VWF:RCo/Ag ratio of 1.20, 1.00 and 0.83, respectively, consistent with the VWF:CB/Ag ratios of 0.80, 1.16, 1.00, thus suggesting a diagnosis of type *1 Vicenza*. Using the ELISA, the VWF:RCo/Ag ratios were 0.24, 0.58, 0.32 suggesting type *2M Vicenza* defects of reduced VWF (Table 2).

Patients #7, 8, and 9 were members of three different families affected by type 2A and 2B VWD, with well known mutations within exon 28. Patient #9 was affected by type 2B VWD, as indicated by an enhanced RIPA. In these cases the VWF:RCo/Ag ratios evaluated by both VWF:RCo assays were always below the normal lower limit even though the values detected by ELISA were lower (Table 2). The VWF:RCo/Ag ratios were below the normal lower limit in both assays, in agreement with the VWF:CB/Ag ratios of 0.4, 0.04, 0.01, respectively, and a loss of the large molecular weight multimers, typical of type 2 VWD.

Table 2. Summary of data obtained in VWD patients (n=9) with VWF:Ag < 15 U/dL.

Test used for diagnosis (initials)	*#1 (CL)*	*#2 (TS)*	*#3 (C.E)*	*#4 (NL)*	*#5 (CD)*	*#6 (MM)*	*#7 (BA)*	*#8 (PP)*	*#9 (FM)*
VWF:Ag (U/dL)	2	3	4	5	6	6	10	11	15
VWF: RCo(U/dL)									
Agglutination	< 6	< 6	< 6	6	6	5	6	< 6	10
ELISA	0.1	0.2	0.4	1.2	3.5	1.9	3.9	2.2	6
VWF: CB (U/dL)	1.8	2.5	3	4	7	6	7	0.5	0.2
VWF:RCo/Ag (ratio)									
Agglutination	1.5	1.00	0.75	1.20	1.00	0.83	0.60	0.54	0.66
ELISA	0.05	0.06	0.10	0.24	0.58	0.32	0.39	0.20	0.40
VWF: CB/Ag (ratio)	0.90	0.83	0.75	0.80	1.16	1.00	0.4	0.04	0.01
Ristocetin Induced Platelet Agglutination (RIPA) (mg/mL)	*> 2.5*	*> 2.5*	*2.4*	*1.2*	*2.0*	*1.6*	*1.8*	*> 2.5*	*0.6*
High molecular weight multimers (low resolution gels):									
present	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
supranormal	No	No	No	Yes	Yes	Yes	No	No	No
Platelet VWF	*Low*	*Low*	*Low*	*Normal*	*Normal*	*Normal*	*Normal*	*Normal*	*Normal*
Mutation analysis:									
Found	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Candidate mutations localized in VWF-exons	31/43	32	31/43	27	27	27	28	28	28
Diagnosis of VWD type using VWF:RCo:									
Agglutination	1	1	1	1Vic	1Vic	1Vic	2A	2A	2B
ELISA	2	2M	2M	2MVic	2MVic	2MVic	2A	2A	2B

Discussion

The high variability and low sensitivity of the commonly used assays for VWF:RCo, a key parameter for the diagnosis of VWD, depend in part on differences in equipment and the reagents used for the test. The use of platelets and the inherent difficulty they pose as a reliable and consistent source of GPIbα are foremost in this problem.

The development of an ELISA for VWF:RCo has satisfactorily addressed both these issues and allowed the measurement of VWF:RCo activity in plasma with the same sensitivity as that provided by the VWF:Ag ELISA.[12] We have established an ELISA assay for VWF:RCo based on the same principles described in a previous publication[12] but with a different recombinant fragment of GPIbα and a different anti-GPIbα antibody.[13-15] We found that this VWF:RCo ELISA assay provides results similar to those of the common platelet agglutination test but with less variability and greatly increased sensitivity.

To determine the potency of this ELISA for VWF:RCo, we applied the method specifically to VWD patients with very low VWF:Ag and found that the greater sen-

sitivity of the assay allowed a more accurate diagnosis and classification of VWD.

Different VWF:RCo/Ag ratios were obtained with the two assays only in type 1 VWD with low VWF:Ag. In the other VWD types this difference was not as evident because VWF:Ag levels are usually >15 U/dL. The diagnosis with this ELISA was more accurate in VWD patients with type 1 *platelet low* (3 cases), type *Vicenza* (3 cases), 2A (2 cases) and 2B (1 case) VWD characterized by VWF:Ag levels lower or equal to 15 U/dL.

Three subgroups of type 1 VWD have been proposed in the past, depending on the content of platelet VWF.[16] The results obtained with the VWF:RCo ELISA test now indicate that VWF is dysfunctional in the so-called *platelet low* type 1 VWD, typically considered to have low levels of normal VWF, and these patients may be more properly considered as having type 2 VWD. In another subtype, type *Vicenza*, the typical phenotype is characterized by relatively low VWF levels and *supranormal* multimers in plasma.[17] Patients with *Vicenza* type VWD show at least one specific mutation, R1205H, located in exon 27 of the VWF gene.[18] Discussion is still open about whether these patients can be classified as having type 1 or 2 VWD.[19] Based on our results in the affected members of three different families, who have VWF:RCo/Ag ratios below the normal lower limit, type 2 disease seems the more appropriate diagnosis. In the case of type 2A or 2B VWD, the diagnosis typically relies on the evidence of a loss of the higher molecular weight multimers or an abnormal response to ristocetin in platelet-rich plasma, decreased in one case (type 2A) and enhanced at a lower concentration of ristocetin in the other (type 2B). Our data illustrate that, in these patients, the VWF:RCo/Ag ratios obtained by ELISA are much lower than those by the platelet agglutination test, enhancing the ability to detect the abnormal VWF.

This ELISA assay for VWF:RCo showed a sensitivity similar to that of the VWF:CB. The two assays are different in that they are based on different VWF functions, binding to collagen via the VWF A3 domain in one case and binding to GPIbα via the A1 domain in the other.[2] When VWF:CB is tested, it should be evaluated in combination with VWF:RCo, because both assays can improve the diagnosis of VWD subtypes, as previously reported.[11] In fact, specific VWF:CB defects have also been identified in the presence of normal VWF:RCo in some patients.[20] The different roles of VWF:RCo and VWF:CB in the diagnosis of VWD are also supported by the discrepant results obtained by these two assays in the small group of patients with low VWF:Ag selected here to determine the potency of the ELISA for VWF:RCo. In fact, the VWF:CB/Ag ratio was always above the normal limit but the VWF:RCo/Ag ratio was low in type 2M and *Vicenza* VWD patients who are characterized by having an apparently normal VWF multimeric structure. Based on these and previous observations, we suggest using both VWF:RCo and VWF:CB normalized with VWF:Ag in the diagnosis of VWD types and then correlating the results of these assays with the pattern derived from the analysis of VWF multimers.[11]

An updated definition of type 2M VWD is still under consideration by the Scientific Standardization Committee on VWF of the International Society of Thrombosis and Haemostasis. Some authors have suggested that patients with a low VWF:RCo/Ag ratio, decreased RIPA and normal or near normal multimeric structure are carriers of qualitatively abnormal VWF and should be classified as having type 2M VWD.[21] In our study we only evaluated a few cases with known mutations in specific VWF exons, but this issue will be further explored in the large, ongoing European study on molecular and clinical markers for diagnosis and treatment of type 1 VWD.

Type 1 VWD, with a complete set of multimers similar to that found in normal individuals, is characterized by both VWF:RCo/Ag and VWF:CB/Ag ratios similar to those in healthy subjects. Conversely, types 2A and 2B, with a relative loss of high molecular weight multimers, always show both VWF:RCo/Ag and VWF:CB/Ag ratios below normal limits. Type 2M VWD patients apparently have a full set of multimers but by definition their VWF is abnormal in its interaction with platelet GPIbα, as a result of specific mutations within the A1 domain. Thus, the VWF:CB/Ag ratio is within the normal range but the VWF:RCo/Ag is below the lower normal limit.[11] In conclusion, our data demonstrate that our ELISA assay for VWF:RCo using rGPIbα correlates well with an *in house* agglutination method but offers the advantages of lower inter- and intra-assay variability (CV<10%) and, above all, a much better sensitivity. Implementation of this assay could improve the diagnosis of VWD patients by providing a clearer distinction between type 2 (abnormal VWF) and type 1 VWD, especially when VWF antigen levels are low.

Contributions: ABF, PMM in Milan and JW, ZMR in La Jolla designed the study and interpreted of the data from the VWD patients obtained in Milan; PM developed the first assay in La Jolla by using the monoclonal antibody and the recombinant Gplba fragment available in La Jolla; MTC and IF introduced minor modifications to the assay, and performed all the assays in Milan. The authors reported no conflict of interest.

This work was supported by grant BM H4-9722 56 from the European Community (to ABF and PMM) and by grants HL-42846, HL-48728 and HL-31950 from the National Institutes of Health of the United States (to ZMR).

Received on June 25, 2003, accepted November 3, 2003.

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