Laboratory diagnosis and monitoring of desmopressin treatment of von Willebrand's disease by flow cytometry

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ABSTRACT

Background and Objectives

von Willebrand's disease (VWD) is a heterogeneous bleeding disorder caused by quantitative or qualitative defects in von Willebrand factor (VWF). The diagnosis of VWD requires several laboratory tests. The aim of our study was to validate a flow cytometric test for the diagnosis of VWD and for monitoring the effects of desmopressin therapy.

Design and Methods

Flow cytometric analysis of ristocetin-induced VWF binding to platelets was performed in platelet-rich plasma (PRP) samples from patients with VWD and from control subjects and in samples of formalin-fixed platelets in the presence of plasma from patients or controls. In 12 VWD patients the test was conducted before and 1 hour after desmopressin infusion. Results were compared with VWF:Ag, VWF:RCo, VWF:CB, RIPA, PFA-100® and the skin bleeding time.

Results

Ristocetin-induced VWF binding to platelets, evaluated by both flow cytometry-based assays, was significantly reduced in patients with type1, 2A and 2M VWD as compared with that in healthy subjects. Patients with type 2B VWD showed reduced binding of VWF to formalin-fixed platelets, but increased binding to autologous platelets in PRP, similar to RIPA. VWF binding to platelets assessed by both flow cytometric assays correlated significantly with VWF:Ag, VWF:RCo, VWF:CB, RIPA, PFA100® and bleeding time. VWF binding to platelets increased after desmopressin infusion.

Interpretation and Conclusions

The measurement of ristocetin-induced binding of VWF to platelets by flow cytometry is a sensitive, simple and rapid test for the diagnosis of VWD and for the monitoring of the effects of desmopressin therapy. The flow cytometric assay performed with autologous platelets is useful in the identification of type 2B VWD patients.

Key words: von Willebrand factor, von Willebrand's disease, flow cytometry, desmopressin, platelets.

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Von Willebrand's disease (VWD) is the most common inherited bleeding disorder and is caused by quantitative or qualitative defects in von Willebrand factor (VWF), a plasma protein essential for primary hemostasis which mediates the adhesion of platelets to the subendothelium.[1] VWD is divided into many types.[2,3] The most common is type 1 VWD, a partial quantitative defect caused by reduced secretion or accelerated clearance of VWF. Type 3 VWD is a rare quantitative disorder characterized by the complete absence of VWF. Type 2 VWD is caused by a defect in the function of VWF and is divided in different variants. Type 2A VWD is characterized by the absence of high molecular weight VWF multimers in plasma and reduced binding of VWF to the platelet glycoprotein complex Ib/IX/V (GP Ib/IX/V). In contrast, in type 2B VWD the loss of high molecular weight VWF multimers is associated with an enhanced affinity of VWF for the GPIb/IX/V complex. In type 2M VWD, the activity of the VWF is reduced, but the distribution of plasma multimers is normal. Type 2N VWD is characterized by a normal distribution of VWF plasma multimers but a reduced capacity to bind FVIII.

Due to its heterogeneity, the diagnosis of VWD is difficult and requires a panel of laboratory tests.[4,5] Screening assays comprise the activated partial thromboplastin time (aPTT), the bleeding time (BT) and platelet function analyzer (PFA-100®). Diagnostic assays include FVIII:C, VWF:Ag, VWF:RCo, and VWF:CB. Ristocetin-induced platelet aggregation (RIPA) and multimeric analysis are confirmatory tests. Some functional assays of VWF are based on an evaluation of the protein's ability to interact with the platelet GP Ib/IX/V complex in the presence of ristocetin, an antibiotic able to cause platelet agglutination through VWF. VWF:RCo, for example, measures ristocetin-induced agglutination of formalin-fixed platelets in the presence of patient's plasma, which is reduced in both quantitative and qualitative defects of VWF.

RIPA, on the other hand, measures ristocetin-induced agglutination of the patient's own platelets in platelet-rich plasma (PRP) and is essential in the diagnosis of type 2B VWD because it is the only test so far available that highlights an increased affinity of VWF for the GP Ib/IX/V complex. VWF:CB is a quantitative and qualitative assay which measures, typically by enzyme-linked immunosorbent assay (ELISA), the ability of VWF to bind collagen and is highly sensitive to the presence of high molecular weight VWF multimers.[6]

Recently many reports have focused on the development of new tests for the diagnosis of VWD[6-11] and for monitoring the effects of treatments.[6,12] Indeed, patients with VWD are often treated with desmopressin (1-deamino-8-arginine vasopressin, DDAVP), a synthetic analog of the antidiuretic hormone vasopressin, which increases the plasma levels of factor VIII (FVIII) and VWF by 3 to 5-fold.[13,14] The variability in the individual response to DDAVP does, however, mean that this treatment needs to be tested before therapeutic use, and its efficacy is monitored by measuring the above mentioned parameters before and after DDAVP infusion. In this report we describe a flow cytometric assay for the evaluation of ristocetin-induced VWF binding to platelet GPIbα and we characterize its usefulness in the diagnosis of VWD and in monitoring DDAVP treatment.

The flow cytometric assay was performed using two different approaches, one assessing the binding of VWF to platelets in the patient's autologous PRP, the other to formalin-fixed platelets in the presence of patient's plasma. Results obtained with the flow cytometric tests were compared with those observed with VWF:Ag, VWF:RCo, VWF:CB, RIPA, PFA-100® and BT assays.

Design and Methods

Materials

The VWF:RCo Kit was purchased from Helena Biosciences Europe (Sunderland, UK). VWF:Ag was determined using an Asserachrom VWF:Ag Kit, Diagnostica Stago (Asnieres, France). The VWF:CB Kit was from Gradipore Ltd. (French Forest NSW, Australia). Ristocetin was purchased from Mascia Brunelli S.p.A. (Milan, Italy). Mouse anti-human VWF antibody (clone 4f9) was purchased from Immunotech (Marseille, France). Fluoroscein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased from Beckman Coulter (Miami, FL, USA). Type V, high gelling temperature (HGT) agarose and type VII, low gelling temperature (LGT) agarose were obtained from Sigma Chemical (St Louise, MO, USA). Horseradish peroxidase (HRP) conjugated rabbit anti-human VWF was purchased from Dako (Copenhagen, Denmark). Formalin-fixed platelets, ristocetin and control plasma for the flow cytometric determination of VWF binding to fixed platelets were from Helena Biosciences Europe. Simplate II devices were purchased from Organon Teknika Corp. (Jessup, MD, USA).

Subjects

We studied 34 healthy subjects (11 men and 23 women, mean age 33.6±15.3 years, range 5 to 66) and 34 patients with VWD (15 men and 19 women, mean age 34.1±19.0 years, range 4 to 70), among whom there were 24 patients from 19 unrelated families with type 1 VWD, three patients from two unrelated families with type 2B VWD, one patient with type 2A VWD, and six patients from three unrelated families with type 2M VWD. Patients were diagnosed according to the criteria of the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis (ISTH).[3] Patients and normal subjects gave their informed consent to participation in the study.

DDAVP administration

Twelve VWD patients, 11 with type 1 VWD and one with type 2M VWD, were treated with an intravenous infusion of 0.3 μg/Kg body weight DDAVP (diluted in 50-100 mL saline). The DDAVP infusion was administered

over 30 minutes. Venous blood was withdrawn at baseline and 1 hour after the end of DDAVP infusion for the performance of all laboratory tests.

Blood drawing

Blood was collected in 3.8% sodium citrate (1:10 v/v). To minimize platelet activation during blood drawing, a 20–gauge butterfly needle was used, the tourniquet was rapidly removed and the first 2 mL of blood were discarded.

Whole blood was centrifuged at 150g or 1000g for 10 minutes to obtain PRP and platelet poor-plasma (PPP), respectively. PRP was used for the determination of RIPA and for the flow cytometric assay with autologous platelets.

Aliquots of PPP were stored at -80°C for subsequent determinations of FVIII:C, VWF:Ag, VWF:RCo, and VWF:CB for multimeric analysis and for the flow cytometric assay of VWF binding using formalin-fixed platelets.

Bleeding time test

A standardized BT test was performed, as previously described,[15] on healthy subjects and patients with VWD by the same operator throughout the study.

Briefly, with the subject lying, a sphygmomanometer cuff was applied to the upper arm and inflated to a pressure of 40 mmHg. After 1 minute, two vertical incisions were made on the volar aspect of the forearm, using an automated Simplate II device. Every 30 seconds, any blood emerging from the wound was removed with filter paper without touching the wound. The mean BT was calculated from the two cuts.

Laboratory tests

RIPA was evaluated with an optical aggregometer (APACT-4, Helena Biosciences Europe, Sunderland, UK) using 0.5 to 2.0 mg/mL of ristocetin. VWF:RCo was assessed using a commercial kit by evaluating the aggregation of control formalin-fixed platelets upon addition of 1 mg/mL of ristocetin in the presence of patients' plasma. VWF:Ag and VWF:CB were evaluated by ELISA. PFA-100® (Dade-Behring, Deerfield, IL, USA) was performed using collagen/epinephrine (C/Epi) cartridges. Multimeric analysis was performed by SDS-agarose gel electrophoresis. Type V HGT agarose was used to prepare the stacking gel (0.8%) while type VII LGT agarose was used for the running gel (1.4%). Plasma samples diluted 1:20 in 0.01M TRIS, 1mM Na_2EDTA, and 2% SDS (pH 8) were run overnight. Separated proteins were transferred onto a nitrocellulose membrane at 1.75 A and 15°C for 4 hours. The nitrocellulose membranes were saturated with 5% fat-free dried milk in phosphate-buffered saline (PBS) and incubated for 2 hours at room temperature with HRP-conjugated rabbit anti-VWF antibody. Blots were developed using the enhanced chemiluminescence assay.

Flow cytometric assay of VWF binding to platelets

Flow cytometry was used to evaluate the binding of VWF induced by ristocetin to either autologous or formalin-fixed platelets. PRP samples from patients or healthy subjects were adjusted to contain 50,000 platelets/μL by adding autologous PPP. Samples of 100 μL were incubated for 3 minutes at 37°C without stirring, with ristocetin (0, 0.75, 1, 1.5, 2, 2.5, 3 mg/mL) and subsequently treated for the flow cytometric analysis as described below.

Alternatively, 200 μL of formalin-fixed platelets (50,000/μL) were incubated for 5 minutes, without stirring, with ristocetin (1 mg/mL) in the presence of 25 μL of three different dilutions of plasma from normal individuals or patients. A standard curve was created using four scalar dilutions of a control plasma containing a known amount of VWF, and the percentage of platelets positive for VWF was plotted as a function of the plasma dilutions.

Ten microliters of the suspensions prepared as described above were fixed for 15 min with PFA 1%, then diluted 1:5 with PBS and labeled with a saturating concentration of the anti-VWF monoclonal antibody and subsequently with FITC-conjugated goat anti-mouse IgG (1:400). Samples were further diluted with 1 mL of PBS and analyzed in an EPICS XL-MCL flow cytometer (Coulter Corporation, Miami, Florida, USA). The instrument was equipped with an argon laser operating at 488 nm. FITC fluorescence was detected using a 525 nm band pass filter. Autologous or formalin-fixed platelets were identified by their light scattering characteristics. A total of 5,000 platelets was analyzed for VWF binding. A negative control consisted of a sample labeled with an isotopic control antibody.

The sensitivities of the assays were calculated as the percentage of VWD patients correctly detected by flow cytometry. Patients were first classified according to VWF:Ag, VWF:RCo, VWF:CB, RIPA and multimeric analysis. The normal values for the flow cytometric assays were calculated as the mean±2SD of the results in the healthy control group and then the percentage of patients with flow cytometric values outside the normal ranges was calculated.

Statistical analysis

Results are expressed as means ± SEM. Differences between controls and patients were analyzed by using ANOVA. A p-value less than 0.05 was considered to be statistically significant. Correlation analyses were carried out using the Spearman's test.

Results

Ristocetin-induced binding of plasma VWF to autologous platelets from healthy subjects and patients with VWD

The binding of plasma VWF to autologous platelets was evaluated by flow cytometry in healthy subjects, after stimulation with various concentrations of ristocetin. In order to avoid platelet agglutination that would

interfere with the flow cytometric analysis of samples, the test was performed, without stirring, on PRP diluted to contain 50,000 platelets/μL. Under these conditions the concentrations of ristocetin able to induce VWF binding to platelets were higher than those used in other functional tests. Soluble VWF bound to platelets in a dose-response manner, with an increase of both the percentage of positive platelets and the mean fluorescence intensity (MFI). MFI increased from 1.8±0.04 in the absence of ristocetin to 13.6±0.29 in the presence of 3 mg/mL of ristocetin (Figure 1). The increase was statistically significant starting from the concentration of 1.5 mg/mL of ristocetin ($p<0.01$ *vs* basal). Binding was never observed in the absence of ristocetin. Intra- and inter-assay coefficients of variation were 2.7 and 4.9%, respectively (n=6). Patients with type 1, 2A and 2M VWD had significantly reduced ristocetin-induced binding of VWF to autologous platelets as compared to that occurring in samples from control subjects. In contrast, patients with type 2B VWD showed increased binding of VWF to platelets (Figure 1). Table 1 summarizes the mean values and ranges of RIPA, VWF:Ag, VWF:RCo, VWF:CB and of the binding of VWF by flow cytometry, in normal subjects and in patients with different types of VWD. Sensitivity was 91.6% considering only patients with type 1 VWD and 85.3% including those with all types of VWD.

Ristocetin-induced binding of plasma VWF to formalin-fixed platelets in healthy subjects and patients with VWD

Soluble VWF bound to formalin-fixed platelets in the presence of ristocetin was detected by flow cytometry. The concentration of VWF in the tested plasma samples was derived from comparing results with a standard curve created by using a control plasma containing known amounts of VWF (Figure 2B). Intra- and inter-assay coefficients of variation were 5.6 and 7.9%, respectively (n=6). The detection limit (2SD from zero) was 2.8 U/dL. As shown in Figure 2 and Table 1, the VWF concentration measured by flow cytometry was 95.1±3.2 U/dL in healthy subjects, 34.8±3.9 U/dL in patients with type 1 VWD, 55.4±5.2 U/dL in patients with type 2B VWD, 49.7 U/dL in the patient with type 2A VWD and 41.2±7.3 U/dL in patients with type 2M VWD. Sensitivity was 91.6% considering patients with type 1 VWD and 88.2% including those with all types of VWD. Data expressed as VWF binding/VWFAg ratios (1.04±0.04 for healthy subjects, 1.22±0.06 for type 1 VWD, 0.63 for type 2A VWD, 0.96±0.03 for type 2B VWD and 0.67±0.17 for type 2M VWD) were less discriminating for the different types than VWF:RCo/VWFAg ratios (0.90±0.03, 0.96±0.09, 0.19, 0.57±0.04 and 0.35±0.03, respectively).

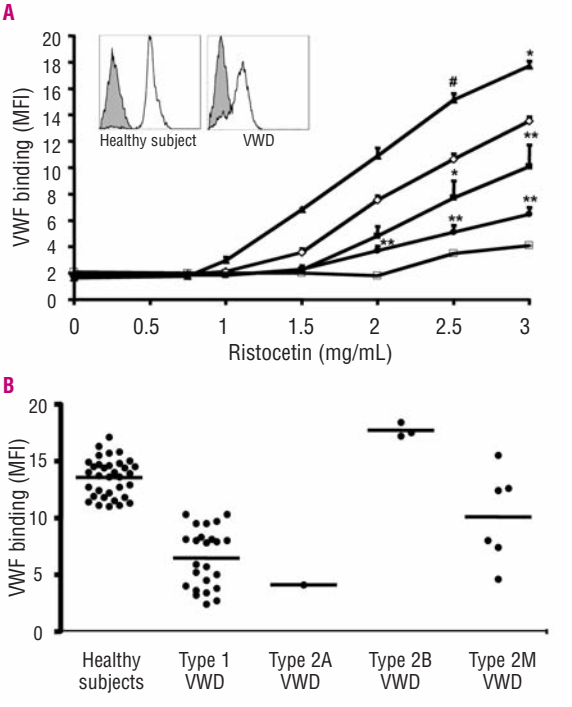

Figure 1. Ristocetin-induced binding of VWF to autologous platelets in healthy subjects and patients with different types of VWD. A. Dose-response curve to ristocetin. The insets show representative flow cytometric histograms of VWF binding to autologous platelets from a healthy subject and from a patient with VWD; filled histogram: no ristocetin; open histogram: 3 mg/mLL ristocetin. (◊: healthy subjects; •: type 1 VWD; m : type 2A VWD; G: type 2B VWD; L: type 2M VWD). B. Scatterplot of individual data points for MFI values obtained with ristocetin 3 mg/mL; #$p<0.05$; *$p<0.01$; **$p<0.001$ *vs* healthy subjects.

Table 1. Mean values and ranges of VWF:Ag, VWF:RCo, VWF:CB and VWF binding to autologous and formalin-fixed platelets in healthy subjects and patients with different types of VWD.

Individuals (n)	*RIPA (mg/mL)*	*VWF:Ag (U/dL)*	*VWF:RCo (U/dL)*	*VWF:CB (U/dL)*	*VWF binding to fixed plts (U/dL)*	*VWF binding to autologous plts (MFI)*
Healthy subjects (n=34)	1.0 (0.75-1.2)	92.7 (55.7-129.6)	82.9 (40.9-124.8)	79.0 (57.0-100.9)	95.1 (57.5-132.6)	13.6 (10.2-16.9)
Type 1 VWD (n=24)	1.4 (1-1.6)	30.1 (6.0-51.8)	24.0 (8.0-52.0)	35.1 (4.8-66.1)	34.8 (7.5-69.4)	6.5 (2.4-10.3)
Type 2A VWD (n=1)	1.4	79.1	15.0	17.5	49.7	4.1
Type 2B VWD (n=3)	0.7 (0.6-0.7)	58.4 (54.3-64.8)	33.3 (32.1-34.1)	55.6 (46.0-61.7)	55.4 (50.0-65.7)	17.7 (17.2-18.4)
Type 2M VWD (n=6)	1.3 (1-1.6)	68.0 (39.4-84.0)	23.5 (16.3-34.2)	45.6 (25.0-62.4)	41.2 (13.4-58.9)	10.1 (4.6-15.5)

Ranges are reported as mean values ±2SD for healthy subjects and as minimum and maximum values for patients with VWD.

Correlation analyses

Correlation analyses between the results of the flow cytometric assays and VWF:Ag, VWF:RCo, VWF:CB, PFA-100®, RIPA and BT were carried out including data from both healthy subjects and VWD patients. The binding of VWF to autologous and formalin-fixed platelets showed a significant correlation with VWF:Ag, VWF:RCo, VWF:CB, PFA-100®, RIPA and BT (Figures 3 and 4).

VWF binding to platelets after DDAVP infusion

After DDAVP infusion, VWF:Ag increased from 26.6±4.0 to 92.2±11.3 U/dL, VWF:RCo increased from 22.6±3.7 to 91.9±11.3 U/dL and VWF:CB from 36.7±6.7 to 77.6±7.0 U/dL. Treatment with DDAVP caused an enhancement of ristocetin-induced binding of VWF, increasing the binding to autologous platelets from 6.5±0.8 to 14.5±2.0 (MFI) and that to formalin-fixed platelets from 32.5±5.7 to 91.4±11.5 U/dL (Figure 5).

Discussion

VWD is the most common inherited bleeding disorder and can cause mucocutaneous and post-operative bleeding of variable severity. The bleeding tendency varies from mild to severe with the type and the degree of the VWF defect.[16] The heterogeneity of VWD and the limitations of the laboratory tests available make it necessary to perform a panel of different laboratory assays, including functional assays, to diagnose VWD[4] and these may require up to a week to be completed. No single test is sufficiently robust to identify definitively the different forms of VWD: for example, VWF:Ag does not detect some type 2 VWD variants while RIPA, which is important for typing VWD, is insensitive for mild quantitative deficiencies. Moreover, functional tests, such as RIPA or VWF:RCo, have other limitations including their high variability and the time the degree of technical skills required for testing. PFA-100®, which is a simple and rapid test, has a good sensitivity but a low specificity for diagnosing VWD.[6]

Flow cytometry is emerging as a useful methodology in the study of platelets and for the diagnosis of thrombocytopenia and platelet disorders.[17] Indeed many hematology or hemostasis laboratories now possess a flow cytometer and dedicated technicians.

The aim of our study was to validate a new flow cytometric method which could facilitate the diagnosis of VWD and monitoring of the efficacy of DDAVP treatment through the determination of ristocetin-induced binding of VWF to platelets. To this purpose we quantified, by flow cytometry, the binding of VWF to platelets from healthy subjects and patients with different types of VWD, using two different approaches.

One approach was aimed at assessing the binding of VWF to platelets in samples of PRP of the patient under study, using fresh autologous platelets from that subject. The other approach consisted in the quantification of VWF binding to formalin-fixed platelets using stored plasma samples from the patient under study. These assays are similar to the RIPA and VWF:RCo, respectively, with the difference that the final end-point is not platelet agglutination but the quantitative assessment of the binding of VWF to platelets by flow cytometry. One flow cytometric assay using fixed platelets was previously described by Lindahl *et al.*[11] Our data are in agreement with those reported by these authors, although our series of patients was larger and we included an evaluation of the effects of DDAVP treatment. When comparing the two different assays (direct and indirect), we found that the assay performed with native patients' platelets was more suitable.

Our results show that both flow cytometric tests are able to detect VWD because patients have altered binding of VWF to platelets as compared to that of healthy subjects.

Furthermore, flow cytometry results correlate well with the results of most currently used laboratory assays, and especially with the functional assays that are partic-

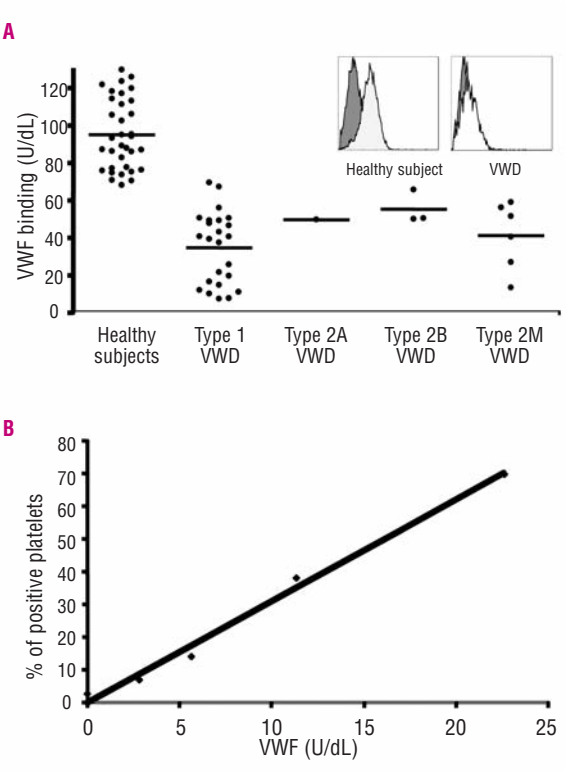

Figure 2. A. Scatterplot of individual data points for ristocetin-induced binding of VWF to formalin-fixed platelets (U/dL) determined by flow cytometry, from healthy subjects and from patients with type 1, 2A, 2B and 2M VWD. The insets show representative flow cytometric histograms of VWF binding to formalin-fixed platelets from a healthy subject and from a patient with VWD. filled histogram: no ristocetin; open histogram: 1 mg/mL ristocetin. B. Example of a standard curve created by serial dilutions of plasma with a known amount of VWF. Flow cytometric data were plotted as a function of plasma dilutions (R^2=0.99).

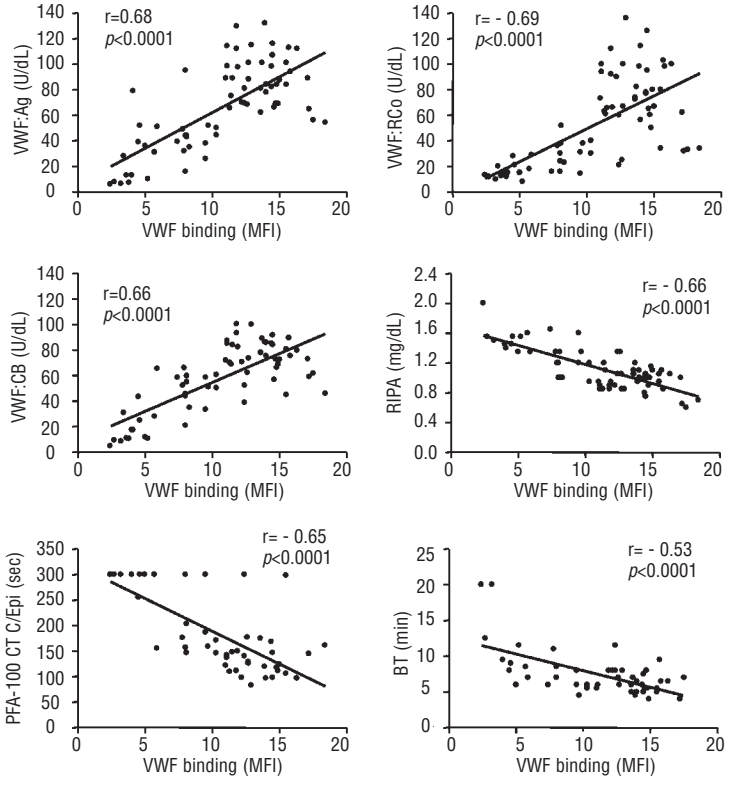

Figure 3. Correlation analyses between ristocetin-induced binding of VWF to autologous platelets and VWF:Ag, VWF:RCo, VWF:CB, RIPA, PFA-100® and BT in healthy subjects and patients with type 1, 2A, 2B and 2M VWD.

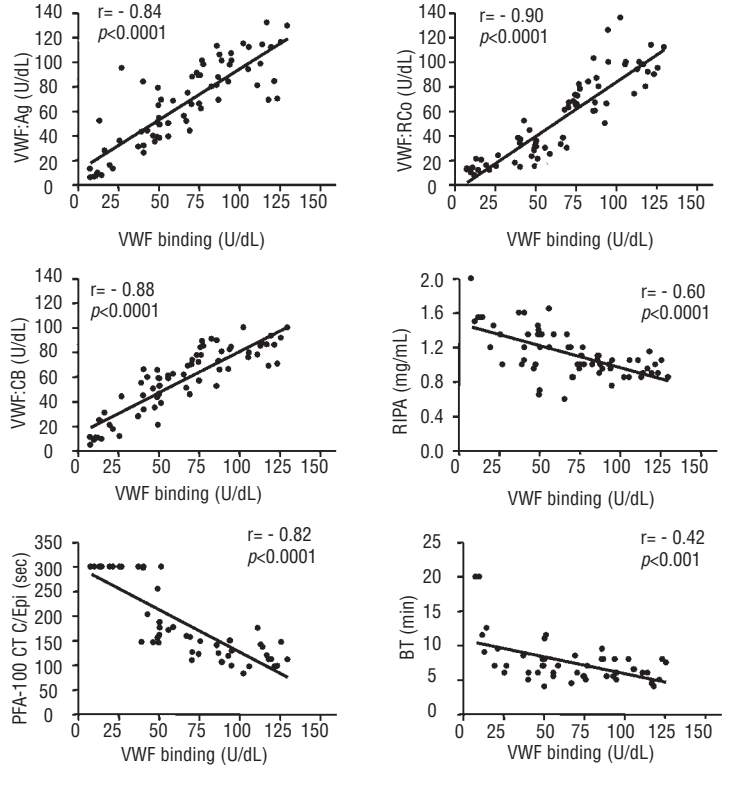

Figure 4. Correlation analyses between ristocetin-induced binding of VWF to formalin-fixed platelets and VWF:Ag, VWF:RCo, VWF:CB, RIPA, PFA-100® and BT in healthy subjects and patients with type 1, 2A, 2B and 2M VWD.

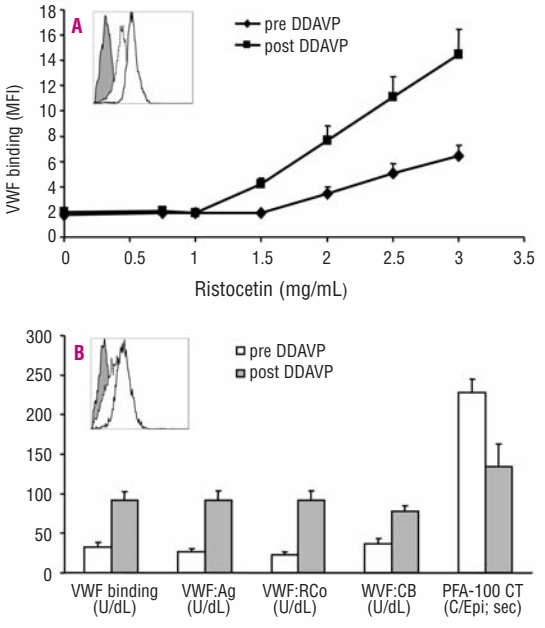

Figure 5. Ristocetin-induced binding of VWF in patients with VWD pre- and post-DDAVP infusion (n=12). Laboratory assays were performed at baseline and 1 hour after the intravenous infusion of 0.3 μg/Kg body weight of DDAVP. A. Dose-response curve of VWF binding to autologous platelets induced by ristocetin. The inset shows representative flow cytometric histograms of VWF binding to autologous platelets in the absence of ristocetin (filled histogram) or in the presence of 3 mg/mL ristocetin (open histograms) before (dotted line) and after (continuous line) an infusion of DDAVP. B. Effect of DDAVP infusion on ristocetin-induced binding of VWF to formalin-fixed platelets, VWF:Ag, VWF:RCo, VWF:CB, PFA-100®. Data represent mean±SEM (n=12). The inset shows representative flow cytometric histograms of VWF binding to formalin-fixed platelets in the absence of ristocetin (filled histogram) or in the presence of 3 mg/mL ristocetin (open histograms), before (dotted line) and after (continuous line) an infusion of DDAVP.

ularly helpful in the detection of type 2 VWF. Concerning the typing of VWD, the best results are those obtained using autologous unfixed platelets in plasma. In fact, this method allows the discrimination of type 2 VWD from type 1 VWD and healthy subjects. The test with autologous platelets is especially useful for the diagnosis of type 2B VWD because it highlights the enhanced affinity of VWF for the platelet GPIb/IX/V complex. The only other test currently performed for the detection of type 2B VWD is RIPA, a time-consuming method; thus, flow cytometry could substitute platelet aggregometry for this purpose.

The assay performed with formalin-fixed platelets is less useful for the diagnosis of type 2 VWD, because some results fall near or within the normal range. Furthermore, this assay does not highlight the enhanced affinity of VWF for the platelet GPIb/IX/V complex in type 2B VWD. The discrepancy between the direct and indirect flow cytometric tests resembles the different behaviour of RIPA and VWF:RCo in the diagnosis of type 2B VWD. VWF in type 2B VWD binds to platelets at ristocetin concentrations that are ineffective in normal plasma. In the presence of excess ristocetin, as in the standard assay for VWF:RCo, it is the overall concentration of bindable VWF that is most likely to affect the extent of platelet agglutination. On the other hand, the typical type 2B abnormality will become apparent irrespective of the VWF concentration when ristocetin becomes the main limiting factor of platelet agglutination, as in the RIPA test.[18]

Finally, flow cytometry is able to discriminate changes in plasma VWF concentration and function after treatment with DDAVP. In fact, our results show an increased binding of VWF to platelets, produced by treatment, comparable to that observed with other assays. Moreover, in one patient treated with VWF-rich factor VIII concentrate we were able to detect an increase of VWF binding to platelets by flow cytometry.

The main advantages of the flow cytometric assay are the small sample volume required and the speed of the assay, which allows several samples to be screened at the same time. The cost of the flow cytometric assays (excluding equipment) does not exceed that of the other currently used functional assays. The limitations are the requirement of trained technical personnel and the cost of the equipment; however, the ever increasing spread of this technology in many hematology and hemostasis laboratories make these limitations less relevant.

In conclusion, flow cytometry is emerging as a methodology to be included in the panel of laboratory tests used in the diagnosis and management of VWD, given its sensitivity, speed and versatility.

Authors' Contributions

PG: designed the study; SG, AMM, ML: carried out all the set of experiments; SG: prepared the manuscript; PG was involved in critically revising the manuscript and gave final approval for its submission.

Conflict of Interest

The authors reported no potential conflicts of interest.

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