Thrombin-generating capacity in patients with von Willebrand's disease

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ABSTRACT

Background and Objectives

von Willebrand's disease (VWD) is the most common hereditary bleeding disorder. Its severity can be classified on the basis of von Willebrand factor (VWF) and factor VIII (FVIII) plasma levels and according to the clinical relevance of bleeding episodes. However, patients with very low VWF activity may exhibit a mild bleeding tendency. The basis for this heterogeneous clinical expression of the deficit is still poorly understood. We investigated the relationship between thrombin generation and levels of factor VIII, VWF and clinical bleeding tendency.

Design and Methods

Thrombin generation was measured in platelet-rich (PRP) and platelet-poor plasma (PPP) from 53 patients with VWD.

Results

We observed a statistically significant higher risk of bleeding in patients with a low thrombin peak in PRP (OR=14.5; 95% CI=5–41.3). Similar results were found in PPP (OR=8.71; 95% CI=3.4–22.3). Two parameters of the thrombin generation curve, peak height and thrombin generation speed (slope), correlated significantly with VWF:RCo and FVIII levels both in PPP and in PRP. Regression analysis showed that thrombin generation was mainly dependent on plasma FVIII activity.

Interpretation and Conclusions

Our results suggest that the thrombin generation test, in combination with routine FVIII and VWF measurements, could be of interest in the assessment of the individual bleeding risk in patients with VWD.

Key words: von Willebrand's disease, factor VIII, thrombin generation assay, bleeding phenotype, von Willebrand factor.

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Von Willebrand factor (VWF) is a plasma protein that plays a critical role in platelet adhesion to the subendothelium and is also required to maintain the plasma level of factor VIII (FVIII). VWF circulates in plasma as an array of molecules ranging from small dimers to high-molecular-weight (HMW) multimers. von Willebrand's disease (VWD), the most common hereditary bleeding disorder, is due to a shortage of functional VWF. VWD is highly heterogeneous. According to the recent classification, type 1 is defined as a partial quantitative deficiency of VWF and type 3 as a complete quantitative deficiency. Type 2 comprises various qualitative abnormalities of VWF. In types 2A and 2B there is a deficit of HMW multimers, with, in type 2B, an increased affinity for platelet GPIb. Type 2M corresponds to the variants with decreased platelet function but with normal multimers. Type 2N refers to variants with a decreased affinity of VWF for FVIII.[1] The clinical expression of VWD is as variable as its pathogenesis. Type I VWD is usually mild whereas types 2 and 3 are more severe.

Mucocutaneous bleeding (epistaxis, menorrhagia) is typical of the disease. The severity of the clinical manifestation is not always correlated with the degree of reduction of VWF:RCo, or with the genetic type,

because it can be very different within the same family. Indeed, bleeding may be absent even in type 3 VWD patients and prophylaxis may be required in some type 1 VWD patients.[2,3] The thrombin generation test (TGT) is a global assay of hemostasis, whose results reflect the overall functional state of the clotting system. It renders the thrombin generation curve (TGC) that has several parameters, the most important of which are the lag-time, the peak, the ascending slope and the area under the curve (endogenous thrombin potential, ETP) (Figure 1). Al Dieri *et al.*[4] showed a correlation between the parameters of the TGT and clinical expression of disease in patients with congenital deficiencies of factors II, V, X and XI. Chantarangkul *et al.*[5] showed a significant correlation between ETP and log-transformed FVIII in a limited series of patients with hemophilia A.

Recently, our group showed a significant correlation between plasma FVIII/FIX levels and ETP, peak and time to peak. In addition, independently of the FVIII/FIX plasma level, a correlation was found between severe clinical bleeding phenotype and ETP.[6] We, therefore, surmised that, although the deficient factor may be rate-limiting in the thrombin generating system, the actual amount of thrombin formed – and therefore the clinical manifestation – could be dependent upon the spectrum of the other clotting factors. We, therefore, investigated whether the measurement of thrombin-generating capacity could contribute to the understanding of the heterogeneity of the clinical expression of VWD.

The aim of the present study was to assess the potential role of the TGT in the diagnosis and management of patients presenting with VWF deficiency and to determine whether the TGT, in combination with routine coagulation tests, could be used as an indicator of bleeding phenotype. To this end we measured thrombin generation in platelet-rich and platelet-poor plasma from 53 patients with VWD.

Design and Methods

Patients

Fifty-six consecutive patients with VWD (mean age±SD=35.4±15.4 years old) visiting the Lyon Comprehensive Hemophilia Care Center from January 2005 to October 2006 were included in the study. The institutional review board approved the study and each patient provided informed consent. Two patients with plasma levels of VWF corrected by infection and another with associated FXI deficiency were excluded. There were 17 males and 36 females; 28 patients had O blood group and 25 other blood groups. VWD was diagnosed and classified according to the clinical and biological criteria recently published by the ISTH Working party on VWD.[1,7] According to these criteria, there were 28 patients with VWD type 1. Of these 28 patients, 27 had normal multimers and one patient who did not undergo multimeric analysis, had a VWF:RCo/VWF:Ag ratio above 0.7. Two patients had type

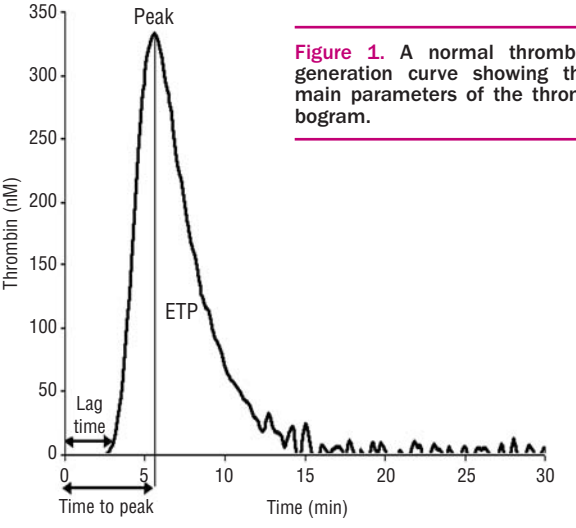


Figure 1. A normal thrombin generation curve showing the main parameters of the thrombogram.

3 VWD without multimers. Finally, 23 patients had qualitative defects of VWF. Among these patients, nine were diagnosed as having VWD type 2A, because they had significantly decreased HMW multimers. Eigtht patients were diagnosed as having VWD type 2B, because they had abnormal ristocetin-induced platelet agglutination (RIPA) with hyperresponssiveness to low concentrations of ristocetin (≤0.6 mg/mL) associated with an abnormal multimeric profile. Two patients had VWD type 2N with a markedly reduced capacity of binding for FVIII. Three patients had a combined VWD type 2A+2N, characterized by a reduced binding capacity to FVIII associated with decreased HMW multimers. Finally, one patient was diagnosed as having VWD type 2M with a VWF:RCo/VWF:Ag ratio < 0.4 and normal multimers. The phenotypic data of the patients are summarized in Table 1. A quantitative bleeding score recently published for use in type 1 VWD was used to assess the severity of bleeding in each patient.[8] The control group comprised 100 healthy volunteer blood donors between 29 and 53 years of age (mean ± SD=42.7±12 year old). There were 56 males and 44 females without a personal history of abnormal bleeding and who were not using drugs known to affect the coagulation system. Among the control group, 49 individuals had O blood group, 50 had other blood groups, and the blood type could not be determined in one control subject. We also studied 21 adult patients with mild hemophilia A who had plasma FVIII concentrations between 9-44 IU/dL.

Blood, platelet-poor plasma and platelet-rich plasma

Peripheral venous blood was collected into Vacutainer tubes (Becton Dickinson, Meylan, France) containing 0.129 mol/L trisodium citrate (1:9, v:v). Following a double centrifugation at 2500×g for 15 min at room temperature, platelet-poor plasma (PPP) was collected from the upper half of the plasma supernatant, quick frozen and stored at -80°C. An ADVIA 120 counter (Bayer Diagnostics, NY, USA) was used to check that the PPP did not contain platelets or leukocytes. Platelet-rich plasma (PRP) was

obtained by centrifugation at 150×g for 10 minutes at room temperature, with collection from the upper half of the PRP fraction. The platelet count was systematically adjusted to 150×10^9/L with autologous plasma. PPP and PRP were always prepared within 30 min after venipuncture and thrombin generation was immediately measured in fresh PRP. In order to minimize contact activation, polypropylene tubes and pipette tips were used throughout.

Phenotypic and coagulation studies

Routine diagnostic tests were carried out on PPP stored at -80°C. Plasma FVIII coagulant activity (FVIII:C) (normal range = 50-150 IU/dL) was measured by a one-stage clotting assay using a Biomérieux-deficient FVIII kit (Biomérieux, Marcy l'Etoile, France) on an MDA II instrument (Biomérieux). VWF:RCo (normal range = 50-150 IU/dL for patients with non O blood group and 40-150 IU/dL for those with O blood group) and VWF antigen (normal range = 50-150 IU/dL) were measured using BC VWF and VWF AG reagents, respectively (Dade Behring, Marburg, Germany) in a BCS Coagulation Analyzer (Dade Behring). Collagen binding capacity (VWF:CB) was measured using the enzyme-linked immunosorbent assay (ELISA) method previously described by Favoloro *et al*.[9] RIPA was measured by mixing, in an aggregometer (Chronolog Corporation, Kordia Life Science, The Netherlands), different concentrations of ristocetin (Diagnostica Stago, France) and PRP of patients. Aggregation at ≤0.6 mg/mL was one of the diagnosis criteria for VWD type 2B. The multimeric structure of plasma VWF was analyzed by electrophoresis with 0.1% sodium dodecyl sulfate and 1.5% agarose gel.[10] The capacity of plasma VWF to bind FVIII (VWF:FVIIIB) was measured as described previously by Caron *et al*.[11] Plasma fibrinogen, prothrombin and antithrombin levels were systematically measured in all patients. Prothrombin was measured using a one-stage assay with a deficient plasma kit (STA deficient II, Diagnostica Stago) (normal range = 50-150 IU/dL). Antithrombin activity was determined using a commercial Biophen Antithrombin 5 kit (Hyphen Biomed, Andrésy, France) (normal range=80-120 IU/dL). Plasma fibrinogen concentration was measured using a Fibriquick kit (Biomérieux) based on Clauss's method (normal range=1.8-4 g/L). Plasma FXI activity was determined using a deficient plasma kit (Dade Behring) (normal range = 40-120 IU/dL).

Reagents for the thrombin generation test

Recombinant human tissue factor (Innovin®) was obtained from Dade Behring and used at a final concentration of 0.5 pM in PRP and 1 pM in PPP samples. Tissue factor concentration was determined using the Actichrome® TF activity assay (American Diagnostica Inc., Greenwich, CT, USA). The phospholipid vesicles used at a final concentration of 4 µM, were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA) and consisted of 20 mol% phosphatidylserine (PS), 20 mol% phosphatidylethanolamine (PE) and 60 mol% phosphatidylcholine (PC) and were prepared by the extrusion method.[12,13] Hepes-buffered saline contained 20 mM Hepes (Sigma Aldrich, l'Ile d'Abeau Chesnes, France), 140 mM NaCl and 5 mg/mL bovine serum albumin (BSA) (Euromedex, Souffelweyersheim, France), pH 7.35. This buffer was stored at -20°C until use. A fresh mixture of fluorogenic substrate and $CaCl_2$ was prepared before each experiment. Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). The mixture of fluorogenic substrate 2.5 mM and $CaCl_2$ 0.1 M was prepared using a buffer containing Hepes 20 mM and 60 mg/mL BSA, pH 7.35. The calibrator with the activity of 600 nM human thrombin was obtained from Thrombinoscope BV (Maastricht, The Netherlands). Transparent, round-bottomed Greiner microtiter plates (Greiner ref 65204, Poitiers, France) were used. VWF concentrate was obtained from LFB (Willfactin®, LFB, Courtaboeuf, France) and recombinant FVIII concentrate (Helixate®) was obtained from ZLB Behring (Marburg, Germany).

Calibrated automated measurement of thrombin generation

Thrombin generation was measured according to the method described by Hemker *et al*.[14] The essential feature of this method is that calibration is done in a parallel sample of the same plasma as that under investigation, thus avoiding errors due to plasma color, substrate consumption, etc. A Fluoroscan Ascent® fluorometer (Thermolab systems OY, Helsinki, Finland) was used, equipped with a dispenser. Fluorescence intensity was

Table 1. VWF and FVIII measurements of the investigated patients (mean±SD).

Patients	*VWF:RCo (IU/dL)*	*VWF:Ag (IU/dL)*	*Ratio VWF:RCo/VWF:Ag*	*VWF:CB (IU/dL)*	*Ratio VWF:CB/ VWF:Ag*	*FVIII:C (IU/dL)*	*Ratio FVIII/ VWF:RCo*
VWD (n=53)	23.3±13.2	37±20	0.7±0.3	27.2±17.2	0.7 ± 0.2	50.5±24.4	2.6±1.6
VWD type 1 (n=28)	26.5±12.5	36.3±16.8	0.8±0.2	27.1±14.6	0.7 ± 0.3	56.1±22.6	2.3±0.9
VWD type 3 (n=2)	0	0	0	0	0	4.3±5	0
VWD type 2A (n=9)	14.8±7.7	36.2±18.6	0.5±0.2	13±4.6	0.5 ± 0.2	57.3±24.7	4.5±2.5
VWD type 2B (n=8)	25.6±12.8	44.6±20.8	0.6±0.3	24.5±12.3	0.5 ± 0.2	59.4±17.9	2.5±0.8
VWD type 2N (n=2)	42±7.1	52.5±6.4	0.8±0	44.5±3.5	0.8 ± 0	17±2.8	0.4±0.1
VWD type 2M (n=1)	32	87	0.4	90	1	100	3.1
VWD type 2A+2N (n=3)	13.3±3.5	34.7±38.5	0.7±0.4	11±7	0.5 ± 0.3	13±7	0.9±0.3

detected at wavelengths of 390 nm (excitation filter) and 460 nm (emission filter). Briefly, 80 µL of PPP or PRP are dispensed into the wells of round-bottomed 96-well microtiter plates. Next, 20 µL of a mixture containing tissue factor and phospholipids are added to the PPP samples and 20 µL of tissue factor added to the PRP samples. The starting reagent (20 µL per well) contains fluorogenic substrate and $CaCl_2$. A dedicated software program, Thrombinoscope® version 3.0.0.26 (Thrombinoscope BV, Maastricht, The Netherlands) enabled the calculation of thrombin activity against the calibrator (Thrombinoscope BV, Maastricht, The Netherlands) and displayed thrombin activity against time. All tests were carried out in duplicate and the measurements usually lasted 90 minutes.

The most important parameters that can be derived from calibrated automated measurement of thrombin generation (CAT) are the lag time, endogenous thrombin potential (corresponding to the area under the CAT curve), peak height of thrombin (corresponding to the maximal amount of thrombin that can be generated by the plasma sample during the thrombin burst) and time to peak (representing the time course of the thrombin generation curve up to the formation of maximal thrombin peak height). It depends upon the lag time and the slope of the thrombin generation curve. In this study, we precisely calculated the slope of the thrombin generation curve, representing the speed of thrombin generation, using the following formula:

$$slope = thrombin\ peak\ (nM) / [time\ to\ peak\ (min) - lag\ time\ (min)]$$

Data analysis

Statistical analyses were performed using Graph Pad Instat 3.0 software (San Diego, CA, USA). Spearman's test was applied for correlation analyses. Results from study groups were compared using Student-t or Mann-Whitney tests. A p value of ≤0.05 was considered statistically significant. Odds ratios (OR) were used to estimate relative risks. Binary logistic regression (program written by JC Pezzullo & KM Sullivan version 05.07.20) was applied to estimate the OR.

Results

Thrombin generation capacity in individuals with VWF deficiency

Thrombin generation was measured in PPP and PRP from 53 patients with VWD. The control group comprised 100 healthy adults. The mean value of TGT results are summarized in Table 2. Prothrombin and antithrombin levels were in the normal range in all patients and controls (normal range = 80–120%). The TGT lag time, peak, time to peak and slope were abnormal in PPP and PRP from patients with VWD. Thrombin generation was significantly decreased and delayed in these patients in comparison with the control group (Figure 2). Using a non-parametric Spearman's correlation test, we found a statistically significant correlation between plasma VWF:RCo and FVIII:C (r=0.572; p<0.0001), VWF:RCo and thrombin peak (r=0.33; p=0.015), and VWF:RCo and TG speed (slope) (r=0.41; p=0.0024) in PPP and in PRP (peak r=0.24; p=0.05 and slope r=0.29; p=0.036). We also confirmed the significant correlation previously shown between plasma FVIII:C levels and thrombin peak (r=0.55; p<0.0001) and slope (r=0.63; p<0.0001) in PPP and in PRP (peak r=0.44; p=0.0011 and slope r=0.51; p<0.0001). A correlation was also found between thrombin peak and VWF:CB in PPP (r=0.31; p=0.025) and in PRP (r=0.26; p=0.05). No statistically significant difference was found between thrombin generation peak and slope of patients with VWD type 1 and VWD type 2 (Mann Whithey test p>0.05).

Relationship between clinical bleeding phenotype and thrombin generation peak

We assessed the clinical bleeding phenotype in 53 patients with VWD according to the criteria published by the ISTH.[2,8] We studied the relationship between low thrombin generation peak values and severe bleeding risk using binary logistic regression (1=low peak, 0=normal peak and 1=severe bleeding phenotype, 0=mild bleeding phenotype). The bleeding phenotype was determined using the clinical score recently published by Tosetto *et al.*[8] This clinical score was retrospectively validated in a multicenter European study in a large number of patients with VWD, their affected and unaffected family members and controls. According to the results of this study, in 195 controls with an available bleeding history, only one female control had a bleeding score of 4, while all the remaining control subjects had a bleeding score lower than 4 with a median score of –1. The family members of VWD patients had a bleeding score higher than control subjects (median score=4) and index cases had the highest bleeding score results (median score=9). In our study, we therefore used a bleeding score ≥5 to indicate a significant risk of bleeding. In our study, 29 patients had a significant bleeding phenotype (score ≥5) whereas 24 had a mild bleeding phenotype (score <5). The normal range (mean±2SD) of the thrombin peak was determined in 100 healthy controls with no personal history of bleeding or thrombosis. A thrombin peak was considered abnormally low if it was below 188.5 nM in PPP and below 55.5 nM in PRP (mean±2SD for peak values obtained in 100 healthy subjects). The results of the binary logistic regression showed that patients whose PRP showed a low thrombin peak had a statistically significant high risk of bleeding (OR=14.5; 95% CI=5-41.3). A similar bleeding risk was also associated with a low thrombin peak in PPP samples (OR=8.71; 95% CI = 3.4-22.3). These results obtained in the first large series of VWD patients are highly relevant in terms of predicting individual bleeding risk. The mean FVIII:C levels were 48.7±21.4 IU/dL in the group of patients with a bleeding score <5 and 50.4±28 IU/dL in the group with a bleeding score ≥5.

Effect of VWF and FVIII on thrombin generation in patients with VWD

We studied the individual role of VWF and FVIII in the thrombin generation reaction and investigated whether or not the two coagulation factors had similar impacts on the capacity to generate thrombin. In the group of patients with VWD type 2, we compared thrombin peak and slope in patients with low plasma FVIII:C (<50 IU/dL) (n=14) and in those with normal FVIII:C (50 to 140 IU/dl) (n=9). In PPP, we observed a significantly decreased peak (Mann Whitney test, p=0.005) and slope in the group with FVIII:C <50%, whereas the thrombin generation peak was in the normal range in the majority of VWD patients with FVIII>50 IU/dL (Figure 3). In PRP there was no significant difference between the two groups. In addition, we compared the thrombin peak values obtained in 23 patients with VWD type 2 with those measured in 21 mild hemophiliacs with similar plasma FVIII:C (FVIII:C= 5-50%). According to our results in VWD described above, the thrombin peak in PPP was below the normal range (335±73.25 nM; mean±2SD) in all the hemophiliacs (FVIII<50 IU/dL) (Figure 3). We also evaluated the effect of reconstituting *in vitro* FVIII and VWF levels in two patients with type 3 VWD who had undetectable plasma VWF:RCo and FVIII <5 IU/dL. Thrombin generation curves were obtained in PPP and PRP samples spiked *in vitro* with VWF or with recombinant FVIII (rFVIII, Helixate®) corresponding to 25-50-100 IU/dL. In PRP and PPP, we observed that rFVIII clearly had a higher capacity of correcting thrombin generation parameters than had VWF (Figure 4). Moreover, after adding rFVIII 100 IU/dL to a PRP sample from a patient with VWD type 3, we spiked the same plasma sample with VWF 25 and 100 IU/dL, in order to investigate whether VWF had an additional role in thrombin generation in the presence of a normal plasma FVIII concentration (Figure 5A). Thrombin generation was similar before and after addition of VWF, since no significant additional effect of VWF on either the thrombin peak or speed of generation was observed. Conversely, we did the same experiment with 25 and 100 IU/dL of rFVIII added to a PRP sample from a patient with VWD type 3 in the presence of VWF 100 IU/dL (Figure 5B). In these conditions, thrombin generation dose-dependently increased in the presence of rFVIII 25 and 100 IU/dL. These results demonstrate that thrombin generation measured by CAT is mainly dependent on plasma FVIII activity. Since FVIII plays a crucial role in thrombin generation in patients with VWD, we compared the thrombin generating capacity of the two patients with VWD type 3 (VWF:Ag/Rco =0 and FVIII=5 IU/dL) with that of two patients with moderate hemophilia A (FVIII:C=6 IU/dL). The mean thrombin peak (25 *vs* 30 nM) and slope (2.7 *vs* 2.6 nM/min) values were similar in the two groups. We also compared two patients with VWD type 2 N with two mild hemophilcs (FVIII:C=15 IU/dL). We found comparable results for mean thrombin peak (101 *vs* 124 nM) and slope (22 *vs* 24 nM/min) in the two groups. Finally, *ex vivo* thrombin generation was measured in a patient with VWD type 3 before and after the infusion of a therapeutic dose of VWF 50 IU/kg (Wilfactin®, LFB,

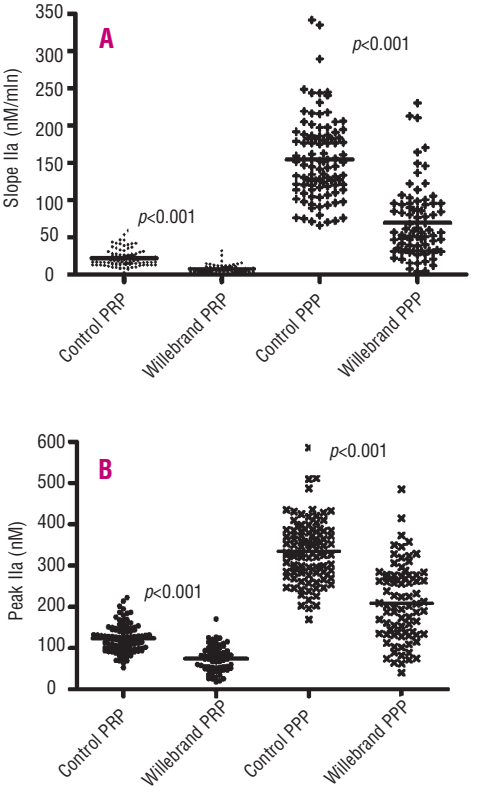


Figure 2. **Thrombin speed and peak values in patients with VWD and controls.**

Table 2. **Comparison of the thrombin-generating capacity between the investigated patients with VWD and the healthy control group.**

		Lag time (min)	*Peak (nM)*	*Time to peak (min)*	*Slope (nM/min)*	*ETP (nM.min)*
PRP	Patients with VWD (n=53)	8.5±2.4 *p*<0.0001	71±32 *p*<0.0001	21±6 *p*<0.0001	6.8±4 *p*<0.0001	1443±487 p=0.83
	Normal controls (n=100)	6.6±1.6	124±34	13±3.2	22±11	1432±229
PPP	Patients with VWD (n=53)	4.7±0.8 *p*<0.0001	195±94 *p*<0.0001	8.6±1.9 *p*=0.0004	60±40 *p*<0.0001	1255±422 p=0.0067
	Normal controls (n=100)	4±0.7	335±73	6.3±0.8	154± 54	1380±214

Courtaboeuf, France). Plasma VWF:RCo, FVIII:C and thrombin peak were measured before and 30 min, 1h, 3h, 6h and 24h after the injection of VWF concentrate. Thirty minutes after the injection, VWF:RCo was in the normal range, and then decreased, while plasma FVIII levels increased progressively throughout the 24 hours following the injection of the VWF concentrate. The kinetics of thrombin-generating capacity paralleled the increase in plasma FVIII. These *ex vivo* results confirmed our *in vitro* data showing that thrombin generation in patients with VWD was mainly dependent on plasma FVIII concentration (Figure 6).

Discussion

VWF contributes to thrombus formation through two main mechanisms. It associates with plasma FVIII, preventing the inactivation and clearance of this factor from plasma, and also mediates the adhesion of platelets to the site of vascular injury.[15] It has also been shown that VWF stimulates thrombin generation in PRP in a fibrin-dependent manner.[16] VWF deficiency is the most common inherited bleeding disorder.[17] In general, the clinical bleeding phenotype correlates with plasma VWF and FVIII concentrations and is usually milder in type 1 VWD than in types 2 and 3. However, there is no clear cut-off between a normal and a pathological level of plasma VWF concentration corresponding to the level of risk of a clinical bleeding event.[18] In addition, it is well known from clinical experience that some patients with similar degrees of VWF deficiency may exhibit variable bleeding phenotypes.[19] Thus, the levels of plasma VWF, FVIII activity and antigen do not predict the individual bleeding risk. In this study, in order to mimic physiological conditions, we measured thrombin generation using a low concentration of tissue factor (TF=1 pM in PPP and 0.5 pM in PRP) and demonstrated that thrombin generation was greatly decreased and delayed in both PPP and PRP from VWD patients. There are very limited and contradictory data on thrombin generation capacity in VWD patients. Quiroga *et al.*[20] found a significant correlation only between ETP and FVIII:C in plasma of VWD patients but they did not show a correlation between VWF and thrombin generation. In the same study they found no significant difference in thrombin generation, in terms of ETP, in PPP, between VWD patients and controls. In our study, we used thrombin peak height instead of ETP and found a highly significant difference between VWD and controls. It has been shown that at the end of the thrombin generation reaction 5-12% of the original prothrombin remains.[21] This suggests that a low thrombin peak, even at normal ETP, can be related to clinical bleeding. It was recently shown by Kyrle *et al.*[22] that a high thrombin peak is associated with a thrombotic tendency. This highlights that the ETP is not the only important parameter of the thrombogram.

Keularts *et al.*[23] showed a concentration-effect relation between VWF and the ETP measured in PRP from VWD patients. They reported that thrombin generation was decreased in PRP from two patients with moderate VWF deficiency, whereas it was normal in their PPP. Our results in PRP and PPP from VWD type 3 patients spiked with VWF and factor VIII alone and in combination confirm

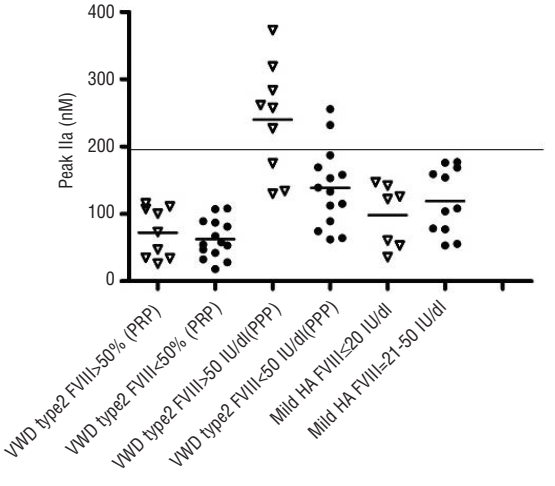
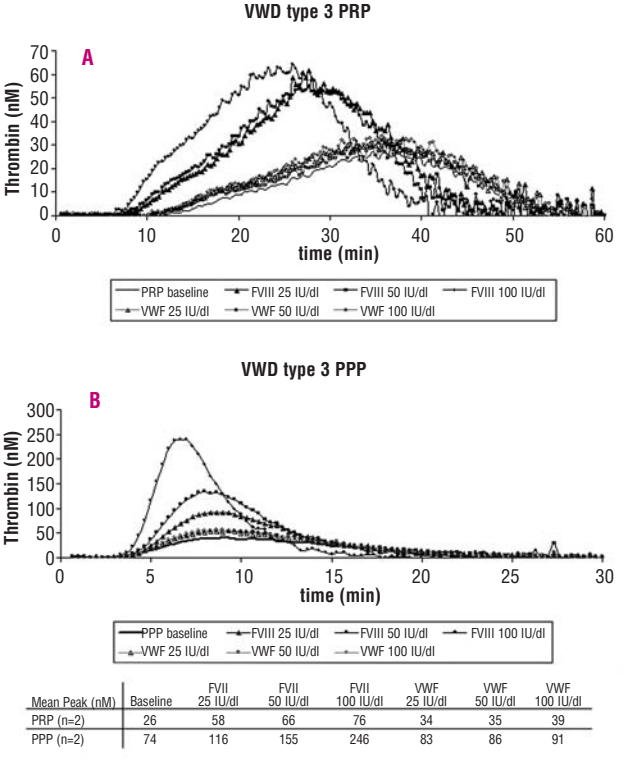


Figure 3. Comparison of thrombin peak values between patients with VWD type 2 with normal or low plasma FVIII:C activity and patients with mild hemophilia A. The horizontal line at 188.5 nM indicates the limit of the normal range (mean±2SD).



Mean Peak (nM)	Baseline	FVII 25 IU/dl	FVII 50 IU/dl	FVII 100 IU/dl	VWF 25 IU/dl	VWF 50 IU/dl	VWF 100 IU/dl
PRP (n=2)	26	58	66	76	34	35	39
PPP (n=2)	74	116	155	246	83	86	91

Figure 4. Representative thrombin generation curves obtained in PRP (A) and PPP (B) samples from patients with VWD type 3, spiked *in vitro* with VWF or with recombinant FVIII (rFVIII) 25, 50 or 100 IU/dL. At baseline FVIII and VWF were undetectable.

the decreased thrombin generation but not the additional effect of VWF in the presence of fibrin and platelets. This discrepancy could be explained by several differences between the thrombin generation methods employed. In the first place Keularts *et al.* used a subsampling method in stirred plasma i.e. at a low shear rate, whereas in the CAT method there is no stress at all. This is a major difference, because the role of VWF in the thrombin-induced procoagulant response of platelets requires at least a minimal shear, as shown by Briedé *et al.*[24] Dörmann *et al.*,[25] measuring thrombin generation on gel-filtered platelets in a system without shear stress, showed that VWF binding to GPIb was not essential to initiate platelet procoagulant activity. In the second place contact activation in the subsampling method is probably higher than in the automated method used in this study.

Our current results suggest that decreased and delayed thrombin generation in VWD can probably be mainly attributed to a decreased plasma FVIII level in patients with this disorder. This also follows from our observation that in VWD type 2 patients (9 with FVIII:C> 50 IU/dL and 14 with FVIII:C< 50 IU/dL) thrombin generation was significantly higher (p=0.005) in those with a normal plasma FVIII concentration (Figure 3). These data are also in accordance with our results obtained in patients with mild hemophilia. The individual role of VWF and FVIII in bleeds in patients with VWD is a still pending question so that the pathophysiology of bleeding in VWD remains unclear. Our *in vitro* and *ex vivo* results clearly showed that thrombin generation was mainly dependent on FVIII activity and VWF probably did not have a major role in the thrombin-generating capacity in PRP and PPP. Béguin *et al.*[16] previously showed that the addition of sufficient rFVIII significantly improved thrombin generation. However, the same authors, having added very low concentrations (2.5% of normal) of exogenous rFVIII or using normal PPP containing both FVIII and VWF, concluded that PPP had a better capacity to restore thrombin generation. In our study, the addition of either rFVIII or VWF to plasma samples allowed a more accurate evaluation of the impact of each component on thrombin generation. Conversely, the addition of VWF to a plasma sample spiked with rFVIII 100 IU/dL did not increase thrombin generation in either PRP or PPP. As discussed above, there are essential differences between the subsampling test used by Béguin *et al.* and the CAT test used in our study. Furthermore, our spiking experiments were conducted in type 3 patients, which has the advantage of detecting all-or-none effects, but would be worth repeating in a larger series, with more concentrations and with and without shear stress. In brief, the relationship between VWF/FVIII levels and bleeding has not been clearly established.

The question of how to predict bleeding risk from laboratory data still remains unanswered. It is unknown which genetic and laboratory data might be useful for predicting the individual bleeding risk in VWD patients. It is also unknown whether the presence of some bleed-

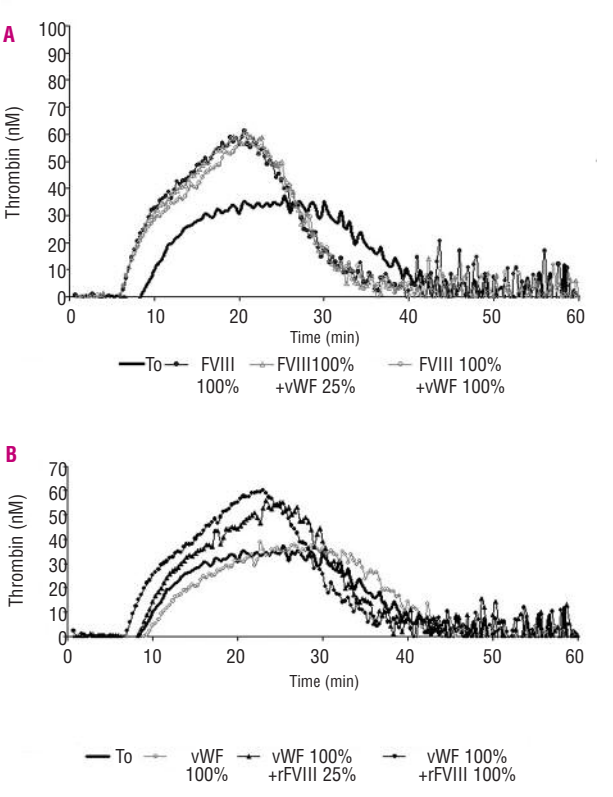


Figure 5. Representative curves showing the additional effect of VWF 25 and 100 IU/dL in a PRP sample from a patient with VWD type 3, spiked with rFVIII 100 IU/dL (A). The same experiment with 25 and 100 IU/dL of rFVIII added to a PRP sample from a patient with VWD type 3 in the presence of VWF at 100 IU/dL (B). At baseline FVIII and VWF were undetectable.

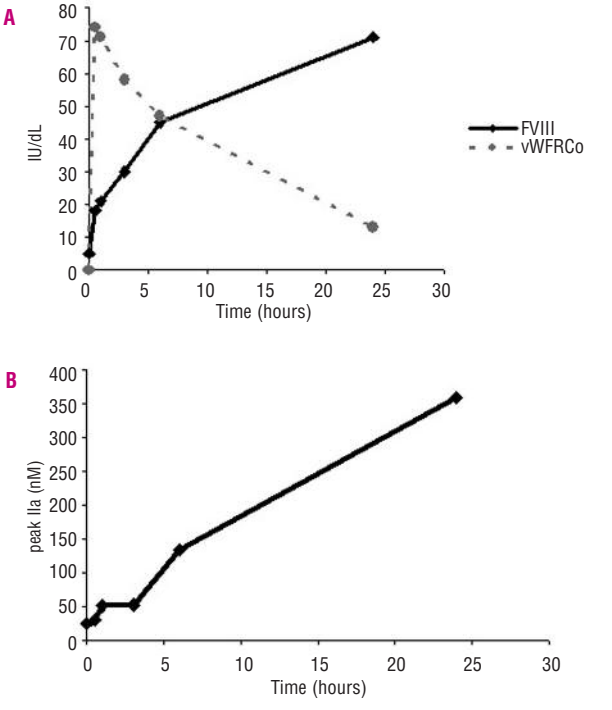


Figure 6. Pharmacokinetics of VWF:RCo, FVIII (A) and thrombin generation peak (B) in PPP from a patient with VWD type 3 before and after administration of 50 IU/kg of VWF concentrate.

ing symptoms may predict the occurrence of hemorrhages in surgical or trauma situations. Our group and others have previously shown that the thrombin generation test could be useful in the assessment of the individual bleeding risk in patients with hemophilia[6,26] and thrombophilia.[22,27] The results of the present study suggest that VWD patients with a low thrombin peak value (<55.5 nM in PRP or <188.5 pM in PPP) might have a higher risk of clinical bleeding. Our results also show a predominant role of FVIII in thrombin generating capacity of VWD. However, the thrombin generation test reflects only a part of the functions of VWF and in order to obtain a complete evaluation of the bleeding risk additional tests studying its role in platelet adhesion/aggregation are required.

Authors' Contributions

LR, SB, HCH, CN, JCB, YD were responsible for the conception and design of the study and initiated the study; LR co-ordinated the trial and participated in writing the paper; YD analyzed and interpreted the data and participated in writing the paper; JCB and BC was responsible for technical support; RF was responsible for administrative and material support; HCH, SB and CN revised the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Sadler JE, Budde U, Eikenboom JC, Favaloro EJ, Hill FG, Holmberg L, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost 2006; 4:2103-14.
2. Sadler JE, Mannucci PM, Berntorp E, Bochkov N, Boulyjenkov V, Ginsburg D, et al. Impact, diagnosis and treatment of von Willebrand disease. Thromb Haemost 2000;84:160-74.
3. Federici AB. Mild forms of von Willebrand disease: diagnosis and management. Curr Hematol Rep 2003;2:373-80
4. Al Dieri R, Peyvandi F, Santagostino E, Giansily M, Mannucci PM, Schved JF, et al. The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding. Thromb Haemost 2002;88:576-82.
5. Chantarangkul V, Clerici M, Bressi C, Giesen PL, Tripodi A. Thrombin generation assessed as endogenous thrombin potential in patients with hyper- or hypo-coagulability. Haematologica 2003;88:547-54.
6. Dargaud Y, Beguin S, Lienhart A, Al Dieri R, Trzeciak C, Bordet JC, et al. Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. Thromb Haemost 2005;93:475-80.
7. Sadler JE, Rodeghiero F, ISTH SSC subcommittee on von Willebrand factor. Thromb Haemost 2005;3: 775-7.
8. Tosetto A, Rodeghiero F, Castaman G, Goodeve A, Federici AB, Batlle J, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). J Thromb Haemost 2006;4: 766-73.
9. Favoloro EJ, Grispo l, Exner T, Koutts J. Development of a simple collagen based ELISA assay aids in the diagnosis of, and permits sensitive discrimination between type I and type II, von Willebrand's disease. Blood Coagul Fibrinol 1991;2:285-91.
10. Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. J Clin Invest 1980;65: 1318-25.
11. Caron C, Mazurier C, Goudemand J. Large experience with a factor VIII binding assay of plasma von Willebrand factor using commercial reagents. Br J Haematol 2002;117: 716-8.
12. Hedegaard E, Jensen B. Nano-scale densitometric quantitation of phospholipids. J Chromatogr 1981;225: 450-4.
13. Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim Biophys Acta 1985;812:55-65.
14. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 2003;33:4-15.
15. Mannucci PM. Treatment of von Willebrand's disease. N Engl J Med 2004; 12;351:683-94.
16. Beguin S, Kumar R, Keularts I, Seligsohn U, Coller BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. Blood 1999;93:564-70.
17. Federici AB, Berntorp E, Lee CA. The 80th anniversary of von Willebrand's disease: history, management and research. Haemophilia 2006;12:563-72.
18. Sadler JE. von Willebrand factor: two sides of a coin. J Thromb Haemost 2005;3:1702-9.
19. Castaman G, Federici AB, Rodeghiero F, Mannucci PM. von Willebrand's disease in the year 2003: towards the complete identification of gene defects for correct diagnosis and treatment. Haematologica 2003; 88:94-108.
20. Quiroga T, Goycoolea M, Giesen PL, Morales M, Munoz B, Aranda E, et al. Thrombin generation in platelet-poor plasma is normal in patients with hereditary mucocutaneous haemorrhages. Pathophysiol Haemost Thromb 2003; 33:30-5.
21. Hemker HC, Willems GM, Beguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. Thromb Haemost 1986;56:9-17.
22. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle PA. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. JAMA 2006;296:397-402.
23. Keularts IM, Hamulyak K, Hemker HC, Beguin S. The effect of DDAVP infusion on thrombin generation in platelet-rich plasma of von Willebrand type 1 and in mild haemophilia A patients. Thromb Haemost 2000;84: 638-42.
24. Briede JJ, Wielders SJ, Heemskerk JW, Baruch D, Hemker HC, Lindhout T. von Willebrand factor stimulates thrombin-induced exposure of procoagulant phospholipids on the surface of fibrin-adherent platelets. J Thromb Haemost 2003;1:559-65.
25. Dormann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. Blood 2000;96:2469-78.
26. Dargaud Y, Lienhart A, Meunier S, Hequet O, Chavanne H, Chamouard V, et al. Major surgery in a severe haemophilia A patient with high titre inhibitor: use of the thrombin generation test in the therapeutic decision. Haemophilia 2005;11:552-8.
27. Dargaud Y, Trzeciak MC, Bordet JC, Ninet J, Negrier C. Use of calibrated automated thrombinography +/- thrombomodulin to recognise the prothrombotic phenotype. Thromb Haemost 2006;96:562-7.