Journal of Thrombosis and Haemostasis, **16**: 104–115 DOI: 10.1111/jth.13881

ORIGINAL ARTICLE

Evaluation of a microfluidic flow assay to screen for von Willebrand disease and low von Willebrand factor levels

M. LEHMANN,* K. ASHWORTH,† M. MANCO-JOHNSON,† J. DI PAOLA,†‡ K. B. NEEVES*† and C. J. NG†

*Chemical and Biological Engineering, Colorado School of Mines, Golden; †Pediatrics, University of Colorado Denver; and ‡Human Medical Genetics and Genomics, University of Colorado Denver, Aurora, CO, USA

To cite this article: Lehmann M, Ashworth K, Manco-Johnson M, Di Paola J, Neeves KB, Ng CJ. Evaluation of a microfluidic flow assay to screen for von Willebrand disease and low von Willebrand factor levels. *J Thromb Haemost* 2018; **16**: 104–15.

Essentials

- von Willebrand factor (VWF) function is shear stress dependent.
- Platelet accumulation in a microfluidic assay correlates with VWF levels.
- The microfluidic assay discriminates type 1 von Willebrand disease from healthy controls.
- The microfluidic flow assay detects responses to therapeutic intervention (DDAVP).

Summary. *Background:* von Willebrand disease (VWD) is a mucocutaneous bleeding disorder with a reported prevalence of 1 in 10 000. von Willebrand factor (VWF) function and platelet adhesion are regulated by hemodynamic forces that are not integrated into most current clinical assays. *Objective:* We evaluated whether a custom microfluidic flow assay (MFA) can screen for deficiencies in VWF in patients presenting with mucocutaneous bleeding. *Methods:* Whole blood from individuals with mucocutaneous bleeding was assayed in a custom MFA. *Results:* Thirty-two patients with type 1 VWD (10/32) or reported mucocutaneous bleeding were enrolled. The platelet adhesion velocity ($r = 0.5978$ for 750 s^{-1} and 0.6895 for 1500 s^{-1}) and the maximum platelet surface area coverage ($r = 0.5719$ for 750 s^{-1} and 0.6633 for 1500 s^{-1}) in the MFA correlated with VWF levels. Furthermore, the platelet adhesion velocity at 750 s^{-1} (type 1 VWD, mean 0.0009761, 95% confidence interval [CI] 0.0003404–0.001612; control, mean 0.003587, 95% CI 0.002455–0.004719) and at 1500 s^{-1} (type 1 VWD, mean 0.0003585, 95% CI 0.00003914–0.0006778; control, mean 0.003132, 95% CI 0.001565–0.004699) differentiated type 1 VWD from controls. Maximum platelet surface area coverage at 750 s^{-1} (type 1 VWD, mean 0.1831, 95% CI 0.03816–0.3281; control, mean 0.6755, 95% CI 0.471–0.88) and at 1500 s^{-1} (type 1 VWD, mean 0.07873, 95% CI 0.01689–0.1406; control, mean 0.6432, 95% CI 0.3607–0.9257) also differentiated type 1 VWD from controls. We also observed an improvement in platelet accumulation after 1-desamino-8-D-arginine vasopressin (DDAVP) treatment at 1500 s^{-1} (pre-DDAVP, mean 0.4784, 95% CI 0.1777–0.7791; post-DDAVP, mean 0.8444, 95% CI 0.7162–0.9726). *Conclusions:* These data suggest that this approach can be used as a screening tool for VWD.

Keywords: hemorheology; hemostasis; microfluidics; von Willebrand disease; von Willebrand factor.

Correspondence: Christopher J. Ng, Department of Pediatrics, University of Colorado and Children's Hospital Colorado, MS 8302, 12800 E. 19th Ave, Aurora, CO 80045, USA
Tel.: +1 303 724 4027
E-mail: christopher.ng@ucdenver.edu

Received: 9 May 2017
Manuscript handled by: S. Kitchen
Final decision: F. R. Rosendaal, 14 October 2017

Introduction

von Willebrand disease (VWD) is an inherited bleeding disorder with a symptomatic prevalence of 1 in 10 000 individuals [1]. Both quantitative and qualitative deficiencies of von Willebrand factor (VWF) predispose individuals to mucocutaneous bleeding [1]. The most common form of the disease is type 1, which is characterized by a mild to moderate deficiency of VWF, and shows variable expressivity and incomplete penetrance [2–4]. There is also a substantial proportion of individuals with low VWF levels who do not meet the criteria for VWD but have clinically significant mucocutaneous bleeding [5]. A third category of patients presents with mucocutaneous bleeding with no abnormal laboratory findings, including normal VWF levels, and we refer to this group as having mucocutaneous bleeding of unknown explanation (MCBUE). In each of these disorders, standard clinical assays of VWF function are unable to predict clinical bleeding [6–8].

VWF function is regulated by the forces imposed on it by blood flow [9]. Thus, it has been hypothesized that assays that mimic physiologic hydrodynamic forces could be a more sensitive measure of VWF function than current clinical assays [10]. In this study, we tested this hypothesis by using a custom microfluidic flow assay (MFA) and the PFA-100 in individuals with VWD, low VWF levels, or MCBUE.

VWF function relies on shear and extensional stresses to expose its A1 domain [11], which, in turn, supports platelet adhesion and aggregation through glycoprotein (GP) 1bα [12]. However, these stresses are not incorporated in most VWF assays. In the VWF ristocetin cofactor (VWF:RCo) assay, ristocetin 'activates' VWF in the absence of shear stress [13]. The VWF collagen-binding (VWF:CB) assay measures the ability of VWF to bind to collagen under static conditions [14,15], but it is unclear whether VWF:CB correlates with clinical bleeding [15,16]. The PFA-100 is the most widely used flow-dependent assay of VWF and platelet function in the clinical setting. In a retrospective study of > 4000 patients, including 213 VWD cases, the PFA-100 outperformed standard VWF:RCo assays in screening for VWF deficiency [17]. However, the system is sensitive to other factors affecting platelet adhesion, such as platelet count, hematocrit, and antiplatelet agents. There are conflicting reports regarding its sensitivity/specificity for screening or confirming the diagnosis of type 1 VWD as compared with other mucocutaneous bleeding disorders [8,17]. In addition, the PFA-100 has a relatively limited linear correlation with VWF levels in individuals with VWD, and does not output real-time platelet adhesion values [18,19].

Flow-based assays can incorporate the hydrodynamic forces that regulate VWF function and platelet adhesion *in vivo* [20,21]. Microfluidic formats of these assays are particularly attractive for the clinical setting, owing to their low blood volume requirements and high throughput [20,22]. Specifically for VWD, early studies in annular or parallel-plate flow chambers to assess VWD showed that platelet adhesion is dependent on VWF levels and shear rate [21,23–26]. These experiments provide an essential framework for investigating the force-dependent function of VWF in VWD, but are limited by relatively small patient numbers and the inability to monitor real-time platelet adhesion rates. More recently, Nogami *et al*. used a commercial microfluidic flow system (T-TAS) to study the bleeding severity of type 1 VWD patients [27]. They reported differences across the patient cohort when they used a collagen-coated microfluidic chip, but, similarly to what occurs with the PFA-100, many patients with VWF:RCo < 10 fell outside the sensitive range of the assay.

We hypothesized that a collagen-based MFA that allows for the real-time evaluation of platelet adhesion and aggregation would correlate with VWF levels in a cohort of individuals with type 1 VWD, low VWF levels, and MCBUE. We also hypothesized that the device would be useful for differentiating those with different clinical diagnoses, and that the results would correlate with clinical bleeding phenotypes as measured with a standardized bleeding assessment tool.

Materials and methods

Materials

Type I collagen, ristocetin and ADP were from Chrono-Log (Havertown, PA, USA). Platelet aggregation was performed on a Chrono-Log Model 700 (Chrono-Log) or a PAP-8E Platelet Aggregometer (Bio/Data Corporation, Horsham, PA, USA). Calcium chloride, magnesium chloride, glutaraldehyde, 3,3′-dihexyloxacarbocyanine iodide and human placental type III collagen were from Sigma-Aldrich (St Louis, MO, USA). (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane was from Gelest (SIT8174.0; Gelest, Morrisville, PA, USA). Tubing (Tygon S-54-HL PVC Medical Tubing; internal diameter, 0.01 inches) was from Cole Parmer (Vernon Hills, IL, USA). Polydimethylsiloxane (Dow Corning Sylgard 184) was from Krayden (Westminster, CO, USA). The photoresist KMPR 1050 was from MicroChem (Newton, MA, USA). HEPES-buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH 7.4), recalcification buffer (750 mM $CaCl_2$ and 375 mM $MgCl_2$ in HBS) and bovine serum albumin buffer (BSA) (BSA buffer; 2 mg mL^{-1} BSA in HBS) were made in-house. The anti-VWF antibodies AvW-1 and 105.4 were gifts from S. Haberichter (BloodCenter of Wisconsin, Milwaukee, WI, USA). The ELISA anti-VWF antibody was from Dako (Santa Clara, CA, USA). Goat anti-rabbit horseradish peroxidase (HRP)-linked antibody was from Fisher Scientific (Hampton, NH, USA).

Study design and patient recruitment

Patients with VWD or reported mucocutaneous bleeding seen at the Hemophilia and Thrombosis Center of the University of Colorado were enrolled after informed consent had been obtained. The study was approved by the University of Colorado Institutional Review Board (COMIRB 09-0816), and was conducted in accordance with the Declaration of Helsinki. Twelve milliliters of citrated whole blood was collected. Whole blood was used for MFAs, the PFA-100, and platelet aggregation; platelet-rich plasma was used for platelet aggregometry; and platelet-poor plasma was used for VWF antigen (VWF:Ag), VWF:GP1bR, and VWF:CBIII. A standardized bleeding assessment tool (BAT) was administered by a research assistant blinded to the specific clinical diagnosis [28]. Type 1 VWD was defined by a VWF:Ag level of < 30 IU dL^{-1} and a VWF:GP1bR/VWF:Ag ratio of > 0.6. Low VWF was defined by a VWF:Ag level of 30–50 IU dL^{-1} and a VWF:GP1bR/VWF:Ag ratio of > 0.6. MCBUE was defined as reported mucocutaneous

bleeding, a VWF:Ag level of > 50 IU dL^{-1}, and no other laboratory evidence of a bleeding diathesis. For those patients who underwent treatment with intranasal 1-desamino-8-D-arginine vasopressin (DDAVP) (Stimate; CSL Behring, King of Prussia, PA, USA), research samples were collected prior to and 1 h following a clinical DDAVP-based challenge. Healthy controls aged > 18 years were recruited at the University of Colorado – Denver, and were screened for the presence of mucocutaneous bleeding symptoms.

VWF:Ag assay

VWF:Ag levels were assessed with a Stago Liatest VWF: Ag Immuno-turbidimetric assay, according to the manufacturer's instructions (Diagnostica Stago, Parsippany, NJ, USA), an ILEX/Instrumentation Laboratory HemosIL Latex enhanced immunoassay, according to the manufacturer's instructions (Ilex Medical, Lexington, MA, USA), or a VWF ELISA. For the VWF ELISA, the level of VWF in each sample was determined by ELISA with two anti-VWF mAbs for capture, i.e. AVW-1 and 105.4, as previously described [29].

VWF:GP1bR assay (VWF functional assay)

VWF:GP1bR levels were assessed with an ILEX/Instrumentation Laboratory HemosIL Latex enhanced immunoassay, according to the manufacturer's instructions [30].

VWF:CB assay

VWF binding to type III collagen was determined with a modified ELISA based on Flood *et al*. [14]. A 96-well plate was coated with 5 μg mL^{-1} type III human placental collagen, and then blocked for 1 h with 1% BSA. Samples were incubated for 1 h at room temperature and washed with phosphate-buffered saline–Tween. VWF was detected with a rabbit anti-human VWF antibody and an HRP-conjugated goat anti-rabbit antibody. Concentrations were calculated by comparing samples with a known range of pooled normal plasma (PNP) dilutions on the same 96-well plate, where the VWF concentration of undiluted PNP was assumed to be 1 U mL^{-1}. VWF:CB ratios were determined by dividing the VWF:CB result by the VWF:Ag result of the same sample.

Platelet aggregation

Platelet aggregation in response to collagen (1 μg mL^{-1} and 5 μg mL^{-1}), ristocetin (1 mg mL^{-1}) and ADP (10 μM) was determined. Platelet aggregation profiles were used to screen individuals for platelet dysfunction. Patients with abnormal platelet aggregometry findings were then excluded from further analysis. Platelet dysfunction was defined as < 50% of control sample aggregation in response to a minimum of two agonists, excluding ristocetin.

MFA

Type I collagen (Chronolog) (500 μg L^{-1}) was patterned into a 250-μm-wide strip by use of a microfluidic channel vacuum-bonded to a glass slide, incubated for 1 h at 30 °C, and then stored at 4 °C up to 3 days [31,32]. A vacuum-bonded microfluidic flow chamber was used according to previous reports [33], and consists of three channels with a width of 500 μm, a height of 50 μm, and a length of 11 mm. Prior to use, citrated whole blood was incubated at 37 °C for 15 min [34], and was then recalcified to 7.50 mM $CaCl_2$ and 3.75 mM $MgCl_2$. Samples were perfused at wall shear rates of 150, 750 and 1500 s^{-1} for 5 min with a syringe pump (Harvard Apparatus; PhD 2000, Holliston, MA, USA). Platelet accumulation was captured by relief contrast microscopy (Olympus IX81, Center Valley, PA, USA, × 20, numerical aperture, 0.45) at 20 frames min^{-1}. After perfusion, the channel was rinsed with HBS and fixed with glutaraldehyde (2%) for 5 min. The total run time for each sample was 25 min from collection to fixation. Images were analyzed with a custom Sobel-based MATLAB edge-finding protocol, which quantifies the surface area coverage of platelets (Matlab File Exchange: EdgeFindRoutine). The lag time was defined as the time required to achieve 5% platelet surface coverage. The velocity was defined with the MATLAB robust fit line function (RANSAC) between achievement of the lag time and 90% of the maximum value. If a given sample did not reach 5% surface coverage ($n = 4$), the slope was taken from 2% coverage to 90% of the maximum. The maximum was defined as the greatest surface coverage during the assay.

PFA-100

Citrated whole blood was assayed in the PFA-100 collagen/epinephrine and collagen/ADP cartridges according to the manufacturer's instructions (Siemens Medical Solutions, Malvern, PA, USA).

Statistical analysis

Statistical analysis was performed with GRAPHPAD PRISM 7 (GraphPad, La Jolla, CA, USA). For demographic data, significance was determined with a one-way ANOVA comparing all groups. For continuous variables, significance was determined with the Spearman correlation coefficient or Pearson's correlation test. The Kruskal–Wallis test with Dunn's *post hoc* test was used to compare cohorts. For pre-DDAVP–post-DDAVP analysis, the one-tailed matched Wilcoxon rank test was used to determine significance under the hypothesis that DDAVP will result in a gain of function in the MFA. An α-value of < 0.05 was

considered to indicate significance. Correlations of MFA outputs (lag time, velocity, and maximum) with VWF:Ag, VWF:RCo, VWF:CB and bleeding score (BS) were a predefined anaylsis, and cohort analysis was determined as a *post-hoc* analysis.

Results

Patient enrollment and characterization

Thirty-five patients with VWD, low VWF or MCBUE were enrolled and evaluated. Three samples were identified as being consistent with a platelet dysfunction disorder, and were excluded from further analysis. On the basis of VWF:Ag, 10 of 32 patients had type 1 VWD, 12 of 32 were patients with low VWF, and 10 of 32 were patients with MCBUE. Table 1 shows the characteristics for each cohort. There was a statistically significant difference in VWF:Ag and VWF:GP1bR between the cohorts, as expected, owing to the *a priori* classification of these patients. There was also a statistically significant difference in clinical BAT score among the three groups ($P = 0.0083$). Collagen type III-binding results demonstrated a slight decrease in the VWF:CBIII/VWF:Ag ratio in the type 1 VWD cohort as compared with the other groups, but the difference was not statistically significant.

Table 1 Descriptive evaluation of patient cohorts (*P*-values shown were analyzed via one-way ANOVA comparing differences in all groups)

Mean	Type 1 ($N = 10$)	Low VWF ($N = 12$)	MCBUE ($N = 10$)	*P*-value
Age (years) [range]	24 [3–60]	14.8 [2–29]	22.8 [9–65]	0.2745
VWF:Ag (IU dL^{-1}) [range]	14.8 [6–26.3]	40.7 [34–47]	71.4 [51–130]	< 0.0001
VWF:GP1bR (IU dL^{-1}) [range]	12.9 [10–27.6]	38.7 [28–52.2]	59.1 [39.3–149.9]	< 0.0001
VWF:CBIII/VWF:Ag ratio [range]	0.77 [0.001–1.6]	0.98 [0.12–1.5]	0.87 [0.58–1.37]	0.4543
BAT score [range]	10.7 [3–19]	4.5 [0–9]	6.2 [0–14]	0.0083

BAT, bleeding assessment tool; MCBUE, mucocutaneous bleeding of unknown explanation; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen-binding.

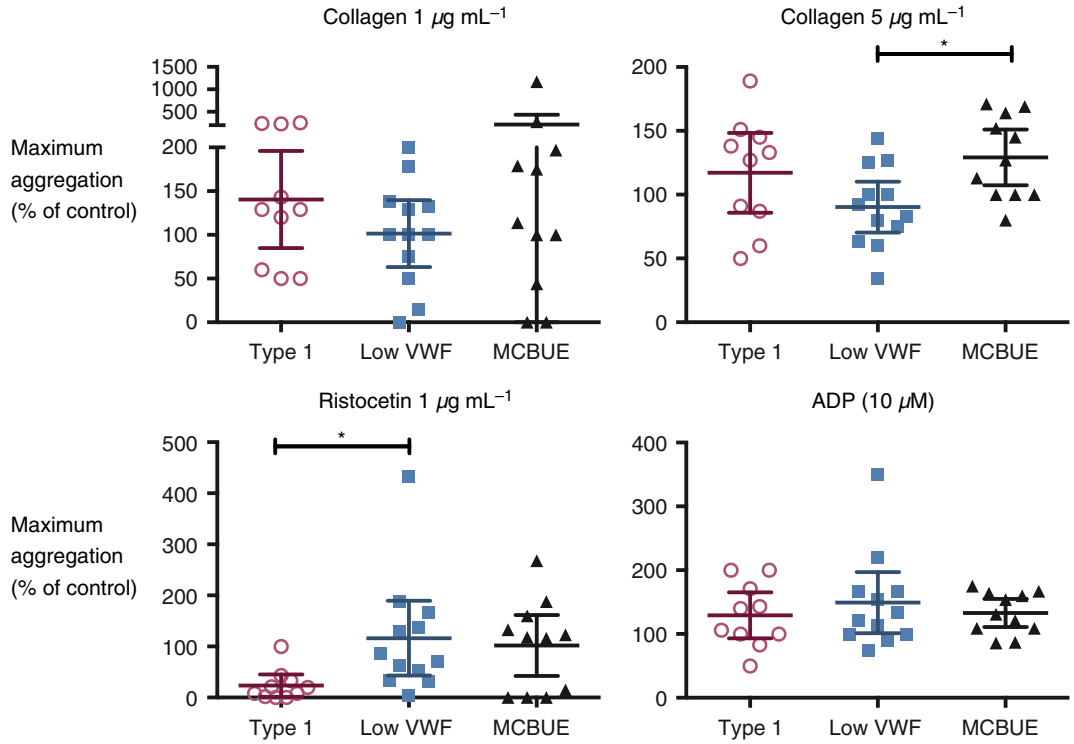

Fig. 1. Platelet aggregometry of type 1 von Willebrand disease, low-von Willebrand factor (VWF) and mucocutaneous bleeding of unknown explanation (MCBUE) patients. Platelet aggregation studies in response to low-dose and high-dose collagen (1 μg mL^{-1} and 5 μg mL^{-1}), ristocetin (1 μg mL^{-1}) and ADP (10 μM) was determined in all patients. Maximum aggregation was compared with control subject maximum aggregation to determine maximum percentage aggregation. Graphs represent mean ± 95% confidence interval. Statistical significance is shown with capped lines (*$P < 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

Platelet aggregometry and VWF–collagen binding

For 5 μg mL^{-1} collagen, there was decreased maximum aggregation in the low-VWF cohort as compared with the MCBUE cohort, and for ristocetin there was decreased maximum aggregation in the type 1 VWD cohort as compared with the low-VWF cohort (Fig. 1). For 1 μg mL^{-1} collagen or ADP, there was no difference in maximum aggregation among cohorts.

Evaluation of velocity, lag time and maximum platelet accumulation in the MFA at shear rates of 750 s^{-1} and 1500 s^{-1}, and correlation with VWF assays and BS

Figure 2 shows representative surface coverage traces for platelets on type I collagen and images from five sample types: control, low VWF before and after treatment with DDAVP, and varying levels of type 1 VWD (see Videos S1–S5). We used the lag time, velocity and maximum to characterize the dynamics of platelet accumulation. In preliminary studies (not shown), we found that saturating the surface with collagen fibers provided larger differences in platelet adhesion at low VWF levels. We therefore used a higher collagen concentration (500 μg mL^{-1}) than is typical in platelet adhesion flow assays (100 μg mL^{-1}) [34]. These metrics were correlated against VWF:Ag (Fig. 3) and VWF:GP1bR (Fig. S1) for each shear rate. Lag time, velocity and maximum values of the MFA correlated with VWF:Ag and VWF:GP1bR levels across the entire cohort at 750 s^{-1} and 1500 s^{-1}. There was not a significant correlation between the MFA metrics and VWF:Ag or VWF:GP1bR at 150 s^{-1}. There was no

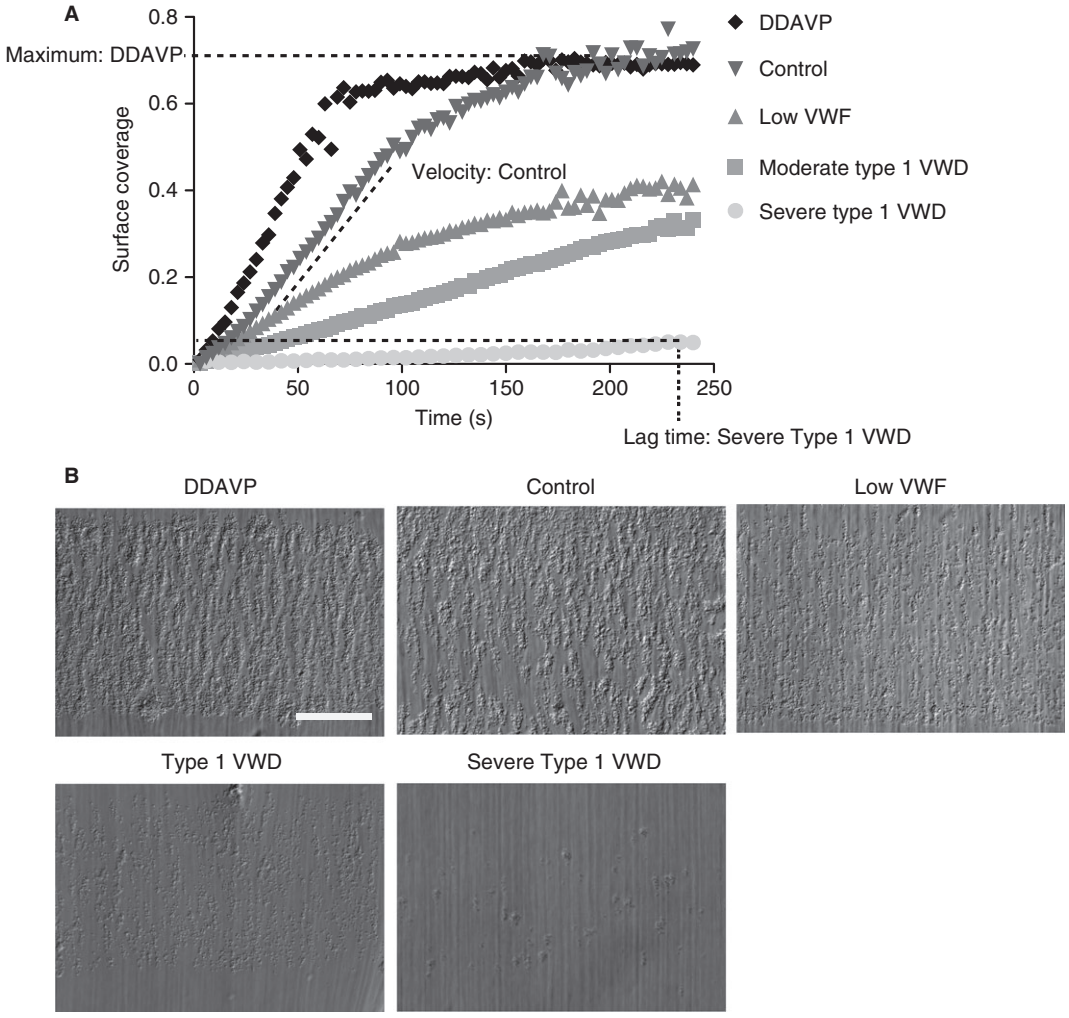

Fig. 2. Platelet accumulation on type I collagen at 750 s^{-1} in the microfluidic flow assay. (A) Representative traces of the kinetics of platelet surface coverage for five samples; severe type 1 VWD (VWF:Ag = 10 UI dL^{-1}), type 1 VWD (VWF:Ag = 17.6 UI dL^{-1}), low VWF before (VWF:Ag = 43 UI dL^{-1}) and after DDAVP treatment (VWF:Ag = 248 IU dL^{-1}), and healthy control. Lag time is defined as the time to 5% surface coverage. Maximum is defined as the highest surface coverage reached in 5 min. Velocity is defined as the slope of the linear growth regime, between the lag time and 90% of the maximum. (B) Relief contrast images (× 20, 0.45 numerical aperture) of the five samples at 150 s^{-1}. Scale bar: 50 μm.

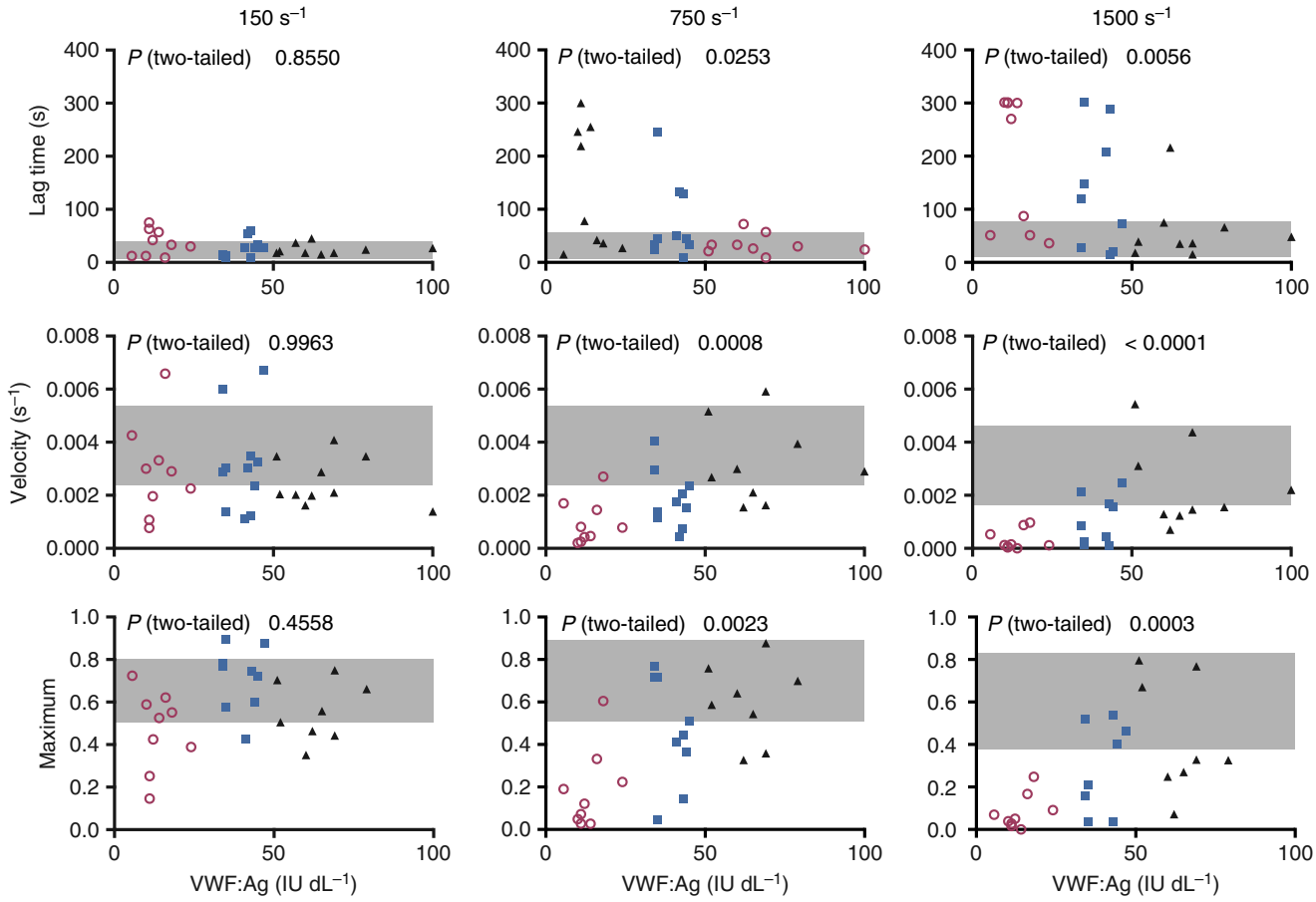

Fig. 3. Correlation of microfluidic flow assay metrics with von Willebrand factor antigen (VWF:Ag) level. The lag times, velocities and maxima of the complete cohort are plotted versus VWF:Ag levels for 150, 750 and 1500 s^{-1}. The cohort is divided into three categories: type 1 von Willebrand disease (magenta circles), low VWF (blue squares), and mucocutaneous bleeding of unknown explanation (black triangles). The mean ± standard deviation of the controls (n = 7) is shown as a gray area. Two-tailed P-values from a Spearman correlation test of the total cohort with a zero-slope null hypothesis are shown on each plot. [Color figure can be viewed at wileyonlinelibrary.com]

significant correlation between the MFA metrics and bleeding score as measured by the BAT (Fig. S2) or VWF:CBIII (Fig. S3).

Recasting the MFA data in terms of clinical groups showed that velocity and maximum metrics were particularly sensitive to shear rate in the type 1 VWD and low-VWF groups (Fig. S4). In the type 1 VWD group and the low-VWF group, there were significant decreases in velocity and maximum at 750 s^{-1} and 1500 s^{-1} as compared with 150 s^{-1}. This result suggests that, in individuals with lower VWF levels (type 1 VWD and low VWF), platelet accumulation velocities and maximal platelet accumulation are impaired as compared with individuals with higher VWF levels.

MFA metrics and PFA-100 correlation with VWF:Ag at levels < 20 IU dL^{-1}

Closure times in the PFA-100 in both collagen/ADP and collagen/EPI cartridges correlated with VWF:Ag levels (Fig. 4). For VWF:Ag < 20 IU dL^{-1}, neither the PFA-100 nor the MFA metrics showed a statistically significant correlation with VWF:Ag levels, but there was a trend for better linear sensitivity in the range of VWF:Ag levels from 5 IU dL^{-1} to 20 IU dL^{-1} for the MFA than for the PFA-100 (Fig. S5). The MFA maxima at 750 s^{-1} and 1500 s^{-1} had an increasing trend with increasing VWF:Ag levels over this range. The closure time for PFA-100 collagen/ADP and collagen/EPI cartridges reached or was close to its maximum value (300 s) with VWF:Ag < 20 IU dL^{-1}.

Discriminatory power of MFA metrics and the PFA-100 on clinical diagnoses

Figure 5 recasts the data in terms of clinical groups. As there was little correlation between VWF:Ag and MFA metrics at 150 s^{-1}, we present the data for 750 s^{-1} and 1500 s^{-1}. Similarly, there was no statistically significant difference in lag time. However, at these shear rates, the type 1 VWD group had significantly decreased velocities and maxima as compared with the control and MCBUE groups. Similarly, the PFA-100 had significantly higher closure times in the type 1 VWD and

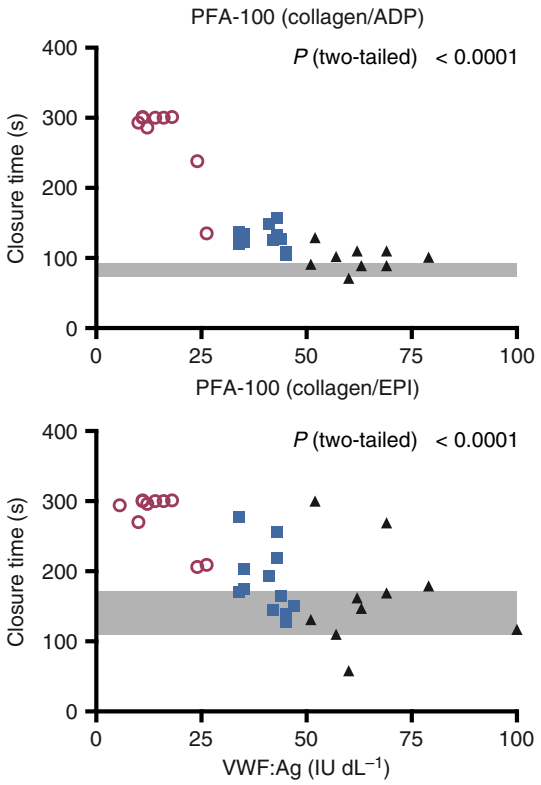

Fig. 4. Correlation of closure times obtained with PFA-100 collagen/ADP and collagen/epinephrine cartridges with von Willebrand factor antigen (VWF:Ag) in the entire cohort. Closure time of the PFA-100 is plotted with respect to VWF:Ag for the complete cohort. The cohort is divided into three categories: type 1 von Willebrand disease (magenta circles), low VWF (aqua squares), and mucocutaneous bleeding of unknown explanation (black triangles). The mean ± standard deviation (SD) of the controls (n = 7) is shown as a gray area. Two-tailed P-values from a Spearman correlation test of the total cohort with a zero-slope null hypothesis are shown on each plot. [Color figure can be viewed at wileyonlinelibrary.com]

low-VWF groups than in the control and MCBUE groups. Neither the MFA nor the PFA-100 could discriminate between the type 1 VWD and low-VWF groups. In further analysis, at 750 s^{-1} a maximum metric of < 0.3455 gave a sensitivity of 88.89% (95% confidence interval [CI] 51.75–99.72) and a specificity of 86.96% (95% CI 66.41–97.22%) for type 1 VWD versus non-type 1 VWD (all low-VWF, MCBUE and control samples). Similarly, at 1500 s^{-1}, a maximum metric of < 0.188 gave a sensitivity of 88.89% (95% CI 51.75–99.72) and a specificity of 80.95% (95% CI 58.09–94.55%) for type 1 VWD. Receiver operating characteristic curves are shown in Fig. S6.

MFA metrics in response to DDAVP therapy

The MFA metrics were evaluated for six patients treated with DDAVP. Paired analyses were performed on blood drawn immediately prior to, and 1 h after, administration of intranasal DDAVP (150 μg for individuals < 50 kg, and 300 μg for individuals > 50 kg) (Fig. 6). At 750 s^{-1}, the lag time decreased but the velocity and maximum did not increase. At 1500 s^{-1}, the lag time, maximum and velocity all significantly improved. There were no statistically significant changes in MFA metrics at 150 s^{-1} before or after DDAVP administration. These results mirrored similar increases in VWF:Ag and VWF:GP1bR seen before and after DDAVP administration (data not shown); one patient with MCBUE did not respond clinically to DDAVP, and for that patient the MFA did not show an increase in function.

Discussion

The force dependence of VWF function and its interactions with platelets has led to the hypothesis that measuring VWF-dependent platelet function under flow conditions will provide additional information beyond that provided by existing clinical assays. In this study, we tested this hypothesis by using a custom microfluidic chamber in a cohort of individuals with type 1 VWD, low VWF, and MCBUE.

Our cohort of individuals was focused on patients presenting symptoms of mucocutaneous bleeding with and without low VWF levels. We have previously demonstrated that a collagen-coated MFA correlates with VWF levels in healthy controls [33], and we sought to extend this analysis to individuals with bleeding symptoms with a specific focus on individuals with lower VWF levels. Although there remains controversy over the classification of patients with type 1 VWD versus low VWF levels, our categories are based on the most recent NHLBI guidelines and the observation that the majority of individuals with VWF levels < 30 IU dL^{-1} have mutations in *VWF*, but the majority of individuals with VWF levels > 40 IU dL^{-1} do not [35,36]. In our cohort, the lack of statistically significant changes in collagen-induced (1 μg mL^{-1}) and ADP-induced (10 μM) aggregation suggests that our results are probably attributable to variability in VWF quantity/function as opposed to intrinsic platelet function. Collagen binding among those in the type 1 VWD, low-VWF and MCBUE cohorts was slightly lower than in controls, similarly to what has been previously reported [14]. Our data also demonstrated that individuals with type 1 VWD had higher BSs, consistent with previous studies [28].

In terms of MFA-based metrics, we demonstrate that the lag time to 5% platelet surface area coverage, the platelet adhesion velocity of the linear portion of the platelet accumulation curve and the maximum platelet surface area coverage are strongly correlated with VWF:Ag and VWF:GP1bR levels at shear rates of 750 s^{-1} and 1500 s^{-1}. This, in conjunction with our earlier publication [33], suggests that a collagen-based MFA is sensitive to a wide range of VWF levels, both in individuals with bleeding and in healthy controls. There

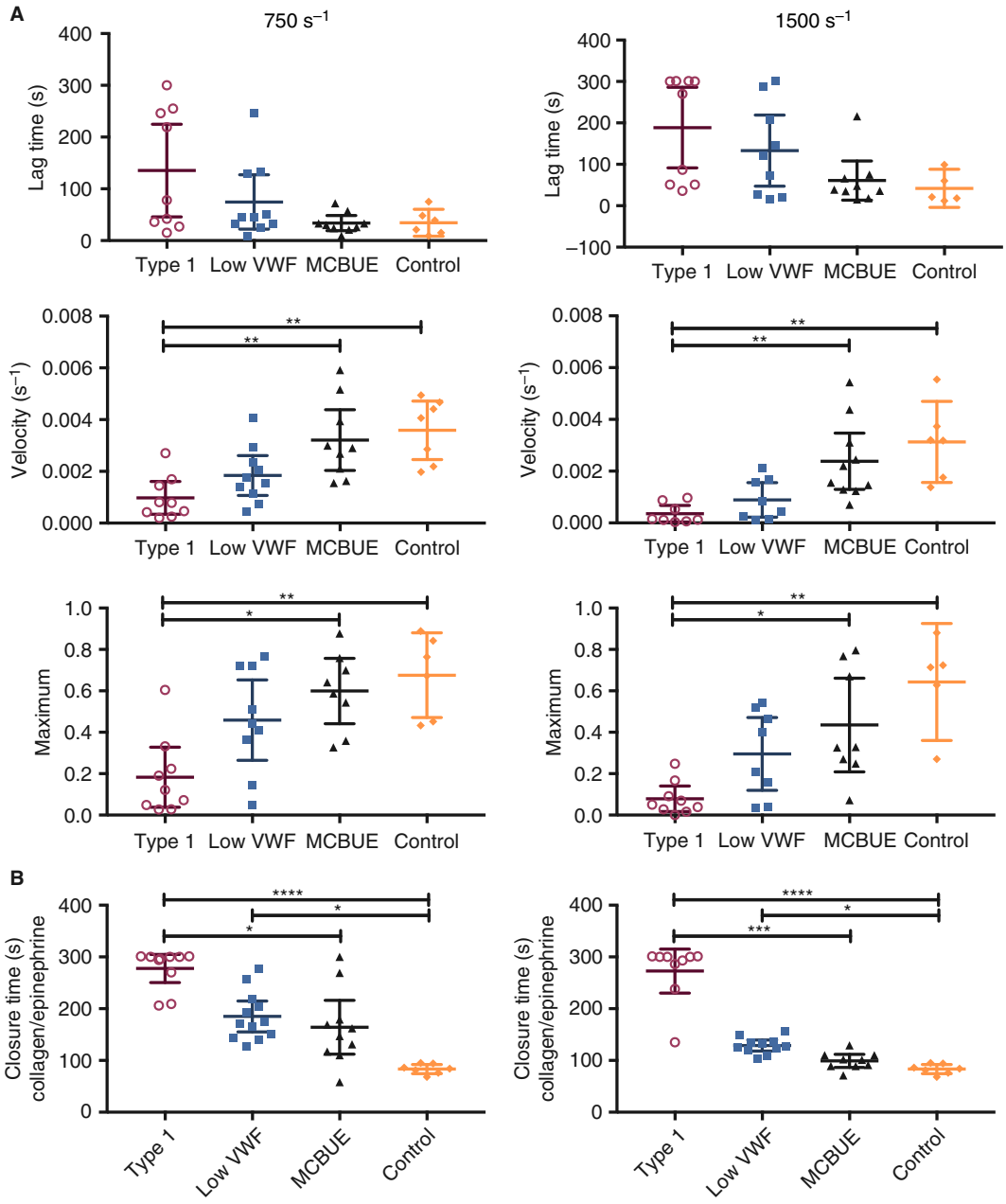

Fig. 5. Microfluidic flow assay (MFA)-based metrics and the PFA-100 have discriminative power in identifying clinical cohorts of patients. The cohort is divided into four categories: type 1 von Willebrand disease (magenta circles), low von Willebrand factor (VWF) (aqua squares), mucocutaneous bleeding of unknown explanation (MCBUE) (black triangles), and controls (yellow diamonds). (A) MFA-based metrics. (B) PFA-100 closure times. A Kruskal–Wallis with Dunn's *post hoc* test was performed to analyze differences between the categories. Graphs represent mean ± 95% confidence interval. Statistical significance is shown with capped lines (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

was no significant correlation at 150 s^{-1}, suggesting that, at this shear rate, platelet accumulation is not strongly dependent on VWF levels. The strong correlative findings in our MFA are similar to those of the PFA-100, whereby PFA-100 closure time was also correlated with VWF:Ag and VWF:GP1bR levels. Future investigation of other shear rates and/or prothrombotic surfaces may provide better differentiation between VWF quantity/function.

In contrast to the correlation with VWF:Ag and VWF:GP1bR levels, our MFA metrics did not correlate with VWF:CBIII/VWF:Ag ratios, suggesting that our MFA metrics were not sensitive to changes in VWF:CB function. This implies that our assay more accurately reflects the platelet-binding function of VWF than its collagen-binding function. This may be attributable to the relatively high amount of immobilized type 1 collagen used in this study.

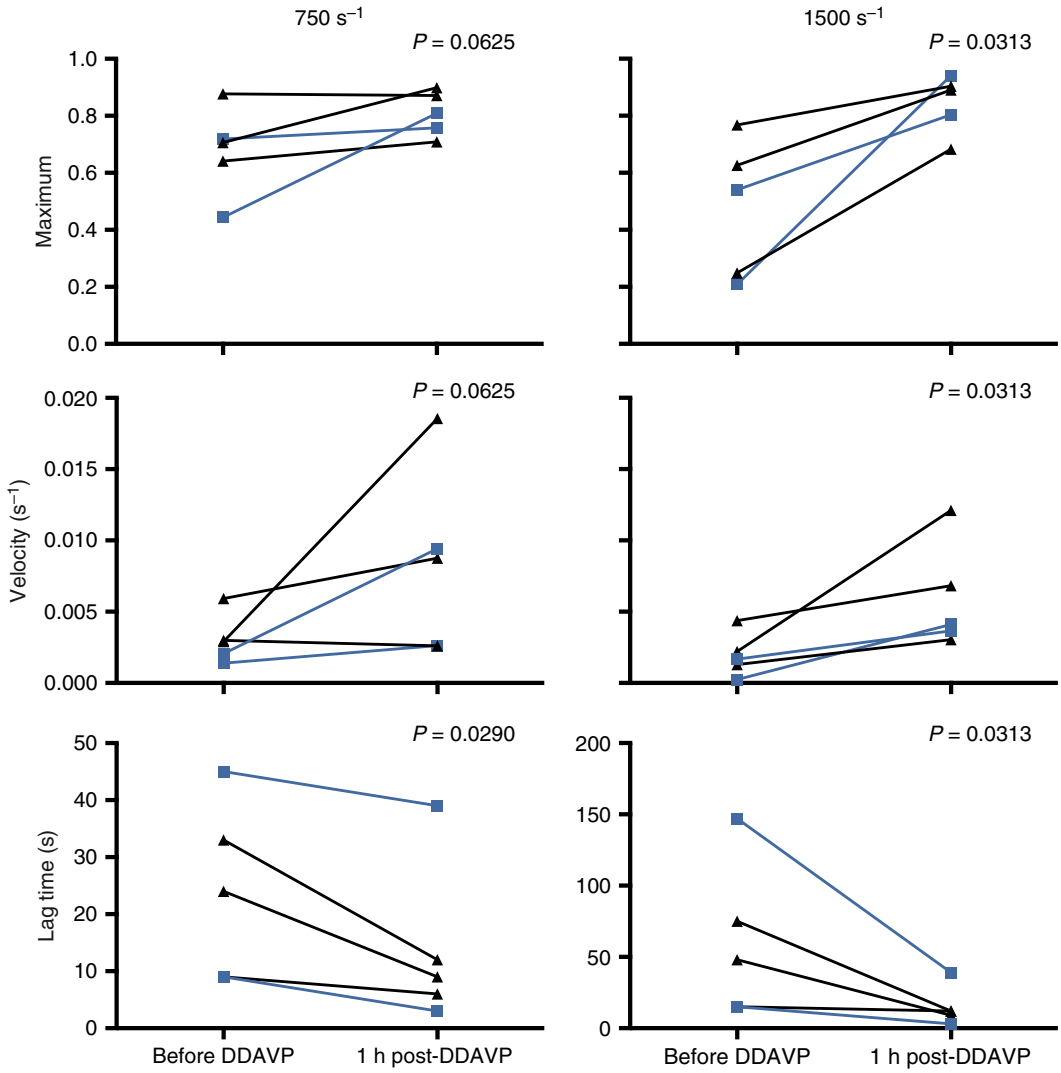

Fig. 6. Microfluidic flow assay (MFA)-based metrics before and after 1-desamino-8-D-arginine vasopressin (DDAVP) treatment in patients with low von Willebrand factor (VWF) and mucocutaneous bleeding of unknown explanation (MCBUE). Plots for immediately prior to and 1 h after treatment show how the MFA responds to DDAVP treatment in patients with low VWF (blue squares) or MCBUE (black triangles). *P*-values are from Wilcoxon signed rank tests performed for each data type. [Color figure can be viewed at wileyonlinelibrary.com]

MFA metrics did not correlate with BSs. Recent studies by Ogiwara *et al.* and Nogami *et al.* used a microfluidic-based approach that monitored pressure gradients to assess thrombus formation, and observed variations among individuals with different BSs, and differentiated high from low BSs on the basis of their thrombus time in collagen/thromboplastin-based devices; however, this difference was seen only in individuals with VWF:RCo < 10 [27,37]. The correlation with BSs in the Nogami study was primarily in a group of 25 individuals with VWF:RCo levels of < 10; in our study, we were able to enroll only seven individuals who met this criterion. The relatively small subgroup in our analysis probably limited the finding of statistical significance in this cohort.

From a categorical perspective, we investigated whether our MFA could reliably differentiate clinical subgroups. Both the platelet adhesion velocity and the maximum platelet adhesion at 750 s^{-1} and 1500 s^{-1} could discriminate type 1 VWD patients from the control and MCBUE cohorts; this is probably because of the significant correlation of these metrics with VWF levels. The discriminative power of the PFA-100 was similar; the PFA-100 was also able to differentiate between the low-VWF and control cohorts. These data suggest that force-dependent assays, and specifically a collagen-based MFA device, have both correlative and discriminative power in the estimation of VWF levels and the screening of VWD.

The MFA detected responses to DDAVP therapy. We demonstrated that an MFA, requiring only 250 μL of blood, showed a universal response to therapy at 1500 s^{-1}. Our results are consistent with previous reports demonstrating that DDAVP can improve platelet

adhesion, bleeding times and PFA-100 closure time in patients with type 1 VWD [26]. Our data support the sensitivity of flow-based models to VWF function at high shear stresses, and suggest the potential use of these assays to model therapeutic response. Additionally, we showed that improvement in VWF function following DDAVP administration was consistently determined only at high shear rates (1500 s^{-1}).

Previous investigations at low shear rates (50–350 s^{-1}) with collagen-coated glass devices or denuded endothelium have demonstrated that there are minimal differences in platelet adhesion between VWD patients and controls [21,23]. At higher shear rates (> 1000 s^{-1}), some investigations have demonstrated minimal to no significant differences in collagen-based platelet adhesion in VWD [21,23], whereas other studies have clearly demonstrated a force-dependent decrease in platelet adhesion [26,38,39]. Our data are consistent with previous observations, when, at 150 s^{-1}, there was no difference between clinical cohorts and no significant correlation with VWF levels, but at 750 s^{-1} and 1500 s^{-1} there was a clear decrease in platelet adhesion velocity and maximal platelet adhesion in individuals with type 1 VWD. We also investigated two additional cohorts, those with low VWF and those with MCBUE. A decrease in platelet adhesion at high shear rates was observed in individuals with low VWF, but not in individuals with MCBUE. This is probably becuase of the strong dependence of our MFA metrics on VWF levels.

There are several limitations of this study. Our MFA metrics were correlated with VWF levels across a broad range of values; however, our MFA metrics were not able to predict a clinical subgroup for each patient. Our cohort size was potentially too small to have sufficient statistical power to discriminate clinical subgroups, and we specifically did not have many patients with VWF < 30 IU dL^{-1}, which was our inclusion criterion for type 1 VWD. Many of our patients with MCBUE were enrolled on the basis of their presenting complaint; however, upon further standardized evaluation, the severity of their bleeding varied significantly. From an assay perspective, the conditions for MFAs are not optimized for linear sensitivity to VWF level and function, but we generally followed the recommendations of the ISTH Subcommittee on Global Assay recommendations [10,40]. Nevertheless, variations in channel cross-sectional area, height/width ratios, shear rates, anticoagulant and collagen type can affect platelet adhesion. Although our data are consistent with VWF having a strong effect on platelet adhesion, there are probably multiple other factors that affect *in vitro* and *in vivo* platelet adhesion, including collagen and platelet receptor densities, hematocrit, and variations in coagulation factor levels. Finally, our post-acquisition analysis was based on a two-dimensional analysis of platelet surface area, and may have underestimated platelet deposition in the vertical plane; further study of thrombus volumes may provide additional details.

In summary, we demonstrate that, in a cohort of individuals with VWD/mucocutaneous bleeding, a collagen-based MFA correlates with VWF levels across a wide spectrum of levels, appropriately groups patients with a diagnosis of type 1 VWD, demonstrates shear-dependent abnormalities in individuals with low VWF, and accurately reflect the response of VWF levels to DDAVP. The relatively small sample size input of our assay (250 μL) and the strong ability to correlate with relevant hemostatic parameters and monitor responses to therapeutic interventions suggest the potential utility of translating microfluidic-based technology to clinical situations.

Addendum

M. Lehmann contributed to the concept and design of the study, analysis and/or interpretation of data, and critical writing or revision of the intellectual content, and gave final approval. K. Ashworth contributed to analysis and/or interpretation of data, and critical writing or revision of the intellectual content, and gave final approval. M. Manco-Johnson contributed to analysis and/or interpretation of data, and critical writing or revision of the intellectual content, and gave final approval. K. B. Neeves contributed to the concept and design of the study, analysis and/or interpretation of data, and critical writing or revision of the intellectual content, and gave final approval. J. A. Di Paola contributed to the concept and design of the study, analysis and/or interpretation of data, and critical writing or revision of the intellectual content, and gave final approval. C. J. Ng contributed to the concept and design of the study, analysis and/or interpretation of data, and critical writing or revision of the intellectual content, and gave final approval.

Acknowledgements

This work was supported by a Hemostasis and Thrombosis Research Society/Novo Nordisk 2013 Mentored Research Award in Hemophilia or Rare Bleeding Disorders (C. J. Ng), an NIH/NCRR Colorado CTSI Grant (UL1 TR001082), the NSF (CBET-1351672), American Heart Association (14GRNT20410094), the National Institutes of Health (R01HL120728), the Health Resources and Services Administration (H30MC24049), and the Postle Chair of Pediatric Cancer and Blood Disorders (J. Di Paola).

Disclosure of Conflict of Interests

The authors state that they no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Correlation of MFA metrics with VWF:GP1bR.
Fig. S2. MFA metrics do not correlate with bleeding history as measured by a standardized bleeding score.
Fig. S3. MFA metrics do not correlate with VWF:CBIII/VWF:Ag ratios.
Fig. S4. Intra-cohort comparison of MFA-based velocity and maximum demonstrated decreased MFA-based metrics with increased shear rate in type 1 VWD and low-VWF patients.
Fig. S5. MFA maxima and PFA-100 closure times for VWF:Ag < 20 at 750 s^{-1} and 1500 s^{-1} and the PFA-100 collagen/ADP and collagen/EPI closure times for VWF:Ag < 20.
Fig. S6. ROC curves for the MFA maxima at 750 s^{-1} and 1500 s^{-1} in determining type 1 VWD.
Video S1. Representative videos of platelet adhesion at 750 s^{-1} for low VWF patient post-DDAVP.
Video S2. Representative video of platelet adhesion at 750 s^{-1} for low VWF patient pre-DDAVP.
Video S3. Representative video of platelet adhesion at 750 s^{-1} for a patient with Type 1 VWD.
Video S4. Representative video of platelet adhesion at 750 s^{-1} for a patient with severe Type 1 VWD.
Video S5. Representative video of platelet adhesion at 750 s^{-1} for a control sample.

References

1 Nichols WL, Hultin MB, James AH, Manco-Johnson MJ, Montgomery RR, Ortel TL, Rick ME, Sadler JE, Weinstein M, Yawn BP. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia* 2008; **14**: 171–232.
2 Ng C, Motto DG, Di Paola J. Diagnostic approach to von Willebrand disease. *Blood* 2015; **125**: 2029–37.
3 Sadler JE. Low von Willebrand factor: sometimes a risk factor and sometimes a disease. *Hematology* 2009; **2009**: 106–12.
4 Sadler JE, Mannucci PM, Berntorp E, Bochkov N, Boulyjenkov V, Ginsburg D, Meyer D, Peake I, Rodeghiero F, Srivastava A. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost* 2000; **84**: 160–74.
5 Quiroga T, Goycoolea M, Belmont S, Panes O, Aranda E, Zúñiga P, Pereira J, Mezzano D. Quantitative impact of using different criteria for the laboratory diagnosis of type 1 von Willebrand disease. *J Thromb Haemost* 2014; **12**: 1238–43.
6 Sadler JE. Von Willebrand disease type 1: a diagnosis in search of a disease. *Blood* 2002; **101**: 2089–93.
7 Quiroga T, Goycoolea M, Panes O, Aranda E, Martínez C, Belmont S, Muñoz B, Zúñiga P, Pereira J, Mezzano D. High prevalence of bleeders of unknown cause among patients with inherited mucocutaneous bleeding. A prospective study of 280 patients and 299 controls. *Haematologica* 2007; **92**: 357–65.
8 Quiroga T, Goycoolea M, Muñoz B, Morales M, Aranda E, Panes O, Pereira J, Mezzano D. Template bleeding time and PFA-100 have low sensitivity to screen patients with hereditary mucocutaneous hemorrhages: comparative study in 148 patients. *J Thromb Haemost* 2004; **2**: 892–8.
9 Savage B, Sixma JJ, Ruggeri ZM. Functional self-association of von Willebrand factor during platelet adhesion under flow. *Proc Natl Acad Sci USA* 2002; **99**: 425–30.
10 Zwaginga JJ, Nash G, King MR, Heemskerk JWM, Frojmovic M, Hoylaerts MF, Sakariassen KS. Biorheology Subcommittee of the SSC of the ISTH. Flow-based assays for global assessment of hemostasis. Part 1: biorheologic considerations. *J Thromb Haemost* 2006; **4**: 2486–7.
11 Schneider SW, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netz RR, Schneider MF. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proc Natl Acad Sci USA* 2007; **104**: 7899–903.
12 Mohri H, Fujimura Y, Shima M, Yoshioka A, Houghten RA, Ruggeri ZM, Zimmerman TS. Structure of the von Willebrand factor domain interacting with glycoprotein Ib. *J Biol Chem* 1988; **263**: 17901–4.
13 Howard MA, Firkin BG. Ristocetin – a new tool in the investigation of platelet aggregation. *Thromb Diath Haemorrh* 1971; **26**: 362–9.
14 Flood VH, Gill JC, Christopherson PA, Wren JS, Friedman KD, Haberichter SL, Hoffmann RG, Montgomery RR. Comparison of type I, type III and type VI collagen binding assays in diagnosis of von Willebrand disease. *J Thromb Haemost* 2012; **10**: 1425–32.
15 Flood VH, Flood VH, Gill JC, Gill JC, Friedman KD, Friedman KD, Christopherson PA, Christopherson PA, Jacobi PM, Jacobi PM, Hoffmann RG, Hoffmann RG, Montgomery RR, Montgomery RR, Haberichter SL; Zimmerman Program Investigators. Collagen binding provides a sensitive screen for variant von Willebrand disease. *Clin Chem* 2013; **59**: 684–91.
16 Flood VH, Christopherson PA, Gill JC, Friedman KD, Haberichter SL, Bellissimo DB, udani RA, Dasgupta M, Hoffmann RG, Ragni MV, Shapiro AD, Lusher JM, Lentz SR, Abshire TC, Leissinger C, Hoots WK, Manco-Johnson MJ, Gruppo RA, Boggio LN, Montgomery KT, *et al*. Clinical and laboratory variability in a cohort of patients diagnosed with type 1 VWD in the United States. *Blood* 2016; **127**: 2481–8.
17 Dean JA, Blanchette VS, Carcao MD, Stain AM, Sparling CR, Siekmann J, Turecek PL, Lillicrap D, Rand ML. von Willebrand disease in a pediatric-based population – comparison of type 1 diagnostic criteria and use of the PFA-100 and a von Willebrand factor/collagen-binding assay. *Thromb Haemost* 2000; **84**: 401–9.
18 Cattaneo M, Federici AB, Lecchi A, Agati B, Lombardi R, Stabile F, Bucciarelli P. Evaluation of the PFA-100 system in the diagnosis and therapeutic monitoring of patients with von Willebrand disease. *Thromb Haemost* 1999; **82**: 35–9.
19 Castaman G, Tosetto A, Goodeve A, Federici AB, Lethagen S, Budde U, Batlle J, Meyer D, Mazurier C, Goudemand J, Eikenboom J, Schneppenheim R, Ingerslev J, Habart D, Hill F, Peake I, Rodeghiero F. The impact of bleeding history, von Willebrand factor and PFA-100® on the diagnosis of type 1 von Willebrand disease: results from the European study MCMDM-1VWD. *Br J Haematol* 2010; **151**: 245–51.
20 Branchford BR, Ng CJ, Neeves KB, Di Paola J. Microfluidic technology as an emerging clinical tool to evaluate thrombosis and hemostasis. *Thromb Res* 2015; **136**: 13–19.
21 Fressinaud E, Sakariassen KS, Rothschild C, Baumgartner HR, Meyer D. Shear rate-dependent impairment of thrombus growth on collagen in nonanticoagulated blood from patients with von Willebrand disease and hemophilia A. *Blood* 1992; **80**: 988–94.
22 Hansen RR, Wufsus AR, Barton ST, Onasoga AA, Johnson-Paben RM, Neeves KB. High content evaluation of shear dependent platelet function in a microfluidic flow assay. *Ann Biomed Eng* 2012; **41**: 250–62.
23 Sugimoto M, Matsui H, Mizuno T, Tsuji S, Miyata S, Matsumoto M, Matsuda M, Fujimura Y, Yoshioka A. Mural thrombus generation in type 2A and 2B von Willebrand disease under flow conditions. *Blood* 2003; **101**: 915–20.
24 Tschopp TB, Weiss HJ, Baumgartner HR. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J Lab Clin Med* 1974; **83**: 296–300.

25 Turitto VT, Weiss HJ, Baumgartner HR. Platelet interaction with rabbit subendothelium in von Willebrand's disease: altered thrombus formation distinct from defective platelet adhesion. *J Clin Invest* 1984; **74**: 1730–41.
26 Sakariassen KS, Bolhuis PA, Blombäck M, Thorell L, Blombäck B, Sixma JJ. Association between bleeding time and platelet adherence to artery subendothelium. *Thromb Haemost* 1984; **52**: 144–7.
27 Nogami K, Ogiwara K, Yada K, Shida Y, Takeyama M, Yaoi H, Minami H, Furukawa S, Hosokawa K, Shima M. Assessing the clinical severity of type 1 von Willebrand disease patients with a microchip flow-chamber system. *J Thromb Haemost* 2016; **14**: 667–74.
28 Tosetto A, Rodeghiero F, Castaman G, Goodeve A, Federici AB, Batlle J, Meyer D, Fressinaud E, Mazurier C, Goudemand J, Eikenboom J, Schneppenheim R, Budde U, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Lethagen S, Pasi J, Hill F, *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.
29 White-Adams TC, Ng CJ, Jacobi PM, Haberichter SL, Di Paola JA. Mutations in the D'D3 region of VWF traditionally associated with type 1 VWD lead to quantitative and qualitative deficiencies of VWF. *Thromb Res* 2016; **145**: 112–18.
30 Bodó I, Eikenboom J, Montgomery R, Patzke J, Schneppenheim R, Di Paola J; von Willebrand factor Subcommittee of the Standardization and Scientific Committee of the International Society for Thrombosis and Haemostasis. Platelet-dependent von Willebrand factor activity. Nomenclature and methodology: communication from the SSC of the ISTH. *J Thromb Haemost* 2015; **13**: 1345–50.
31 Lehmann M, Wallbank AM, Dennis KA, Wufsus AR, Davis KM, Rana K, Neeves KB. On-chip recalcification of citrated whole blood using a microfluidic herringbone mixer. *Biomicrofluidics* 2015; **9**: 064106.
32 Neeves KB, Maloney SF, Fong KP, Schmaier AA, Kahn ML, Brass LF, Diamond SL. Microfluidic focal thrombosis model for measuring murine platelet deposition and stability: PAR4 signaling enhances shear-resistance of platelet aggregates. *J Thromb Haemost* 2008; **6**: 2193–201.
33 Neeves KB, Onasoga AA, Hansen RR, Lilly JJ, Venckunaite D, Sumner MB, Irish AT, Brodsky G, Manco-Johnson MJ, Di Paola JA. Sources of variability in platelet accumulation on type 1 fibrillar collagen in microfluidic flow assays. *PLoS One* 2013; **8**: e54680.
34 Van Kruchten R, Cosemans JMEM, Heemskerk JWM. Measurement of whole blood thrombus formation using parallel-plate flow chambers – a practical guide. *Platelets* 2012; **23**: 229–42.
35 Goodeve A, Eikenboom J, Castaman G, Rodeghiero F, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Schneppenheim R, Budde U, Ingerslev J, Habart D, Vorlova Z, Holmberg L, Lethagen S, Pasi J, Hill F, Hashemi Soteh M, Baronciani L, *et al*. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood* 2007; **109**: 112–21.
36 James PD, Notley C, Hegadorn C, Leggo J, Tuttle A, Tinlin S, Brown C, Andrews C, Labelle A, Chirinian Y, O'Brien L, Othman M, Rivard G, Rapson D, Hough C, Lillicrap D. The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood* 2007; **109**: 145–54.
37 Ogiwara K, Nogami K, Hosokawa K, Ohnishi T, Matsumoto T, Shima M. Comprehensive evaluation of haemostatic function in von Willebrand disease patients using a microchip-based flow chamber system. *Haemophilia* 2015; **21**: 71–80.
38 Sakariassen KS, Cattaneo M, vander Berg A, Ruggeri ZM, Mannucci PM, Sixma JJ. DDAVP enhances platelet adherence and platelet aggregate growth on human artery subendothelium. *Blood* 1984; **64**: 229–36.
39 Turitto VT, Weiss HJ, Baumgartner HR. Decreased platelet adhesion on vessel segments in von Willebrand's disease: a defect in initial platelet attachment. *J Lab Clin Med* 1983; **102**: 551–64.
40 Heemskerk JWM, Sakariassen KS, Zwaginga JJ, Brass LF, Jackson SP, Farndale RW; Biorheology Subcommittee of the SSC of the ISTH. Collagen surfaces to measure thrombus formation under flow: possibilities for standardization. *J Thromb Haemost* 2011; **9**: 856–8.