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Von Willebrand Factor Multimer Densitometric Analysis: Validation of the Clinical Accuracy and Clinical Implications in Von Willebrand Disease

Johan Boender[1], Ferdows Atiq[1], Marjon H. Cnossen[2], Johanna G. van der Bom[3,4], Karin Fijnvandraat[5,6], Joke de Meris[7], Moniek P. M. de Maat[1], Karin P. M. van Galen[8], Britta A. P. Laros-van Gorkom[9], Karina Meijer[10], Jeroen Eikenboom[11,12], Frank W. G. Leebeek[1], for the WiN study group*

Correspondence: Frank W. G. Leebeek (F.leebeek@erasmusmc.nl).

Abstract

Von Willebrand factor (VWF) multimer analysis is important in the classification of von Willebrand disease (VWD). Current visual VWF multimer analysis is time consuming and inaccurate in detecting subtle changes in multimer patterns. Although VWF multimer densitometric analysis may be useful, the accuracy needs further investigation before it can be widely applied. In this study we aimed to validate VWF multimer densitometric analysis in a large cohort of VWD patients and to identify patient characteristics associated with densitometric outcomes. Patients were included from the Willebrand in the Netherlands (WiN) study, in which a bleeding score (BS) was obtained, and blood was drawn. For multimer analysis, citrated blood was separated on an agarose gel and visualized by Western blotting. IMAGEJ was used to generate densitometric images and medium-large VWF multimer index was calculated. We included 560 VWD patients: 328 type 1, 211 type 2, and 21 type 3 patients. Medium-large VWF multimer index performed excellent in distinguishing visually classified normal VWF multimers from reduced high-molecular-weight (HMW) multimers (area under the curve [AUC]: 0.96 [0.94-0.98], $P < 0.001$), normal multimers from absence of HMW multimers (AUC 1.00 [1.00-1.00], $P < 0.001$), and type 2A and 2B from type 2M and 2N (AUC: 0.96 [0.94-0.99], $P < 0.001$). Additionally, higher medium-large VWF multimer index was associated with lower BS in type 1 VWD: $\beta = -7.6$ (-13.0 to -2.1), $P = 0.007$, adjusted for confounders. Densitometric analysis of VWF multimers had an excellent accuracy compared with visual multimer analysis and may contribute to a better understanding of the clinical features such as the bleeding phenotype of VWD patients.

[1]Department of Hematology, Erasmus MC, University Medical Center Rotterdam, The Netherlands
[2]Department of Pediatric Hematology, Erasmus MC-Sophia Children's Hospital, University Medical Center Rotterdam, The Netherlands
[3]Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
[4]Jon J van Rood Center for Clinical Transfusion Medicine, Sanquin Research, Leiden, The Netherlands
[5]Pediatric Hematology, Amsterdam UMC, Emma Children's Hospital, University of Amsterdam, The Netherlands
[6]Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands
[7]Netherlands Hemophilia Society, Leiden, The Netherlands
[8]Van Creveldkliniek, University Medical Center, University Utrecht, The Netherlands
[9]Department of Hematology, Radboud University Medical Center, Nijmegen, The Netherlands
[10]Department of Hematology, University of Groningen, University Medical Center Groningen, The Netherlands
[11]Department of Internal Medicine, Division of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, The Netherlands
[12]Einthoven Laboratory for Vascular and Regenerative Medicine, Leiden University Medical Center, Leiden, The Netherlands.
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Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder and is characterized by reduced levels or an abnormal function of von Willebrand factor (VWF).[1] Platelet plug formation is disturbed in patients with VWD because VWF has a crucial role in platelet adhesion and aggregation.[2] An additional function of VWF is prevention of factor VIII (FVIII) degradation by serving as a carrier protein of FVIII.[2,3] Consequently, VWD patients also have reduced FVIII levels, contributing to their hypocoagulable state.[1,4]

VWF is synthesized in endothelial cells or in α-granules of platelets.[5] VWF is formed as monomers and subsequently dimerized in the endoplasmatic reticulum.[1,5] Subsequently, VWF is multimerized in the Golgi apparatus to form high-molecular-weight (HMW) multimers, after which it is packed into Weibel-Palade bodies and excreted into the circulation.[1,5] In the circulation, VWF is cleaved by ADAMTS13 into smaller, hemostatically less active, multimers.[1,5]

VWD is divided into 3 types: reduced VWF levels without change of VWF function is classified as type 1, an abnormal function of VWF is classified as type 2, and complete absence of VWF is defined as type 3 VWD.[1] Type 2 VWD is subdivided in type 2A, 2B, 2M, and 2N based on the specific VWF activity defect, which is caused by a specific *VWF* gene mutation.[1,6] Of these subtypes, type 2A and type 2B VWD are characterized by loss of HMW VWF multimers, which leads to a more severe bleeding phenotype compared with type 2M VWD in which

patients have a normal VWF multimer pattern.[1,7,8] Therefore, VWF multimer analysis is an essential step for the correct classification of types and subtypes of VWD. It may also have important therapeutic consequences, for instance, in distinguishing type 2A and 2B from other type 2 VWD subtypes, since desmopressin that is used as treatment for many VWD patients is contraindicated in type 2B patients.[4]

VWF multimer patterns are currently analyzed with visual examination of agarose gels.[9,10] Multimer patterns are classified as normal, reduced HMW multimers, or absence of HMW multimers.[9] In type 3 VWD, there is a lack of VWF multimers because of a complete absence of VWF.[9] Sometimes, a smear of multimers can be present, making multimeric analysis impossible.[11,12] Lastly, some VWD patients have a specific triplet structure of VWF multimers, in most cases caused by an increased VWF cleavage by ADAMTS13.[13,14]

Although visual examination of VWF multimers may be sufficient in clinical practice, subtle changes in multimer pattern may be missed.[9] Moreover, visual examination of multimers may be observer-dependent and time-consuming, and does not allow the quantification of HMW multimers.[9] Therefore, studies have recently investigated VWF multimer quantification using densitometric analysis.[15-20] These studies have validated the technical merits of the method and confirmed that VWF multimer densitometric analysis is able to detect differences between types and subtypes of VWD.[15-20] However, the performance of VWF multimer densitometric analysis in clinical practice requires further investigation before it can be widely used. Also, it is not known yet which factors, besides the specific type of VWD and genetic mutations, influence VWF multimer patterns in VWD patients. Lastly, subtle differences in HMW VWF multimers may have important implications for remaining functionality of VWF, thereby potentially explaining some of the variability of bleeding phenotype in VWD patients. Therefore, we hypothesized that VWD patients with relatively more HMW VWF multimers, which are more hemostatically active, may bleed less than VWD patients with less HMW VWF multimers.

Given the limited data on VWF multimer densitometry analysis in VWD, we performed VWF multimer densitometry analysis in a large, well-defined cohort of VWD patients. The aim of the study was to validate the diagnostic accuracy of VWF multimer densitometric analysis by comparing it to visual examination. Secondly, we aimed to identify patient characteristics associated with VWF multimer densitometry outcomes in type 1 and type 2 VWD patients. We also investigated the association between VWF multimer densitometry outcomes and different VWF activity assays. Lastly, we investigated whether type 1 and type 2 VWD with less HMW VWF multimers had a more severe bleeding phenotype.

Methods

We included patients from the nationwide Willebrand in the Netherlands (WiN) study.[21,22] The WiN study is a cross-sectional study in all hemophilia treatment centers in the Netherlands, which enrolled patients between 2007 and 2009. The inclusion criteria of the WiN study were a personal hemorrhagic diathesis or family history of VWD and historically lowest VWF antigen (VWF:Ag), VWF activity (measured with the monoclonal antibody assay [VWF:Ab]), or VWF collagen binding (VWF:CB) ≤0.30 IU/mL or FVIII activity (FVIII:C) ≤0.40 IU/mL in case of type 2N VWD.[21,22] Patients with other bleeding disorders or acquired VWD were excluded. We also excluded patients in whom no citrated blood was available, pregnant women, and patients who were less than 3 days before blood sampling treated with desmopressin or VWF containing concentrates.

Assessment methods

At inclusion in the WiN study, patients filled in an extensive questionnaire containing a condensed self-administered Tosetto bleeding score (BS) and blood was drawn.[22] The assessment methods of the WiN study have been reported in detail previously.[21-23] Also, VWF:Ag, several VWF activity assays, such as the monoclonal antibody assay (VWF:Ab), VWF ristocetin cofactor activity (VWF:RCo), activity assay using ristocetin and recombinant GP1b fragments (VWF:GPIbR), activity assay using recombinant GPIb fragments and 2 gain-of-function mutations (VWF:GPIbM), VWF:CB, FVIII:C, and VWF propeptide (VWFpp) were centrally measured as previously described.[21-24] Of note, VWF:CB was measured with an in-house ELISA, in which collagen type 1 (Sigma-Aldrich, St Louis, MO) was used for capturing and horseradish peroxidase-conjugated anti-human VWF antibody (DakoCytomation) for detection.[22]

VWF multimer analysis

Samples were separated on 0.9% precast agarose gel (Bio-Rad Laboratories, Hercules, CA) and visualized by Western blotting, in which we used rabbit anti-human VWF polyclonal antibodies (Dako A/S, Glostrup, Denmark). VWF:Ag was used to ensure uniform application of samples on the agarose gels. Multimer patterns of all included patients were visually examined by 2 independent and blinded experts (JE and FWGL), who did not know which multimer pattern was from which patient. In case of disagreement about the classification of the multimeric pattern of a patient, they discussed the results until consensus was reached.

Densitometric analysis of VWF multimers

For densitometric analysis, IMAGEJ software (developed by the National Institutes of Health) was used to generate densitometric images and to calculate the intensity of multimer bands. The 5 smallest bands on densitometric images were selected by a blinded researcher (JB) and defined as small multimers (Figure 1). Likewise, next 5 bands were defined as medium multimers and the remaining bands were defined as large multimers (Figure 1). Some previously reported studies that used VWF multimer densitometric analysis reported the percentage of HMW multimers, intermediate MW multimers, and low MW multimers.[15,17,18] Others focused on the proportion of HMW multimers compared with other multimers.[25,26] In this study, we report the proportion of HMW VWF multimers, because, besides the clinical relevance, it is also more suitable for statistical analysis due to its continuous nature as a single number. Therefore, medium-large VWF multimer index was calculated according to de Jong et al[25] by dividing the patient's medium-large multimer ratio (intensity of the medium and large multimers divided by the total intensity of all multimers) by the medium-large multimer ratio of a normal control in the same western blot (Figure 1). Large VWF multimer index was calculated according to Tamura et al[26] by dividing the patient's large multimer ratio (intensity of the large multimers divided by the total intensity of all multimers) by the large multimer ratio of a normal control in the same western blot (Figure 1). Thus, VWF multimer indices below 1 indicate reduced medium-large or large VWF multimers compared with small VWF multimers. If no VWF multimers could be observed in a patient, the VWF multimer index was set at 0 to indicate the complete absence of VWF. Lastly, the VWF multimer index (ie, medium-large index versus large index), which performs best in distinguishing normal VWF multimers from reduced and absent HMW VWF multimers, was selected as primary variable to investigate the objectives of this study with.

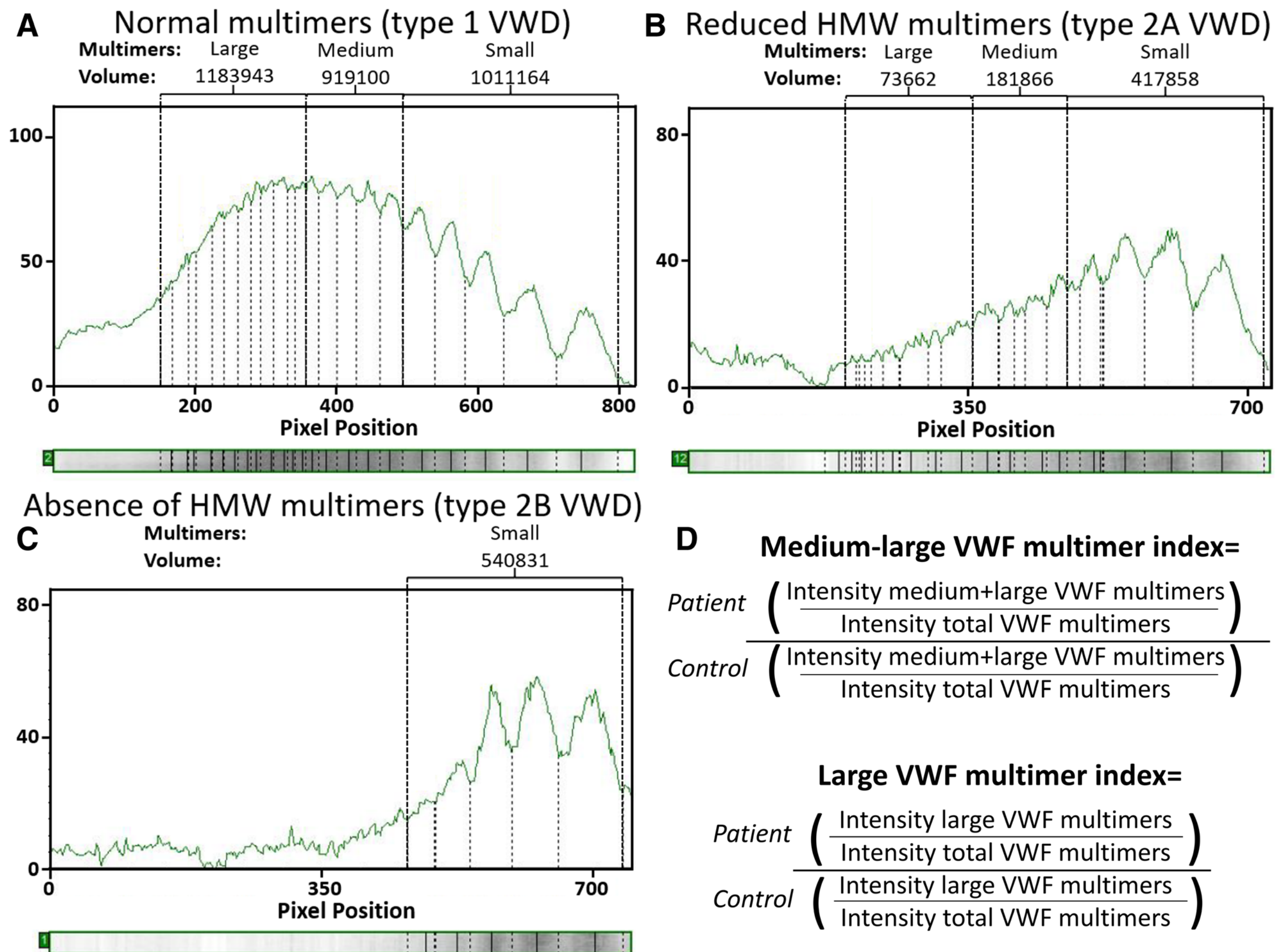

Figure 1. Typical examples of densitometric images (A-C) and calculation of medium-large and large multimer indices (D). HMW = high-molecular-weight; VWD = von Willebrand disease; VWF = von Willebrand factor.

We analyzed the assay variance of 64 controls and found a mean medium-large multimer ratio of 0.61 with a SD of 0.06, variance of 0.003, and intra-run coefficient of variation (CV) of 3.5%, whereas for large multimer ratio, the mean was 0.28, SD was 0.06, variance 0.003, and intra-run CV of 8.9%. The overall inter-run CV for controls was 6.5%.

Definitions

Type 1 VWD was defined as VWF:Ab/VWF:Ag ratio > 0.6.[1] Type 2 VWD was defined as VWF:Ab/VWF:Ag ratio ≤ 0.6.[1] Type 3 VWD was defined as VWF:Ag and VWFpp ≤0.05 IU/mL.[1] Of note, if mutation analysis revealed VWF mutations that were previously consequently reported in literature as a specific type of VWD, we classified them accordingly. An increased clearance of VWF was defined as a VWFpp/VWF:Ag ratio of ≥2.2.[23]

Statistical analyses

Continuous data are described as median and interquartile range (IQR) and categorical data as number and percentage. In case of more than 30 patients per group, groups were compared with parametric tests (central limit theorem). In case of missing data on a secondary variable in a patient, the patient was excluded from the analysis with that secondary variable.

Receiver operating characteristic (ROC) curves were used to analyze the accuracy of VWF multimer indices to, for instance, differentiate between visually classified normal multimers and reduced multimers. Outcomes of ROC curves are presented as area under the curve (AUC) with 95% confidence interval and *P* value. Based on the ROC curves, we selected cutoff values for medium-large VWF multimer index to differentiate visually classified normal multimers from reduced HMW and absent HMW multimers, aiming for high sensitivity and specificity, and easy clinical application. To analyze the correlation between medium-large VWF multimer index and VWF functional measurements and ratios of VWF functional measurements (divided by VWF:Ag), Spearman correlation analysis was used. In these analyses, we excluded outliers with VWF activity >1.50 IU/mL and VWF activity/VWF:Ag ratio above 1.5. Outcomes of Spearman correlation analysis are presented as ρ and *P* value.

To investigate which patient characteristics are independently associated with medium-large VWF multimer index, multiple regression analysis was performed in which age, sex, blood group, increased clearance (defined as VWFpp/VWF:Ag ratio ≥2.2) versus no increased clearance of VWF, VWFpp, and ADAMTS13 were included as independent variables. In type 2 VWD, the subtype was also added as a variable in the analysis (type 2A and 2B versus 2M and 2N). Regression analyses were also used to investigate the association between medium-large VWF multimer index and the bleeding phenotype. In this analysis, the medium-large VWF multimer index was adjusted for age, sex, blood group, type of VWD, VWF:Ag, VWF:Ab, and VWF:CB. Outcomes of regression analysis are presented as unstandardized beta (β), 95% confidence interval, and *P* value. We compared medium-large VWF

multimer index between type 1 VWD patients with and without a *VWF* gene mutation using an independent *t* test. In this analysis, we only included patients in whom mutation analysis was performed. Linear regression analysis was used to adjust for relevant confounders. Statistical analyses were performed with SPSS Statistics version 25 (IBM Corp., Armonk, NY). A *P* value below 0.05 was considered significant.

Results

Characteristics of the study population

From the total WiN cohort of 834 patients, we performed VWF multimer densitometry analysis in 663 patients from whom citrate blood was available. Pregnant women and patients who recently used VWF concentrates or desmopressin were excluded from this analysis (n = 17). In addition, 28 patients were excluded because of technical problems with the run and 58 because of insufficient quality of the agarose gels. Therefore, we included a total of 560 VWD patients (328 type 1, 211 type 2, and 21 type 3 patients). The median age was 44 (IQR 29-58), 351 patients (62.7%) were female and 336 patients (60.4%) had blood group O (Table 1). Figure 1 illustrates typical densitometric outcomes of a type 1 VWD patient with normal VWF multimers (A), type 2A patient with reduced HMW VWF multimers (B), and type 2B patient with absence of HMW multimers (C). Medium-large VWF multimer index was 1.06 (0.99-1.12) in type 1 and 0.53 (0.29-0.89) in type 2 VWD, whereas large VWF multimer index was, respectively, 1.23 (1.04-1.40) and 0.20 (0.00-0.92). As expected in type 3 VWD, no VWF multimers were detected, and both indices were 0.00 (0.00-0.00). All the baseline characteristics in Table 1 were significantly different among the 3 types of VWD.

Validation of VWF multimer densitometric analyses

To assess the diagnostic value of VWF multimer densitometric analyses, densitometric results were compared with the visual examination of the multimer patterns on agarose gels. Medium-large VWF multimer index was in patients who were visually classified as normal, reduced, and absent HMW VWF multimers, respectively, 1.07 (1.02-1.12), 0.84 (0.71-0.91), and 0.31 (0.20-0.44) ($P < 0.001$; Figure 2A). Large VWF multimer index was in patients visually classified as normal, reduced, and absent VWF HMW multimers, respectively, 1.27 (1.12-1.41), 0.79 (0.54-0.97), and 0.00 (0.00-0.00) ($P < 0.001$; Figure 2B). A triplet structure of VWF multimers was visually identified in 31 patients, of whom 22 had been classified as type 1 VWD, 7 as type 2A VWD, and 2 as type 2B VWD. Type 2A and 2B patients with a triplet structure had a higher medium-large VWF multimer index compared with type 2A and 2B patients without triplet structure of multimers, respectively, 0.93 (0.66-1.12) versus 0.40 (0.26-0.69) ($P < 0.001$). Because a triplet structure of multimers resembles a distinct group of VWD patients with a specific pathogenesis and a specific multimer pattern, which needs to be analyzed with a different agarose gel and cannot be automatically detected by densitometric analysis, patients with a triplet structure were excluded from the remaining analyses. Likewise, 23 patients with a smear multimer pattern were also excluded because there could no distinct multimers be identified, and subsequently, the multimer index could not be reliably calculated. Of note, from the 11 patients with genotypically diagnosed type 1 Vicenza (R1205H), 6 had a triplet structure and 5 had a smear pattern. Although medium-large VWF multimer index ($P = 0.136$) and large VWF multimer index ($P = 0.773$) were similar between type 1 Vicenza patients and other type 1 VWD patients, these patients were excluded because of the triplet and smear patterns.

Compared with visual examination, medium-large VWF multimer index had a very good accuracy in distinguishing normal VWF multimers from reduced VWF HMW multimers: AUC of 0.96 (0.94-0.98, $P < 0.001$). Large VWF multimer index also had a good accuracy in distinguishing normal VWF multimers from reduced VWF HMW multimers: 0.91 (0.87-0.95, $P < 0.001$). However, this was less sensitive than the medium-large VWF multimer index. Medium-large VWF multimer index and large VWF multimer index could make a distinction between normal VWF multimers and absence of VWF HMW multimers with 100% accuracy (AUC 1.00 [1.00-1.00], $P < 0.001$). Medium-large VWF multimer index was able to distinguish reduced HMW VWF multimers from absence of HMW multimers with an AUC of 0.95 (0.92-0.97, $P < 0.001$), whereas large multimer index had an AUC of 0.91 (0.87-0.95, $P < 0.001$) in making this distinguishing. Since medium-large VWF multimer index had a better accuracy than large VWF multimer index, we only used medium-large VWF multimer index in the remaining analyses.

Table 1

Baseline Patient Characteristics.

Characteristics	Type 1, N = 328	Type 2, N = 211	Type 3, N = 21	Total, N = 560
Age	45 (30-58)	45 (30-59)	26 (12-54)	44 (29-58)
Female, n (%)	223 (68.0)	116 (55.0)	12 (57.1)	351 (62.7)
Blood group O, n (%)	228 (70.2)	100 (47.6)	8 (38.1)	336 (60.4)
VWF:Ag	0.35 (0.21-0.51)	0.25 (0.17-0.35)	0.00 (0.00-0.01)	0.29 (0.17-0.44)
VWF:Ab	0.43 (0.20-0.67)	0.08 (0.03-0.16)	0.00 (0.00-0.00)	0.21 (0.08-0.51)
VWF:RCo	0.35 (0.18-0.59)	0.06 (0.06-0.06)	0.06 (0.06-0.06)	0.18 (0.06-0.45)
VWF:GPIbR	0.39 (0.21-0.59)	0.10 (0.06-0.15)	0.03 (0.03-0.03)	0.20 (0.09-0.45)
VWF:GPIbM	0.42 (0.22-0.67)	0.12 (0.08-0.19)	0.02 (0.02-0.08)	0.22 (0.12-0.49)
VWF:CB	0.41 (0.19-0.63)	0.07 (0.05-0.14)	0.00 (0.00-0.00)	0.19 (0.07-0.49)
FVIII:C	0.63 (0.43-0.86)	0.37 (0.27-0.48)	0.01 (0.01-0.03)	0.49 (0.32-0.70)
Medium-large multimer index	1.06 (0.99-1.12)	0.53 (0.29-0.89)	0.00 (0.00-0.00)	0.98 (0.56-1.08)
Large multimer index	1.23 (1.04-1.40)	0.20 (0.00-0.92)	0.00 (0.00-0.00)	1.04 (0.22-1.31)
Tosetto bleeding score	9 (5-14)	12 (8-17)	19 (11-23)	11 (6-16)

Data are presented as median (interquartile range), unless otherwise specified. All variables were significantly different among the 3 types of VWD: VWF:Ag, VWF:CB VWF, and FVIII:C. Different types of VWF activity assays: VWF:Ab, VWF:RCo, VWF:GPIbR, and VWF:GPIbM.

FVIII:C = factor VIII activity; VWF:Ab = von Willebrand factor monoclonal antibody assay; VWF:Ag = von Willebrand factor antigen; VWF:CB = von Willebrand factor collagen binding; VWF:GPIbM = von Willebrand factor recombinant GPIb fragments and 2 gain-of-function mutations; VWF:GPIbR = von Willebrand factor ristocetin and recombinant GP1b fragments; VWF:RCo = von Willebrand factor ristocetin cofactor activity.

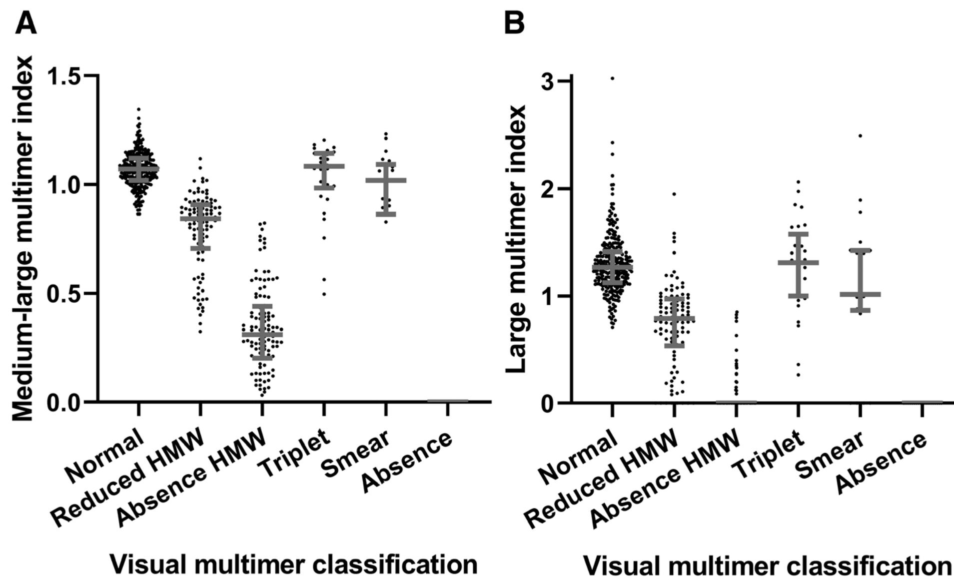

Figure 2. Medium-large VWF multimer index (A) and large VWF multimer index (B) compared with visual examination of multimers. Data are presented as median with interquartile range. HMW = high-molecular-weight; VWF = von Willebrand factor.

Based on the ROC curves, medium-large VWF multimer index of 1.00 was selected as cutoff value to distinguish visually classified normal multimers from reduced HMW VWF multimers, whereas 0.65 was selected as cutoff value to distinguish visually classified reduced HMW multimers from an absence of HMW VWF multimers. With these 2 cutoff values, medium-large VWF multimer index classified 83.0% of all patients similarly as visual examination. Patients who were visually classified as absence of HMW VWF multimers were either classified by medium-large VWF multimer index as having absence of HMW multimers (92.1%) or reduced HMW VWF multimers (7.9%). In patients who were visually classified as reduced HMW VWF multimers, medium-large VWF multimer index classified 93.8% of patients as reduced or absence of HMW VWF multimers, whereas only 6.3% were classified as normal multimers.

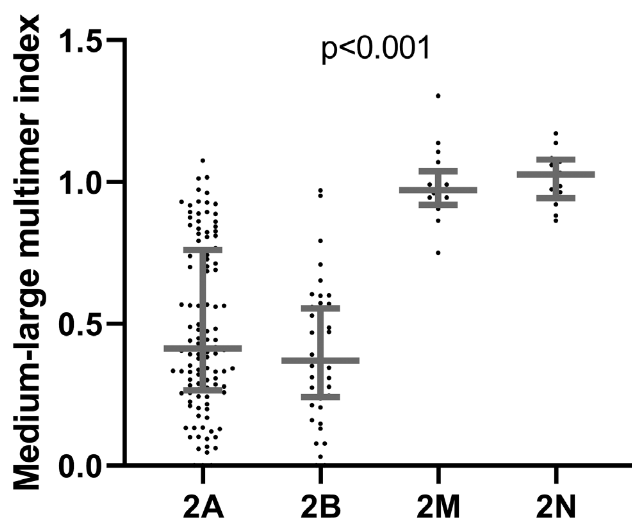

Figure 3. Medium-large VWF multimer index in subtypes of type 2 VWD. Data are presented as median and interquartile range. VWD = von Willebrand disease; VWF = von Willebrand factor.

VWF multimeric densitometry in type 2 VWD

Medium-large VWF multimer index was 0.42 (0.26-0.76) in patients previously classified as type 2A (n = 132), 0.37 (0.24-0.55) in 2B (n = 47), 0.97 (0.92-1.04) in 2M (n = 19), and 1.03 (0.94-1.08) in 2N (n = 13) VWD ($P < 0.001$; Figure 3). Medium-large VWF multimer index had an excellent accuracy in distinguishing type 2A and type 2B VWD patients from type 2M and type 2N (AUC of 0.96 [0.94-0.99], $P < 0.001$).

Genetic data of the *VWF* gene was available in 97 type 2A VWD patients. Medium-large VWF multimer index was much lower in 58 patients with mutations in the VWF A2 domain (0.32 [0.20-0.42]) compared with 39 type 2A patients with mutations in other domains (0.77 [0.57-0.86], $P < 0.001$). In 43 type 2B patients, medium-large multimer index was comparable between 23 patients with R1306W (0.37 [0.28-0.55]), 15 patients with R1306C (0.28 [0.21-0.60]), 4 patients with R1341P, and 1 with R1341Q (0.39 [0.22-0.52], $P = 0.378$).

Correlation between large VWF multimer index and functional VWF measurements

We found that VWF:Ag, VWF:Ab, VWF:RCo, VWF:GPIbR, VWF:GPIbM, and VWF:CB were all nonlinearly highly correlated with large VWF multimer index (Table 2). From the functional measurements, large VWF multimer index was strongest correlated with VWF:CB ($\rho = 0.79$, $P < 0.001$; Table 2). This correlation was stronger in patients with type 2 VWD, than in type 1 VWD, respectively, $\rho = 0.66$, $P < 0.001$ and $\rho = 0.36$, $P < 0.001$. Also, the ratio of the various functional VWF measurements (divided by VWF:Ag) was highly correlated with large VWF multimer index. The strongest correlation was again found for VWF:CB/VWF:Ag ratio ($\rho = 0.80$, $P < 0.001$; Table 2). Also, this correlation was stronger in patients with type 2 VWD, than in type 1 VWD, respectively $\rho = 0.79$, $P < 0.001$ and $\rho = 0.57$, $P < 0.001$.

Patient characteristics associated with medium-large VWF multimer index

In type 1 VWD, we found that an increased clearance of VWF was independently associated with lower medium-large VWF

Table 2

Correlation Between Medium-large VWF Multimers and VWF Measurements.

VWF Measurements	Medium-large VWF Multimers
VWF:Ag	ρ = 0.42, $P < 0.001$
VWF:Ab	ρ = 0.68, $P < 0.001$
VWF:RCo	ρ = 0.71, $P < 0.001$
VWF:GPIbR	ρ = 0.73, $P < 0.001$
VWF:GPIbM	ρ = 0.73, $P < 0.001$
VWF:CB	ρ = 0.79, $P < 0.001$
VWF:Ab/VWF:Ag	ρ = 0.67, $P < 0.001$
VWF:RCo/VWF:Ag	ρ = 0.73, $P < 0.001$
VWF:GPIbR/VWF:Ag	ρ = 0.76, $P < 0.001$
VWF:GPIbM/VWF:Ag	ρ = 0.73, $P < 0.001$
VWF:CB/VWF:Ag	ρ = 0.80, $P < 0.001$

Outcomes of Spearman correlation analysis. Outliers with VWF activity >1.50 IU/mL and VWF activity/VWF:Ag ratio >1.5 were excluded from these analyses.
FVIII:C = factor VIII activity; VWF = von Willebrand factor; VWF:Ab = von Willebrand factor monoclonal antibody assay; VWF:Ag = von Willebrand factor antigen; VWF:CB = von Willebrand factor collagen binding; VWF:GPIbM = von Willebrand factor recombinant GPIb fragments and 2 gain-of-function mutations; VWF:GPIbR = von Willebrand factor ristocetin and recombinant GP1b fragments; VWF:RCo = von Willebrand factor ristocetin cofactor activity.

multimer index (β = -0.10 [-0.13 to -0.06], $P < 0.001$) and female sex was independently associated with higher medium-large VWF multimer index (β = 0.04 [0.01-0.08], $P = 0.021$) using multiple regression analysis with age, sex, blood group, pathophysiology of reduced VWF levels (increased clearance of VWF defined as VWFpp/VWF:Ag ratio ≥2.2 versus no increased clearance of VWF), VWFpp, and ADAMTS13 as independent variables.

Mutation analysis of *VWF* gene was performed in 162 type 1 VWD patients. In 94 (58%) of these patients, a *VWF* gene mutation was found. Type 1 patients with a *VWF* gene mutation had relatively lower medium-large VWF multimer index compared with type 1 patients without a *VWF* gene mutation, respectively, 1.03 (0.95-1.10) versus 1.08 (1.04-1.12) ($P < 0.001$). After adjustment for age, sex, blood group, pathophysiology of reduced VWF levels, VWFpp, and ADAMTS13, type 1 patients with a *VWF* gene mutation still had lower medium-large VWF multimer index compared with patients without a mutation: β = -0.06 (-0.10 to -0.03), $P = 0.001$. Furthermore, in type 1 VWD, there was no significant difference in the medium-large multimer index between patients with blood group O (1.06 [1.01-1.11]) and non O (1.04 [0.92-1.12], $P = 0.172$).

In type 2 VWD, we added the subtype of VWD (type 2A and 2B versus type 2M and 2N) in the multiple regression analysis and found that type 2A and 2B versus type 2M and 2N, and VWFpp were independently associated with lower medium-large VWF multimer index, respectively, β = -0.52 (-0.62 to -0.42), $P < 0.001$ and β = -0.09 (-0.15 to -0.02), $P = 0.009$.

Medium-large VWF multimer index and the bleeding phenotype

Interestingly, higher medium-large VWF multimer index was associated with lower BS in type 1 VWD patients: β = -7.6 (-13.0 to -2.1), $P = 0.007$, but not in type 2 VWD patients: β = -1.9 (-7.0 to 2.8), $P = 0.427$, both adjusted for age, sex, blood group, VWF:Ag, VWF:Ab, and VWF:CB.

Discussion

In this large study in a well-defined cohort of VWD patients, we demonstrate that densitometric analysis of VWF multimers has an excellent accuracy in clinical practice. Medium-large VWF multimer index was as accurate as visual observation of 2 blinded-independent experts and could precisely distinguish type 2A and 2B from type 2M and type 2N VWD. Furthermore, from all functional measurements of VWF, VWF:CB, and VWF:CB/VWF:Ag were strongest associated with medium-large VWF multimer index. In type 1 VWD, an increased clearance of VWF was independently associated with a lower medium-large VWF multimer index, whereas female sex was independently associated with a higher medium-large VWF multimer index. Also, type 1 VWD patients with a *VWF* gene mutation had lower medium-large VWF multimer index compared with type 1 patients without a *VWF* gene mutation. In type 2 VWD, type 2A and 2B, and VWFpp were independently associated with lower medium-large VWF multimer index. Lastly, in type 1 VWD, higher medium-large VWF multimer index was associated with lower BS, whereas there was no association in type 2 VWD.

In accordance with previous studies, we found that densitometric analysis of VWF multimers is an accurate tool to analyze VWF multimers and to distinguish the different types of VWD. Previous studies already showed positive results in the technical validation of this method.[15-20] In this study, we found that this method has a good performance in clinical practice. We have analyzed the utility of both medium-large multimer index and large multimer index and found that medium-large multimer index had a better performance in clinical practice. The downside of densitometric VWF multimer analysis is that the analysis cannot automatically detect triplet pattern of multimers or a smear pattern. Therefore, the technique should be optimized to detect such structures. Until then, a quick visual analysis by technicians at the multimeric patterns on the agarose gel itself remains necessary.

Medium-large VWF multimer index had a good correlation with VWF activity measurements. Especially, with VWF:CB/VWF:Ag ratio, which was also found in several previous studies, some even suggested that VWF:CB could be used as an initial test to screen for VWF multimer defects instead of performing time-consuming visual VWF multimer analysis.[18,27] However, this may depend on the type of collagen used in the VWF:CB assay. In the current study, we used collagen type 1, and we acknowledge that the correlation between medium-large VWF multimer index and VWF:CB might be different for VWF:CB measurements in which another type of collagen is used.

In type 1 VWD, an increased clearance of VWF was independently associated with a lower medium-large VWF multimer index. In accordance, Haberichter et al[15] found loss of HMW multimers in patients with type 1C VWD (C1130Y and W1144G mutations), which is characterized by an increased clearance of VWF. In the current study, we confirm an association between an increased clearance of VWF and lower HMW multimers for type 1 VWD patients, irrespective of their specific mutation. Unfortunately, all type 1 Vicenza patients in our cohort had a smear or triplet structure of VWF multimers and could therefore not reliably be analyzed. Furthermore, we found that type 1 patients with a *VWF* gene mutation had lower medium-large VWF multimer index than type 1 patients without a *VWF* gene mutation. This was irrespective of the pathophysiology of reduced VWF levels (ie, increased clearance of VWF versus no increased clearance of VWF). Possibly, some mutations in the *VWF* gene, which lead to a laboratory phenotype of type 1 VWD, may cause a relatively lower VWF multimer index.

Lastly, medium-large VWF multimer index was associated with the BS in type 1 VWD patients. It is known that VWF levels do not fully explain bleeding phenotype in VWD patients.[1] Although patients with VWF levels below 0.10 IU/mL have a more severe bleeding phenotype compared with those with VWF levels above 0.10 IU/mL, there is no linear association observed in patients with historically lowest VWF levels between 0.10 and 0.50 IU/mL.[22,28,29] Thus, the bleeding phenotype of patients is partly determined by other factors, such as age, sex, specific genetic mutations, presence of comorbidities, body weight, and

hemostatic response during hemostatic challenges.[21,22,28-32] In the current study, we found that relative differences in HMW VWF multimers are also associated with the bleeding phenotype of type 1 patients. Additionally, although it was previously shown that type 2A and 2B patients who have reduced HMW multimers have a more severe bleeding phenotype compared with patients with type 2M who have normal multimers, we found in the current study no independent association between HMW multimers and the bleeding phenotype of type 2 VWD patients.[7]

Although several studies have been performed previously to investigate VWF multimer densitometric analysis in VWD patients, to our knowledge, this is the largest study to date in a cohort of well-defined VWD patients, including data on different VWF activity assays and genetic variants. However, there were some potential limitations. Firstly, with the used agarose gel concentration of 0.9% and our analysis method in which we merely focused on the proportion of HMW VWF multimers, we were unable to reliably investigate triplet patterns of VWF multimers. Therefore, those patients were excluded from the remaining analysis. The technique of densitometric VWF multimer analysis should be optimized in the future so it can detect triplet and smear multimer patterns. Secondly, all included patients were already diagnosed with VWD. The next step is to perform VWF multimer densitometric analysis in a large cohort of individuals referred to the hospital with suspected VWD. By performing such a study, 1 can investigate the additional value of densitometric analysis in diagnosing patients with VWD and classifying patients with the (sub)type of VWD. Lastly, the bleeding phenotype was assessed with the BS, which gives an indication of all bleeding during life time. We acknowledge that a prospective evaluation of bleeding phenotype would have been more accurate.

To conclude, VWF multimer densitometric analysis has an excellent accuracy in clinical practice and may have an additional value in providing a better understanding of the clinical features including the bleeding phenotype of VWD patients.

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WiN study group memebers: Amsterdam UMC, Amsterdam, The Netherlands: K. Fijnvandraat, M. Coppens, A. Kors, S. Zweegman; Netherlands Hemophilia Society, Nijkerk, The Netherlands: J. de Meris; Amphia Hospital, Breda, The Netherlands: G. J. Goverde, M. H. Jonkers; Catharina Hospital, Eindhoven, The Netherlands: N. Dors; Maxima Medical Center, Eindhoven, The Netherlands: L. Nieuwenhuizen; University Medical Center Groningen, Groningen, The Netherlands: K. Meijer, R. Y. J. Tamminga; Kennemer Gasthuis, Haarlem, The Netherlands: P. W. van der Linden; HagaZiekenhuis, The Hague, The Netherlands: P. F. Ypma; Leiden University Medical Center, Leiden, The Netherlands: J. G. van der Bom, J. Eikenboom, F. J. W. Smiers; Maastricht University Medical Center, Maastricht, The Netherlands: B. Granzen, E. Beckers, F. C. J. I. Heubel-Moenen; Radboud University Medical Center, Nijmegen, The Netherlands: P. Brons, B. A. P. Laros-van Gorkom; Erasmus University Medical Center, Rotterdam, The Netherlands: M. H. Cnossen, F. W. G. Leebeek (principal investigator), J. Boender, F. Atiq; and Van Creveld Kliniek, University Medical Center, Utrecht, The Netherlands: E. P. Mauser-Bunschoten (chairman steering committee), K. P. M. van Galen.

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