Received: 18 February 2020 | Revised: 28 May 2020 | Accepted: 29 May 2020

DOI: 10.1002/rth2.12408

ORIGINAL ARTICLE

# Improving diagnosis of von Willebrand disease: Reference ranges for von Willebrand factor multimer distribution

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**Handling Editor:** Pantep Angchaisuksiri

## Abstract

**Background:** Phenotypic von Willebrand disease (VWD) classification requires multiple tests including analysis of multimeric distributions von Willebrand factor (VWF) and evaluation of its structure. VWF multimer analysis is labor intensive, nonstandardized, and limited to specialized laboratories. A commercial semiautomatic assay, HYDRAGEL VW multimer assay (H5/11VWM, Sebia), has become available.

**Objectives:** Establishment of reference ranges for H5/11VWM to improve VWD classification.

**Methods:** Implementation validation, establishment and validation of normal and pathological reference intervals (NRIs/PRIs), comparison with in-house method using 40 healthy volunteers and 231 VWD patients.

**Results:** Qualitative and quantitative validation of NRI obtained sensitivity of 88% and 79%, respectively, for type 2. Comparison of the two methods showed an overall concordance of 86% with major conflicting results in all atypical 2B (n = 7) and 50% 2M-GPIb (n = 41) showing quantitative and qualitative multimeric loss, that was not detected with in-house method. We were able to use established PRIs, with 73% validity in type 2 cases, to distinguish individual type 2A subtypes (IIA, IIC, IID, IIE) from 2M and 2B.

**Conclusion:** H5/11VWM could be used for all clinical purposes because its reliability and its rapid and accurate diagnostic ability and reduced observer bias. Although H5/11VWM cannot evaluate triplet structures, we were able to define 2A subtypes by stripping back to the percentage of intermediate/high-molecular-weight multimers. H5/11HWM could be an efficient and widely available alternative for the "gold standard" technique.

KEYWORDS

classification, densitometry, multimers, quantification, reference values, von Willebrand disease

**Essentials**

- Current multimeric assay is not standardized, which influences von Willebrand disease subtyping.
- A new multimeric assay, Hydragel VW multimer analysis, is evaluated.
- We provide pathological reference intervals.
- This new assay can be used to classify von Willebrand disease.

## 1 | INTRODUCTION

von Willebrand disease (VWD), an inherited bleeding disorder caused by a quantitative or qualitative defect in von Willebrand factor (VWF), can be divided into three major types (ISTH Scientific and Standardization Committee [SSC] classification).[1] Type 1 is characterized by partial quantitative deficiency of functionally normal VWF and type 3 with a complete absence. Type 2 VWD is characterized by a qualitatively defective VWF, which results into an asymmetric decrease in VWF activity and VWF antigen (VWF:Ag). Type 2 VWD is subdivided into 2A, 2B, 2M, and 2N based on the specific functional defect(s). Type 2A VWD is further classified into four subgroups (IIA, IIC, IID, and IIE), based on the unique multimeric patterns that occur due to differences in sensitivity to cleavage, dimerization, or multimerization assembly of ADAMTS-13.[2]

Initial VWD diagnosis is made through the "routine" tests that are based on key functions of VWF protein, such as VWF:Ag, factor VIII clotting factor activity (FVIII:C) and VWF:activity (VWF:ristocetin cofactor activity [VWF:RCo] or VWF:glycoprotein Ib [GPIb] binding activity). More specialized tests that can provide additional information about VWD such as ristocetin-induced platelet aggregation (RIPA), VWF:collagen-binding capacity (VWF:CB), VWF propeptide, VWF:FVIII binding capacity (VWF:FVIIIB), VWF multimer assay (VWF:MM) and genetics,[3,4] are commonly restricted to specialized laboratories. The VWD characterization is complicated due to intraindividual variations in VWF:Ag, FVIII:C, VWF:activity, and VWF:FVIIIB (necessary for type 2N identification) levels (ie, stress, inflammation), as well as type and percentage of coefficient of variation (CV) of each assay used. Genetic analysis is not influenced by intraindividual variations. In our experience, VWF:MM (required for subtyping type 2 into 2A/2B and 2M) is also not influenced by intraindividual variations.

VWF is a large multimeric glycoprotein. It is present in plasma composed of a variable number of subunits (500-10 000 kDa) that are linked through disulfide bridges. VWF stored in Weibel-Palade bodies (endothelial cells) or alpha granules (megakaryocytes), is rich in extremely large multimers, and gets cleaved into smaller multimers of different size by ADAMTS-13, after secretion.[5]

Under normal conditions, VWF circulates in the plasma in its globular form. Under high shear stress or vascular injury, VWF unfurls and exposes platelet GPIb receptor binding sites located in its A1 domain.[6] This induces platelet GPIbα interaction, which is responsible for platelet adhesion and aggregation. Therefore, multimers are crucial for normal functioning of platelet. Quantitative and/or qualitative abnormalities in multimers result in defective hemostasis, as hemostatic activity of multimers is directly proportional to their size.

Using electrophoretic protein separation, VWF multimers can be separated into following classes based on their molecular weight (MW): low (LMWM), intermediate (IMWM) and high MW (HMWM). A normal pattern with all multimers and no aberrant triplet structure is seen in types 1, 2M, and 2N, although recently some slight multimeric abnormalities have been observed in types 1 and 2M.[1,7,8] Types 2A and 2B show abnormalities in pathognomonic multimeric pattern and loss of multimers, which cause bleeding.

The VWF multimeric method based on luminographic detection,[9,10] is considered the "gold standard" for diagnosis VWD. It evaluates of the multimer distribution and the triplet structure, which is essential for subtyping 2A into 2A/IIA, IIC, IID, and IIE.[2,3,11-13] The analysis is time consuming, labor intensive, and highly sensitive to many variables (technique, home-made reagents, temperature, working skills, and interpretation experience),[14] which complicates the interpretation of the test and results in high external quality assessment (EQA) error rates (10%-52%)[15,16] and leads to incorrectly influencing VWD subtyping.

Recently, a quick semiautomatic technique called HYDRAGEL-5 or −11 VW multimers (H5/11VWM, Sebia, Lisses, France), has been developed for implementation with an HYDRASYS-2 SCAN system (Sebia).[17-22] It is a more standardized and ready-to-use assay, aimed at making more accurate distinction between normal multimer distribution (types 1, 2M, and 2N) and absence of multimers (types 2A and 2B), with the added benefit of being able to calculate percentage of area under the curve (%AUC).[19]

We evaluated the H5/11VWM technique for its implementation into our laboratory as a screening test. We also wanted to evaluate the ability of H5/11VWM to (sub)classify VWD using quantitative densitometry results.

## 2 | PATIENTS AND METHODS

### 2.1 | Study design

*Part I:* A validation procedure of H5/11VWM for routine use, in line with current laboratory practices and guidelines of Clinical and Laboratory Standards Institute was performed (CLSI: H57-P, EP5-A2, and EP28-A3c). Normal reference intervals (NRIs) were established directly from %AUC values of 40 healthy volunteers and were

**TABLE 1** VWD population

| VWD type | All VWD samples n = 231 (%) | VWD samples for establishment PRI n = 100 (%) | VWD samples for validation PRI n = 131 (%) |
|---|---|---|---|
| Type 1 | 43 (18.6) | 7 (7.0) | 36 (27.5) |
| Type 2A | 104 (45.0) | 58 (58.0) | 46 (35.1) |
| 2A/IIA | 43 (18.6) | 21 (21.0) | 22 (16.8) |
| 2A/IIC | 2 (0.9) | 2 (2.0) | … |
| 2A/IID | … | … | … |
| 2A/IIE | 50 (21.6) | 35 (35.0) | 15 (11.5) |
| 2A-U (unclassified) | 9 (3.9) | … | 9 (6.9) |
| Type 2B | 22 (9.5) | 16 (16.0) | 6 (4.6) |
| Typical 2B | 15 (6.5) | 9 (9.0) | 6 (4.6) |
| Atypical 2B | 7 (3.0) | 7 (7.0) | … |
| Type 2M | 43 (18.6) | 13 (13.0) | 30 (22.9) |
| 2M-GPIb | 41 (17.7) | 11 (11.0) | 30 (22.9) |
| 2M-CB | 1 (0.43) | 1 (1.0) | … |
| 2M-U (unclassified) | 1 (0.43) | 2 (1.0) | … |
| Type 2N | 7 (3.0) | 5 (5.0) | 2 (1.5) |
| Homozygous 2N | 3 (1.3) | 3 (3.0) | … |
| Compound heterozygous with null-allele | 4 (1.7) | 2 (2.0) | 2 (1.5) |
| Type 3 | 12 (5.2) | 1 (1.0) | 11 (8.4) |

*Note:* The study population exists out of 231 patients with genetically documented von Willebrand Disease (VWD) and was used for validation of normal reference intervals. One hundred of 231 samples were used for defining the PRIs, which were validated using the remaining 131 samples. VWD classification was done according to the current ISTH Scientific and Standardization Committee classification[1] with an additional subdivision of type 2A into types 2A/IIA. 2A/IIC. 2A/IID, and 2A/IIE.[2,3,11-13] Atypical type 2B patients are those with enhanced responsiveness to low-dose ristocetin. *VWF* mutation in the A1 domain but with normal VWF activity to VWF:Ag ratios results in normal VWF multimers.[28-30] Type 2M are characterized by a reduced VWF activity to VWF:Ag ratio; (2M-GPIb and 2M-Unclassified (U) with a VWF:GPIb defect and a VWF:CB defect for 2M-CB).

Abbreviations: PRI, pathological reference interval; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor:antigen; VWF:CB, von Willebrand factor:collagen-binding capacityVWF:GPIb, von Willebrand factor:glycoprotein Ib.

validated with 231 genetically documented VWD samples (Table 1). Specificity and sensitivity for type 2 VWD were determined using visual evaluation (qualitative) and densitometry %AUC results (quantitative). Effectivity of the in-house method and H5/11VWM was evaluated by comparing all VWD samples. Additionally, we performed the analysis on 4 patients with acquired Von Willebrand syndrome (aVWS), both "immunological" (monoclonal IgG, IgM, and benign lymphocytosis) and "mechanical" (essential thrombocythemia).

*Part II:* Pathological reference intervals (PRIs) for each separate VWD (sub)type (cfr. ISTH/SSC VWD classification[1] with an additional subclassification of type 2A into 2A/IIA, IIC, IIE and IID[2,3,11-13] were established using 100 VWD samples and were validated using another 131 VWD samples (Table 1). If validated, adjustment of PRI was done based on the total VWD cohort (n = 231).

*Part III:* Subclassification of VWD (solely) using H5/11VWM %AUC results was performed using all VWD samples (Table 1). Correlation with the final VWD classification and laboratory phenotype (based on FVIII:C, VWF:Ag, VWF:GPIb).

## 2.2 | VWD classification

ISTH/SSC VWD classification[1] with subdivision of type 2A into 2A/IIA, 2A/IIC, 2A/IID and 2A/IIE[2,3,11-13] "extended ISTH/SSC VWD classification."

Type 1: Equally reduced VWF parameters, normal multimers.

Type 3: VWF:Ag and VWF:GPIb < 5.0IU/dL.

Type 2: VWF:GPIb/VWF:Ag and/or VWF:CB/VWF:Ag < 0.60[23-26] (except 2N).

Type 2A: VWF:GPIb/VWF:Ag and VWF:CB/VWF:Ag < 0.60,[23] multimeric loss and aberrant triplet structure.[2]

- 2A/IIA: pronounced first subband and mutation in A2 domain.
- 2A/IIC: pronounced protomer and mutation in D2 domain.
- 2A/IIE: absence of triplet structure and mutation in D3 domain.
- 2A/IID: absence of triplet structure and odd number of monomers and mutation in CK-terminal.
- 2A-U: no mutation in A2, D2, D3, CK.

Type 2B:

- Typical 2B: enhanced responsiveness to low dose (0.6 mg/mL) RIPA,[27], VWF:GPIb/VWF:Ag and VWF:CB/VWF:Ag < 0.60, multimeric loss and mutation in A1 domain.
- "Atypical" type 2B with enhanced responsiveness to low dose (0.6 mg/mL) RIPA,[27] normal VWF:GPIb/VWF:Ag and VWF:CB/VWF:Ag, normal VWF multimeric pattern.[28-30]

Type 2M: normal VWF multimers.[31,32]

- 2M-GPIb: VWF:GPIb/VWF:Ag < 0.60 and mutation A1 domain.[11,33,34]
- Type 2M-U: VWF:GPIb/VWF:Ag < 0.60 and mutation outside A1 domain.
- Type 2M-CB: VWF:GPIb/VWF:Ag > 0.60, VWF:CB/VWF:Ag < 0.60 and mutation in A3 domain.

Type 2N: FVIII:C/VWF:Ag < 0.60, defective VWF:FVIIIB and mutation in D′-D3.

Where the laboratory phenotype (based on the three most common assays: FVIII, VWF:Ag, and VWF:GPIb), VWF:CB, and the multimeric and genetic (Sanger sequence and Muliplex ligation-dependent probe amplification) results were not concordant, *VWF* mutation (restricted to specific domain) and multimeric pattern were considered more important for final VWD classification.

## 2.3 | In-house method

Our VWF:MM assay currently in use has been duplicated from the method developed by Schneppenheim et al[7] and Budde et al,[9,10] with subtle modifications done in our laboratory. Visualization by chemiluminescene was performed on an Optigo-750 CCD photo Imager (Isogen Life Science, Utrecht, the Netherlands) with TotalLab-100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to evaluate substructures of the different multimers, triplet structures, and their distribution. The visual number of multimeric bands was determined visually, with guideline values of 18-22 multimers defining a normal distribution.[9] Densitometry curves were routinely created but the software was not able to quantify AUC.

## 2.4 | HYDRAGEL VW multimer assay

The HYDRAGEL VW multimer assay was performed on the HYDRASYS-2 SCAN system (Sebia) using the HYDRAGEL 5- or 11-VW multimer kit (H5/11VWM, Sebia) and was visualized with the PHORESIS software program version 8.63 (Sebia), according to the manufacturer's instructions,[22] with results available within 1 day. Electrophoretic VWF multimer separation according to their MW was carried out on a 2% agarose gel and the separated proteins were immunoprecipitated with a specific anti-VWF antiserum. Different bands were visualized using a peroxidase-labeled antibody and a specific substrate. The HYDRASYS-2 SCAN (Sebia) performed all steps, including visualization, with regular manual interventions from the technician. Apart from the visual evaluation, the PHORESIS CORE software also offers quantification of the densitometry. After defining the multimer fractions (LMWM: peak 1-3, IMWM: peak 4-7 and HMWM: peak > 7), the AUC was calculated automatically and expressed as relative AUC, for example, total of HMWM divided by the overall AUC of all bands (%AUC). A normal plasma control (NPC) was performed on each gel, similar to the one that is performed for the in-house method.

## 2.5 | Ethical issues

The study used plasma samples from healthy volunteers and from the Antwerp VWD biobank. The use of normal donor plasma was approved by the Antwerp University Hospital Ethics Committee, and volunteers signed an informed consent form. Samples from Antwerp-VWD Biobank were used with the approval of the Antwerp University Hospital Ethics Committee, which permitted the use of residual patient plasma. The database and biobank (plasma and DNA) are registered with the Antwerp University Hospital data protection authority.

## 2.6 | Statistical analysis

Statistical analysis was performed using IBM SPSS statistics software, version 21.0 (SPSS Inc, IBM Corporation US, Armonk, NY, USA). Results were expressed in mean values and CV. Statistical tests for normality of distribution using SPSS (ie, Kurtosis-risk, Pearson's chi-squared test, Shapiro-Wilk test, and Kolmogorov-Smirnov test) were performed. All reference intervals were established by using the 95% confidence interval. As our established NRIs were verified with previously published NRIs,[17] our use of 40 individuals was methodologically justified.[35]

# 3 | RESULTS

## 3.1 | Part I: Assay validation

The HYDRAGEL 5- or 11- VW multimer assay (H5/11VWM) within- and between-run variability and intraindividual variations resulted in acceptable repeatability with comparable multimeric patterns and CVs < 10% (Table S1). Lower limit of detection was set at 5 IU/dL (Figure S1).

NRIs for LMWM, IMWM, and HMWM were defined using 40 healthy volunteers (Table 2). All of them demonstrated normal multimeric distribution with equal intensity of bands when compared with that of NPC (Table S2). No significant difference was seen for %AUC

**TABLE 2** NRIs and PRIs and mean %AUC for each VWD (sub)type

| Type VWD | LMWM mean %AUC (95% CI PRI) | IMWM mean %AUC (95% CI PRI) | HMWM mean %AUC (95% CI PRI) |
|---|---|---|---|
| Normal population (n = 40) | 17.4 (10.2-24.6) | 30.6 (24.0-37.)2 | 52.0 (40.8-63.2) |
| Type 1 (n = 43) | 19.6 (18.0-21.2) | 26.8 (26.7-27.8) | 53.6 (51.7-55.6) |
| Type 2A (n = 104) | 55.8 (34.1-84.9)[a] | 25.7 (15.0-42.3)[a] | 18.4 (0-34.3)[a] |
| Type 2A/IIA (n = 43) | 81.0 (77.1-84.9) | 16.9 (15.0-18.9) | 1.9 (0-4.6) |
| Type 2A/IIC (n = 2) | 35.4 incalculable | 34.8 incalculable | 29.9 incalculable |
| Type 2A/IID (n = 0) | No patients included | No patients included | No patients included |
| Type 2A/IIE (n = 50) | 37.9 (34.6-41.1) | 30.6 (29.1-32.2) | 31.5 (28.7-34.3) |
| Type 2A-U (n = 9) | 39.1 (34.1-44.2) | 38.5 (34.7-42.3) | 21.6 (17.9-25.3) |
| Type 2B (n = 22) | 49.5 (25.5-65.9)[a] | 31.5 (26.8-34.3)[a] | 18.9 (6.3-42.4)[a] |
| Typical 2B (n = 15) | 58.8 (51.6-65.9) | 30.6 (26.8-34.3) | 10.6 (6.3-14.9) |
| Atypical 2B (n = 7) | 29.7 (25.5-33.9) | 33.4 (29.6-37.2) | 36.8 (31.3-42.4) |
| Type 2M (n = 43) | 27.8 (24.5-31.8)[a] | 28.6 (27.0-30.3)[a] | 43.9 (39.6-47.3)[a] |
| Type 2M-GPIb (n = 41) | 28.2 (24.5-31.8) | 28.7 (27.0-30.3) | 43.4 (39.6-47.3) |
| 2M-CB (n = 1) | 26.0 incalculable | 27.8 incalculable | 46.2 incalculable |
| 2M-U (n = 1) | 13.5 incalculable | 26.0 incalculable | 60.5 incalculable |
| Type 2N (n = 7) | 17.3 (14.2-20.4) | 27.2 (24.5-29.8) | 55.5 (50.3-60.7) |

*Note:* Final adjusted PRIs were established out of 231 VWD samples for densitometry fromLMWMs (peak 1-3), IMWMs (peak 4-7), and HMWMs (peak > 7).

Abbreviations: %AUC, percentage of area under the curve; CI, confidence interval; HMWMs, high-molecular-weight multimers; IMWMs, intermediate-molecular-weight multimers; LMWMs, low-molecular-weight multimers; NRIs, normal reference intervals; PRIs, pathological reference intervals; VWD, von Willebrand disease.

[a]PRIs for types 2A, 2B, and 2M as group were composite reference intervals. PRIs could not be calculated for types 2A/IIC, 2A/IID, 2M-CB, and 2M-U because of low/absent numbers.

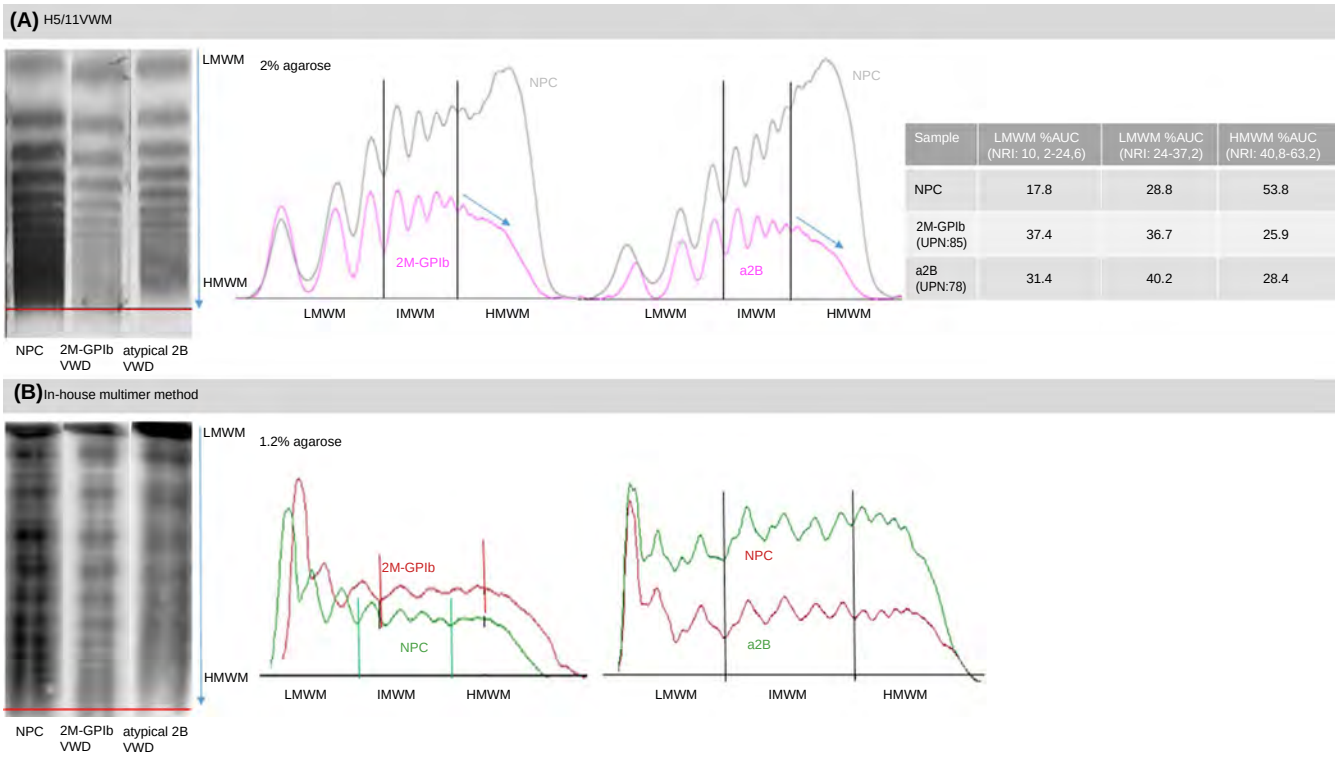
between blood groups O (n = 19) and non-O (n = 21) (Δ = 0.58%; 95% CI, −83.2 to −80.9).

H5/11VWM results of all VWD samples (Table 1) were evaluated visually (qualitative) and by densitometry analysis (quantitative) (Table S3; unique patient number [UPN] 1-231). All type 1 (n = 43) and 2N (n = 7) patients demonstrated qualitatively normal multimers, and densitometry results were within the estimated NRI. Both of these types were distinguished from normal samples due to their low intensity of bands, which indicated decreased VWF levels. No multimeric pattern was seen in type 3 VWD (n = 12).

Multimeric loss in 149 of 169 type 2 samples (non–type 2N) could be detected visually, while a normal distribution was observed for 20 of 169 type 2 patients, resulting in 88.2% sensitivity and 100% specificity for type 2 diagnosis (subjected to observer's experience). Quantitative densitometry results reached 79.3% sensitivity and 100% specificity for type 2, which was lower than that seen in visual interpretation by a trained technician. Thirty-five of 169 type 2 patients (20.7%) showed normal HMWM %AUC results, but 15 of these patients had a visual loss by naked eye: 9 type 2A/IIE (n = 50), 1 type a2B (n = 7), 4 type 2M-GPIb (n = 41), and 1 type 2M-CB (n = 1).

H5/11VWM results of all 231 VWD samples were compared with those obtained using the in-house method. Qualitative evaluation revealed identical results in both methods for 199 of 231 (86.1%) samples: all cases of type 1 (n = 43), type 2A/IIA (n = 43), type 2A/IIC (n = 2), type 2B (n = 15), type 2A-U (n = 9), type 2N (n = 7), and type 3 (n = 12) and for 48 type 2A/IIE (n = 50), 19 type 2M-GPIb (n = 41), and 1 type 2M-U (n = 1). However, for 32 of 231 (13.9%) a discrepancy in results was seen between both methods: 22 of 41 type 2M-GPIb, 7 of 7 atypical 2B (a2B), 2 of 50 type 2A/IIE and 1 of 1 2M-CB (Table S3).

For all a2B (UPN 65, 74-78 and 80), the H5/11VWM densitometry results suggested loss of multimers as seen during visual observation, where it was not the case for the in-house method (Figure 1). This was also confirmed quantitatively for 6 of 7 patients.




| Sample | LMWM %AUC (NRI: 10, 2-24,6) | LMWM %AUC (NRI: 24-37,2) | HMWM %AUC (NRI: 40,8-63,2) |
|---|---|---|---|
| NPC | 17.8 | 28.8 | 53.8 |
| 2M-GPIb (UPN:85) | 37.4 | 36.7 | 25.9 |
| a2B (UPN:78) | 31.4 | 40.2 | 28.4 |

**FIGURE 1** Comparison of multimeric patterns between the Hydragel von Willebrand multimer (H5/11VWM) and in-house method for patients with type 2M-GPIb and atypical 2B (a2B) von Willebrand disease (VWD). (A, B) Example of von Willebrand factor multimeric electrophoresis and densitometry of a patient with type 2M-GPI and atypical 2B (a2B) VWD (patients: UPN 85 and 78, respectively). Densitometry from low-molecular-weight multimers (LMWMs, peak 1-3), intermediate-molecular-weight-multimers (IMWMs, peak 4-7) and high-molecular-weight multimers (HMWMs, peak > 7). (A) Results for Hydragel VW multimers (H5/11VWM). Compared with the normal plasma control (NPC), a qualitative loss of HMWM was seen in both patients (marked by an arrow) and confirmed with a quantitative multimeric loss with a percentage of area under the curve (%AUC) below the normal reference interval (NRI), individual results are illustrated in the table. (B) Results for the in-house multimeric method. No multimeric loss was seen between both VWD types and the NPC

For approximately half (22/41) of type 2M-GPIb and 2M-CB cases, the H5/11VWM suggested a qualitative multimeric loss, while the in-house method showed a quantitative normal distribution (Figure 1). In 17 of 22 type 2M-GPIb (UPN 83-86, 91, 117, 118, 164, 169, 170, 173, 212, 213, 217, 221-223) this was also confirmed by a quantitative multimeric loss. 5/22 2M-GPIb (UPN 88-90, 92, and 168) and the 2M-CB (UPN 93) showed a "normal" %AUC.

Two of 50 type 2A/IIE cases showed qualitative normal multimer distribution with H5/11VWM, which were not detected by the in-house method (UPN 18 and 34). Quantitatively, both methods showed "normal" %AUC. We were unable to find an explanation for this phenomenon.

All patients with aVWS, either suffering from immunologically (one IgG, one IgM, and one benign lymphocytosis) or mechanically (one essential thrombocythemia) mediated VWS, showed a qualitative and quantitative loss of multimers with both VWF:MM methods.

## 3.2 | Part II: PRIs

PRIs for each VWD (sub)type (cfr. "extended ISTH/SSC VWD classification[1-3,11-13]) were established with 100 of 231 VWD samples and were validated with the other 131 samples (Table S3; UPN 1-100 and 101-231), which were used to generate the final adjusted PRI for each (sub)type based on the total VWD population (Table 2). Ranges could not be calculated for type 2A/IIC, 2A/IID, 2M-CB, and 2M-U because of low or absent numbers. However, we observed the typical pattern with pronounced protomer for type 2A/IIC (Figure 2).

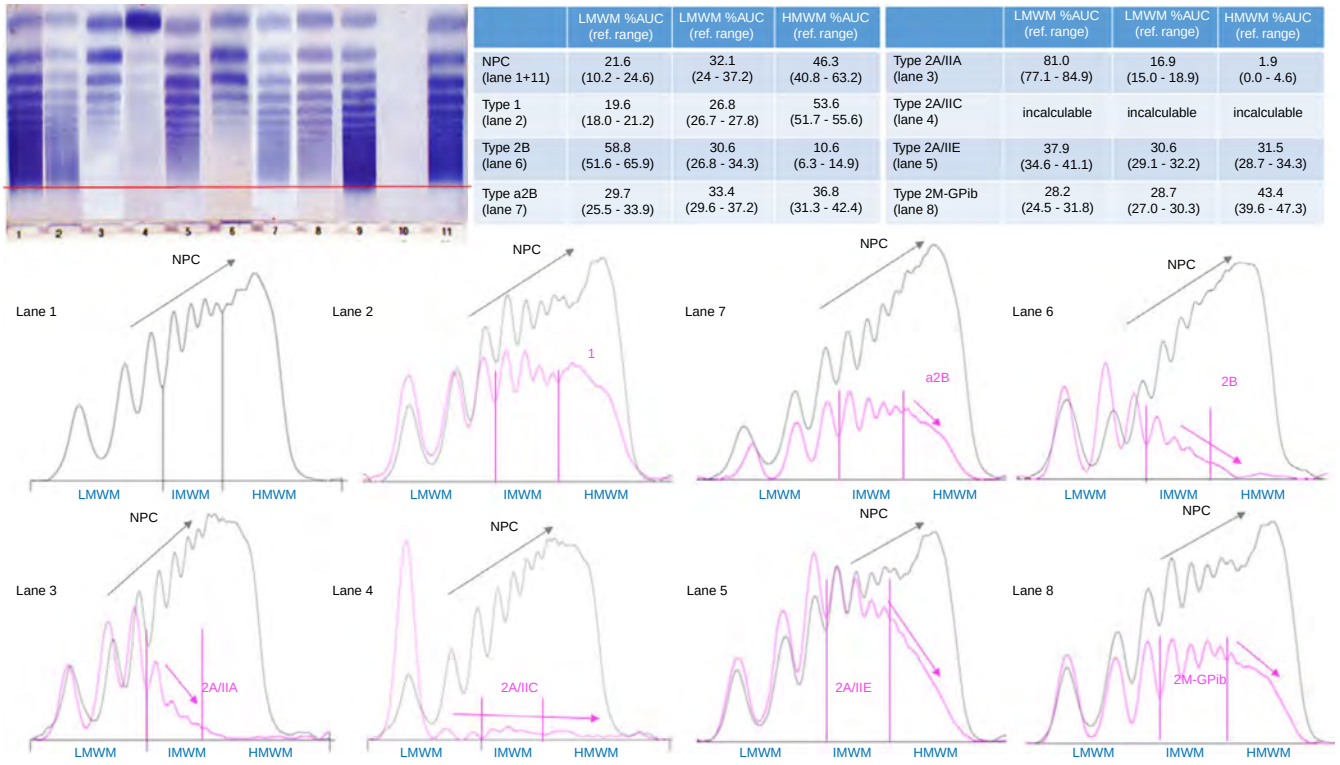
As they have qualitative and quantitative normal multimers, reference ranges of type 1 and 2N VWD overlapped with those of normal population. A partial overlap with the normal population was seen for type 2M-GPIb and a2B as well. Based on current ISTH/SSC VWD classification,[1] these PRIs successfully distinguished type 2A (as group) from type 2M but were unable to distinguish them from type 2B (Figure 3A). However, due to the establishment of PRIs for individual subtypes of 2A (IIA, IIE), and division of 2B (typical and atypical), no overlap was seen among typical type 2B and the individual subtypes of type 2A (IIA and IIE) (Figure 3B).

## 3.3 | Part III: VWD (sub)classification

As shown in Part I, no multimer abnormalities were seen for type 1, 2N, and 3 VWD samples. The remaining 169 (non–type

2N) type 2 (type 2A (n = 104), (2A/IIA (n = 43), 2A/IIC (n = 2), 2A/IIE (n = 50), 2A-U (n = 9)), 2B (n = 15), a2B (n = 7), 2M-GPIb (n = 41), 2M-CB (n = 1), and 2M-U (n = 1)) were selected for further evaluation.

By using only the three most commonly available assays (FVIII:C, VWF:Ag, and VWF:GPIb), VWD classification was limited to its main "primary level" types (types 1, 2 [without further subdivision], and 3) and 123 of 169 (73%) of all type 2 samples were classified correctly (Figure 4).




| | LMWM %AUC (ref. range) | LMWM %AUC (ref. range) | HMWM %AUC (ref. range) |
|---|---|---|---|
| NPC (lane 1+11) | 21.6 (10.2 - 24.6) | 32.1 (24 - 37.2) | 46.3 (40.8 - 63.2) |
| Type 1 (lane 2) | 19.6 (18.0 - 21.2) | 26.8 (26.7 - 27.8) | 53.6 (51.7 - 55.6) |
| Type 2B (lane 6) | 58.8 (51.6 - 65.9) | 30.6 (26.8 - 34.3) | 10.6 (6.3 - 14.9) |
| Type a2B (lane 7) | 29.7 (25.5 - 33.9) | 33.4 (29.6 - 37.2) | 36.8 (31.3 - 42.4) |

| | LMWM %AUC (ref. range) | LMWM %AUC (ref. range) | HMWM %AUC (ref. range) |
|---|---|---|---|
| Type 2A/IIA (lane 3) | 81.0 (77.1 - 84.9) | 16.9 (15.0 - 18.9) | 1.9 (0.0 - 4.6) |
| Type 2A/IIC (lane 4) | incalculable | incalculable | incalculable |
| Type 2A/IIE (lane 5) | 37.9 (34.6 - 41.1) | 30.6 (29.1 - 32.2) | 31.5 (28.7 - 34.3) |
| Type 2M-GPib (lane 8) | 28.2 (24.5 - 31.8) | 28.7 (27.0 - 30.3) | 43.4 (39.6 - 47.3) |

**FIGURE 2** Overview of different von Willebrand disease (VWD) patterns with the Hydragel von Willebrand multimer (H5/11VWM). Example of Hydragel VW multimers (H5/11VWM) electrophoresis and densitometry of a normal plasma control (NPC) and different types of VWD. Densitometry from low-molecular-weight multimers (LMWMs, peak 1-3), intermediate-molecular-weight multimers (IMWMs, peak 4-7) and high-molecular-weight multimers (HMWMs, peak > 7). For each different type of VWD, the mean percentage of area under the curve (%AUC) for LMWM, IMWM, and HMWM and pathological reference intervals (PRI) are illustrated in the tables. Lanes 1 and 11, NPC; lane 2, patient with type 1 VWD; lane 3, type 2A/IIA VWD; lane 4, type 2A/IIC VWD; lane 5, type 2A/IIE; lane 6, type 2B VWD; lane 7, atypical 2B (a2B); lane 8, type 2M-GPIb VWD; lane 9, type 2N VWD; lane 10, type 3 VWD. No densitometry was given for lanes 9 and 10

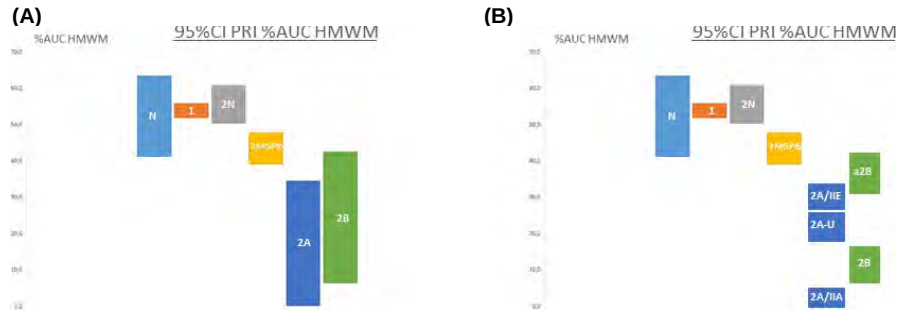



**FIGURE 3** Boxplots of 95% confidence interval (CI) pathological reference intervals (PRIs) for each von Willebrand disease (VWD) type. (A, B) Plots of 95% CI PRIs of percentage area under the curve of high-molecular-weight multimers (%AUC HMWMs) for each VWD type. (A) Boxplots for VWD classification based on current ISTH Scientific and Standardization Committee (SSC) VWD classification (1) "secondary level." Not unexpectedly, reference ranges of types 1 and 2N VWD overlapped with the normal population, since they have qualitative and quantitative normal multimers. Type 2A (as group) can be distinguished from type 2M-GPIb but not from type 2B (as group). A partial overlap with the normal population was seen for types 2M-GPIb and 2B. (B) Boxplots for each VWD type based on current ISTH/SSC VWD classification (1) with additional subdivision of type 2A into subclasses (2A/IIA, IIC, IID and IIE), 2B into typical and atypical 2B (a2B) "tertiary level." At this level, there is no overlap between typical 2B and the individual subtypes of type 2A (IIA and IIE). A partial overlap of type 2M-GPIb and a2B with the normal population is present and between 2M-GPIb and a2B as well

Adding the VWF:CB to this basic phenotype panel allowed for subclassification of type 2[8,32] into types 2A, 2B, and 2M (current classification by Sadler et al[1]), and 67% (114/169) of type 2 samples were correctly classified. This lower percentage could be attributed to the fact that it was impossible to distinguish type 2A from 2B by using FVIII:C, VWF:Ag, VWF:GPIb, and VWF:CB, as it needed (low dose) RIPA to differentiate them correctly. Replacing VWF:CB with the multimeric results as an addition to the basic laboratory phenotype, showed a concordance with the final classification in 67% (113/169) and 69.8% (118/169) for the H5/11VWM and in-house methods, respectively. In view of the similar results it could be supposed VWF:CB and VWF:MM reflected the same cases. However, addition of VWF:CB and multimeric results to the basic laboratory tests, led to an increase in the concordance (79% and 88% for H5/11VWM and in-house methods, respectively), confirming that both tests represented separate information.

Use of the "extended ISTH/SSC VWD classification" yielded concordant results between the H5/11VWM %AUC (along with the laboratory phenotype) and final classification in 55% of all type 2, and 70% with the gold standard technique. Adding VWF:CB test results resulted in accurate classification of 73% with H5/11VWM and 79% with the in-house method.

### 3.4 | Additional analysis on intraindividual variation

In view of our results, we were anxious to see there was an issue with intraindividual variation of the multimeric analysis and we went back to de Antwerp-VWD Biobank to perform the H5/11VWM assay on eight different patients (four patients with congenital VWD and four with aVWS) with blood drawn at two different times. We did not find any differences between the two time points (Table S1).

## 4 | DISCUSSION

The strict validation of the semiautomated Hydragel 5 or 11 VW multimer technique (H5/11VWM) showed findings that are in line

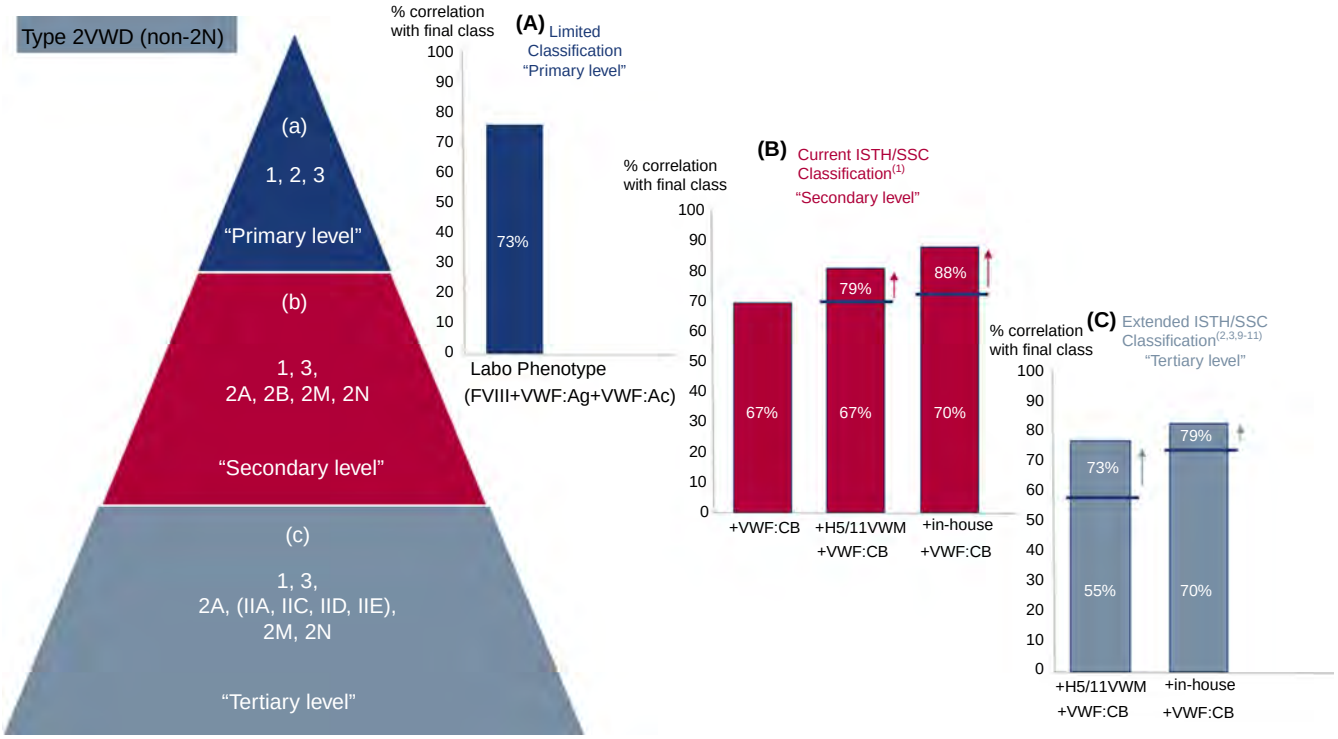



**FIGURE 4** Correlation of von Willebrand Factor (VWF) results obtained with different (combination) VWF assays with the final given classification of von Willebrand disease. (A) Using only the three most common assays (factor VIII clotting factor activity [FVIII:C], VWF antigen [VWF:Ag], and VWF:glycoprotein Ib [VWF:GPIb]), the VWD classification is limited to its main types, "primary levels" (types 1, 2 [without subdivision], and 3), with a correct classification of 73% of all type 2 samples. (B) Adding the VWF:CB to this basic phenotype panel allows subclassification according to the current classification, Sadler et al[1] "secondary levels." At this moment, 67% of type 2 samples were correctly classified with a major remark that type 2A cannot be distinguished from 2B as the low-dose RIPA is required. Adding the multimeric results to the phenotype laboratory instead of VWF:collagen-binding capacity (VWF:CB), showed a concordance in 67% (H5/11VWM) and 69.8% (in-house). Adding both VWF:CB and multimeric results to the phenotype laboratory, the concordance will increase up to 79% (H5/11VWM) and 88% (in-house). (C) Concordant results were obtained in 55% (H5/11VWM) and 70% (in-house) when only multimeric testing is added to the laboratory phenotype and 73% (H5/11VWM) and 79% (in-house) if VWF:CB is added in the "extended" ISTH Scientific and Standardization Committee VWD classification

with those reported by Bowyer et al.[17] A total of 231 VWD samples were analyzed using in-house and H5/11VWM methods, and showed an overall concordance of 86.1%. Apart from some minor differences, major conflicting results were observed in all atypical 2B (a2B) and 50% of all type 2M-GPIb VWD, with qualitative and quantitative loss of HMWM seen with H5/11VWM but not with the in-house method. This discordance could be a result of analytical differences between the methods or suboptimal execution of the in-house method, which is prone to manual errors.

As general laboratories can perform only the three most common assays (FVIII, VWF:Ag, and VWF:GPIb), they can only classify VWD into primary levels (1, 2, and 3). The addition of VWF:CB to this basic assay panel,[8,32] allows for further classification according to the current ISTH/SSC classification (type 1, 2A/2B, 2M, 2N, and 3 VWD, secondary levels) and in our study this could be done correctly in 67% all type 2. In the absence of VWF:CB (as is the case in the United States[16]), the multimeric analysis is the only mean to distinguish 2A/2B from 2M from 1, although the low-dose RIPA is required to differentiate type 2A from 2B.[27] The H5/11VWM achieved a concordance of 67% with the final classification for all type 2 VWD cases, and addition of VWF:CB assay increased the concordance to 80%. These results demonstrate the relative importance of multimeric analysis in first line, and this was more easily achieved with H5/11VWM than with the more cumbersome in-house method. Furthermore, the VWF:MM did not show any intraindividual variation, which was evaluated additionally in this study.

The established NRIs, tested with normal and known VWD samples, were broadly similar to those reported by Bowyer et al,[17] in our study with more type 2 cases both in absolute numbers and as a percentage of the VWD group, and with a sensitivity of 88% based on visual evaluation (highly dependent on observers' skills) and 79.3% for quantitative evaluation (removing observer bias).

We also established PRIs for different VWD types based on a cohort of 231 VWD patients, which we believe could be used in clinical practice. It is important to state that there was an overlap between the type 2A and 2B (as a group), but this could be resolved by low-dose RIPA,[14] division of 2B and atypical 2B and the use of individual PRIs for 2A subtypes (with some reservations for 2A/IIC and IID where PRIs could not be established because of low patient numbers).

The multimer analysis can subclassify type 2A VWD into its subclasses (2A/IIA, IIC, IID, and IIE) on the basis of differences in triplet structure, which can be nicely visualized with the in-house method. Although the H5/11VWM was not able to detect triplet abnormalities (due to unadaptable agarose concentration), it was able to define these 2A subtypes (with reservations for 2A/IIC and IID) by stripping back to the percentage of IMWM and HMWM, thereby demonstrating that for the hemostatic capacity the volume of IMWM and HWMW outweighs the importance of the structural triplet abnormalities.

Major differences between H5/11VWM and in-house method were seen within the types 2M-GpIb and a2B groups. H5/11VWM was able to detect qualitative and also quantitative multimeric loss in 50% of types 2M-GpIb and all a2B, where this was, except for some subtle changes, "classically" not thought to be the case.[1,28-30] Although the definition of these types was based on the conventional method, we believe that this method is less sensitive to "subtle" multimeric losses, and that conceivably H5/11VWM results could detect a subtle loss of HMWM that participates in the pathophysiological process in these subtypes. Although "subtle" loss of multimers in these types has been seen in some studies,[1,7,36] we believe that our results have reopened the discussions about types 2M-GpIb and a2B.

Multimeric abnormalities observed within our type 2M-GPIb patients were linked to different A1 domain mutations, with p.Arg1315Cys and p.Arg1374Cys having the highest prevalence. Both variants are described to be linked to more than one VWD type, eg, type 2A and 2M. Casonato et al,[36] Ribba et al,[37] Doruelo et al,[38] and Bowyer et al[17] have identified patients with these variants and have classified them as type 2M patients with an aberrant multimerization.

Favaloro et al[20] and Oliver et al[21] have also reported the presence of all HMWM in their type 2M patients, but their studies do not provide any information about its quantification and the diagnosis of type 2M. On the other hand, Bowyer et al[17] and a recent report by Favaloro et al[39] published quantitative HMWM loss in some of the type 2M patients. However, Crist et al[18] and Pikta et al[19] do not mention type 2M VWD in their studies about H5/11VWM.

Compared with these previous publications[17-21], the strength of our analysis lies in the larger number of samples (n = 275:40 normal, 231 known VWD and 4 acquired VWS), and the extensive VWD typing with all available laboratory techniques including final classification done by an expert panel according to the ISTH/SSC classification[1], and subclassification of types 2A (IIA, IIC, IIE)[2,3,11-13], 2B (2B, a2B[28-30]), and 2M (2M-GpIb,[11,33,34] 2M-U). These results indicate that the normal and pathological reference intervals are scientifically well substantiated.

One prominent weakness of our analysis lies in the comparison with the "traditional" in-house electrophoresis method, which is very sensitive to technical and observer issues. We acknowledge the possibility that different results could be seen by another observer in another experimental setup, but this does not affect the findings from the H5/11VWM.

We wanted to evaluate suitability of H5/11VWM as a screening technique that could select the samples, which would go forward for further evaluation with the gold standard technique to decrease the workload of the latter. In practice, however, we are now using the H5/11VWM for all clinical purposes because of its simple introduction, reliability, short duration, and cost effectiveness. Our work offers the possibility of quantitative evaluation, reduction in observer bias, and has standardized a "visual" technique with PRIs that could simplify VWD classification. The results were comparable with those obtained using in-house method, where expert interpretation is a requirement. For research practice, we recommend using both assays: our in-house method to obtain information about triplet structure and to understand more about the

underlying mechanisms, and the H5/11VWM for the quantification of the multimers.

## ACKNOWLEDGEMENTS

The authors thank Bayer Healthcare Belgium for provision of the HYDRASYS analyzer.

## RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

IV and AG designed the study. IV collected subject data. IV performed the experiments and wrote the manuscript. AG was responsible for review and approval of the final manuscript for submission.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Vangenechten I, Gadisseur A. Improving diagnosis of von Willebrand disease: Reference ranges for von Willebrand factor multimer distribution. *Res Pract Thromb Haemost*. 2020;00:1–11. https://doi.org/10.1002/rth2.12408