DOCTORAL THESIS

Implementation of Innovative Techniques in the Diagnostic Work-up of von Willebrand Disease

Marika Pikta

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TAL TECH PRESS

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Marika Pikta

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Innovaatiliste meetodite juurutamine von Willebrandi tõve diagnostikas

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II **Pikta M,** Zemtsovskaja G, Bautista H, Nouadje G, Szanto T, Viigimaa M, Banys V. (2018). Preclinical evaluation of a semi-automated and rapid commercial electrophoresis assay for von Willebrand factor multimers. Journal of clinical laboratory analysis, *32*(6), e22416. https://doi.org/10.1002/jcla.22416

III **Pikta M,** Szanto T, Viigimaa M, Lejniece S, Balode D, Saks K, Banys V. (2021). Evaluation of a new semi-automated Hydragel 11 von Willebrand factor multimers assay kit for routine use. Journal of medical biochemistry, *40*(2), 167–172. https://doi.org/10.5937/jomb0-26008

IV **Pikta M**, Saks K, Varik M, Hytti M, Ilves K, Ross M. (2021). Assessment of the von Willebrand factor multimers profile in patients referred for bleeding tendency evaluation in Estonia: a preliminary report of the von Willebrand disease diagnostics project. Int. J. Med. Lab. Res., 6,1, 17–27. http://doi.org/10.35503/IJMLR.2021.6103

V **Pikta M**, Banys V, Szanto T, Joutsi-Korhonen L, Vaide I, Varik M, Lehtinen A. E, Giangrande P, Laane E. (2021). Von Willebrand Factor Multimeric Assay in Acquired von Willebrand Disease Diagnosis: A Report of Experience from North Estonia Medical Centre. Journal of laboratory physicians, *13*(3), 195–201. https://doi.org/10.1055/s-0041-1730818

VI **Pikta M,** Vasse M, Smock K. J, Moser K. A, van Dievoet M.A, Lejniece S, Szanto T, Bautista H, Nouadje G, Banys, V. (2022). Establishing reference intervals for von Willebrand factor multimers. Journal of medical biochemistry, *41*(1), 115–121. https://doi.org/10.5937/jomb0-31941

Author's Contribution to the Publications

Contribution to the papers in this thesis are:

I Study concept and design, interpretation of data, manuscript drafting, study supervision.

II Study concept and design, interpretation of data, manuscript drafting, study supervision.

III Study concept and design, recruitment of healthy volunteers, supervising the study, interpreting data, and drafting the manuscript.

IV Study concept and design, data acquisition, statistical analysis, manuscript drafting, study supervision, and critical revision of the manuscript for important intellectual content.

V Study concept and design, data acquisition, statistical analysis, data analysis and interpretation, manuscript drafting, study supervision, and critical revision of the manuscript for important intellectual content.

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Approbation

- **Pikta M**, Szanto T, Banys V. Evaluation of within-subject variation of VWF multimers assay. In proceedings of the 30th Congress of the International Society on Thrombosis and Haemostasis (ISTH), London, England, U.K., July 9–13, 2022.
- **Pikta M**, Szanto T, Reimal R, Muliin P, König H, Blinova G, Banys V. (2021). Analysis of Coagulation Factors in Fresh-frozen Plasma. In proceedings of the XXIX Congress of the International Society on Thrombosis and Haemostasis (ISTH), Philadelphia, U.S., July 17–21, 2021.
- Balode D, **Pikta M**, Lejniece S. von Willebrand factor multimer assay as a more accurate diagnostic tool for distinguishing between von Willebrand disease types. In proceedings of Rīga Stradiņš University (RSU) Research Week 2019. Rīgas Stradiņa universitāte.
- **Pikta M**, Banys V, Vaide I, Varik M, Saks K, Lepik K, Hein M, Joutsi-Korhonen L, Szanto T, Lassila R, Armstrong E, Giangrande P, Laane, E. Development of diagnostic algorithm for von Willebrand disease within WFH the Twinning Tallinn-Helsinki program. In proceedings of the World Federation of Hemophilia World Congress, Glasgow, Scotland, May 20–24, 2018.
- **Pikta M**, Laane E, Zolotareva V, Titova T, Banys V. In Freezer Storage of Plasmas for Delayed Coagulation Factors Testing Does Not Affect the Results. In proceedings of the 14th Baltic Congress of Laboratory Medicine, May 10–12, 2018, Vilnius, Lithuania.
- **Pikta M.** Improvement in von Willebrand disease laboratory diagnostics as a result of Tallinn-Helsinki WFH Twinning Program. Presented (oral presentation) at the 14th Baltic Congress of Laboratory Medicine, Vilnius, Lithuania, 12.05.2018.
- **Pikta M.** Diagnostic algorithm for von Willebrand disease. Presented (oral presentation) at the laboratory seminar in North Estonia Medical Centre, Tallinn, Estonia, 09.03.2018.
- **Pikta M.** Laboratory activities: the Baltic VWD perspective. Presented (oral presentation) at the WFH Twinning Program meeting, Tallinn, Estonia, 21.11.2017.
- **Pikta M**. Developments in North Estonia Medical Centre coagulation laboratory during Tallinn-Helsinki WFH Twinning program. Presented (oral presentation) of the 50th Nordic Coagulation Meeting, 31.08–02.09.17, Helsinki, Finland.
- **Pikta M.** How to diagnose von Willebrand disease? Presented (oral presentation) at the meeting of Estonian Society of Laboratory Medicine, Tallinn, Estonia, 16.03.2017.
- **Pikta M**, Zolotareva V, Vaide I, Laane E, Kleinson I, Pulk R, Banys V. Pre-analytical variables in coagulation testing: focus on high hematocrit. Proceedings of the 4th EFLM-BD European Conference on Preanalytical Phase, Amsterdam (NL), 24–25 March 2017.
- **M. Pikta**, E. Laane, I. Vaide, K. Saks,J. T Õnne, V. Zolotareva, V. Banys. Modified Assay for Quantification of Low FVIII:C Values. Proceedings of the 10th Annual Congress of the European Association for Hemophilia and Allied Disorders, Paris, France, 1–3 February 2017.

- **Pikta M,** Saks K, Lepik K, Zemtsovskaja G,Villemson K, Bautista H, Nouadje G, Viigimaa M, Banys V. Assessment of biological response to desmopressin using von Willebrand factor multimers analysis. Proceedings of the 22nd IFCC-EFLM European Congress of Clinical Chemistry and Laboratory Medicine "EuroMedLab Athens 2017" Athens, Greece, on June 11–15 2017.
- **Pikta M**, Banys V, Laane E, Vaide I, Zolotarjova V, Titova T, Karp K. Evaluation of von Willebrand factor activity assay in comparison with "gold standard" assay. Proceedings of the 9th Annual Congress of the European Association for Hemophilia and Allied Disorders, Malmö, Sweden, February 3–5 2016.

Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder first described in 1926 by Erik von Willebrand, with 0.1% of the population are symptomatic and 1% are affected as symptoms are often mild and diagnosis of VWD can be challenging (Berntorp et al., 2022). Thus, the level of primary care, approximately 1 in 1000 individuals, are affected and require medical attention for bleeding (James et al., 2021).

VWD is a heterogeneous disorder caused by a deficiency (type 1 or 3) or defect (type 2A, 2 B, 2N, or 2M) in the von Willebrand factor (VWF) (P. D. James et al., 2021). The most common symptoms include mucocutaneous bleeding, epistaxis, bruising, menorrhagia, heavy blood loss after trauma, childbirth, or surgery (Nichols et al., 2008). Another important condition called acquired von Willebrand syndrome (AVWS), is a rare and potentially underdiagnosed bleeding disorder that may be misdiagnosed as congenital VWD (Tiede et al., 2011). However, differentiating between the two conditions is important as the treatment options and prognoses may differ (Shetty et al., 2011; Tiede et al., 2011).

Underrecognition of VWD can lead to increased patient morbidity and mortality, and may result in increased uncorrected bleeding complications in patients who undergo invasive medical procedures, surgery (Colonne et al., 2021), dental extractions (Baghaie et al., 2021), or postpartum hemorrhage (PPH) in patients with VWD (Brignardello-Petersen et al., 2022). Overdiagnosis of VWD, in turn, can lead to increased patient morbidity and healthcare system burden, and can result in unnecessary evaluations for patients who require an invasive procedure or elective surgery (Colonne et al., 2021).

Therefore, an accessible and accurate diagnosis is important for the appropriate management of VWD (Boender et al., 2018). However, assays for an accurate diagnosis are often available only at few specialized centers and are time-consuming and costly. Hence, new assays to support a simple and accurate diagnosis are necessary (Berntorp et al., 2022).

The rationale for the study

Prior to 2016, confirming a suspected case of VWD or AVWS in Estonia was not possible owing to the limited available VWF assays. Thus, the prevalence of VWD in Estonia remains unclear. The diagnosis of VWD is incomplete without assessment of VWF activity. However, routine laboratories face challenges in selecting suitable assays for VWF activity (VWF:Ac). In particular, not all in vitro diagnostics (IVD) manufacturers offer a possibility to measure VWF:Ac. Moreover, several assays have been developed as an alternative to the imprecise and insensitive ristocetin cofactor activity (VWF:RCo) assay, which is traditionally considered as a “gold standard” for evaluating VWF function (Boender et al., 2018).

One of commercially available alternative automated assays (INNOVANCE® VWF Ac by Siemens Healthcare Diagnostics, Germany) is based on the spontaneous binding of VWF to a gain-of-function mutant GPIb fragment and does not require ristocetin. **However**, analytical performance characteristics of this assay have been most extensively evaluated, specifically for Sysmex coagulation instruments (Patzke et al., 2014), and data on its clinical validation in patients with VWD remains limited (Bodó et al., 2015). Currently, the Estonian hemophilia treatment centres use the STA-R Evolution platform (Stago, France) for coagulation testing.

Historically, VWF multimer (VWF:MM) analysis has been performed only by a limited number of expert laboratories as it is technically difficult, laborious, non-standardized, and time-consuming (Torres et al., 2012). In-house techniques are considerably difficult to standardize, and a relatively high proportion of laboratories generate unreliable VWF:MM results (E. J. Favaloro et al., 2014). To overcome technical difficulties and attempt to standardize the method, Sebia (Lisses, France) introduced a simplified, same-day semi-automated assay (Hydragel 5 von Willebrand multimers) in 2015, which provides visualization of VWF multimers on a gel, making quantification of multimer band patterns based on densitometric data possible. **However**, the analytical performance of WVF:MM was not evaluated in detail when this study began (2016), and information on clinical validation was not available. Furthermore, no reference values were provided by the manufacturer. Owing to the lack of reference values for VWF multimer fractions, the interpretation of results can be challenging in some VWD cases. VWF multimer interpretation using the new VWF multimer assay (Sebia) in clinical practice is largely based on individual decisions, and there is currently no consensus.

The international standard EN ISO 15189 (Medical laboratories – Requirements for Quality and Competence) requires laboratories to evaluate the performance of new, non-standard, laboratory-designed methods.

This thesis aimed to perform a preclinical and clinical evaluations of new VWF activity and VWF multimer assays for the diagnosis of VWD and AVWS.

In the first part of the study, the analytical performance characteristics of the new VWF activity assay (Publication I) and the new VWF multimer assay (Publications II–III) were investigated. These became the first reports of a new VWF multimer technique evaluation in Northern Europe and Baltic countries, and one of the few published data from Europe. Additionally, reference intervals for VWF multimers were established (Publication VI) in a multicenter trial in Estonian, USA and French centers.

The second part of the study (Publications IV–V) focused on the validation of a clinical-laboratory approach for the diagnosis of VWD and AVWS. The main objective was to analyze the value added by the incorporation of new VWF activity and new VWF multimer assays into the initial approach for the diagnosis of VWD and AVWS, thereby improving the detection of bleeding disorders. These were the first reports in Baltic countries to provide insight into the potential clinical significance of using new VWF activity and VWF multimer assays for the diagnosis of VWD and AVWS.

Abbreviations

APTT	Activated partial thromboplastin time
AT	Autoimmune thyroiditis
AVWS	Acquired von Willebrand syndrome
BAT	Bleeding assessment tools
BT	Bleeding time
CNP	Commercial lyophilized pooled normal plasma
CV	Coefficient of variation
DDAVP	Desmopressin
EQA	External quality assurance
ET	Essential thrombocythemia
FVIII	Factor VIII
FVIII:C	Factor VIII coagulant activity
GPIb	Platelet glycoprotein Ib
HA	Hemophilia A
HMWM	High-molecular-weight multimers
H5VW	Hydragel 5VWF multimer assay kit (Sebia)
H11VW	Hydragel 11VWF multimer assay kit (Sebia)
IMWM	Intermediate molecular weight multimers
IRP	In-house reference plasma
ISTH	International Society on Thrombosis and Haemostasis
LMWM	Low molecular weight multimers
MGUS	Monoclonal gammopathy of undetermined significance
NHL	Non-Hodgkin's lymphoma
PFA	Platelet function analyzer
PLT	Platelet
PT	Prothrombin time
PV	Polycythemia vera
RIPA	Ristocetin induced platelet aggregation
RistoHigh	von Willebrand Factor and glycoprotein Ib-dependent platelet aggregation (high concentration)
SD	Standard deviation
TA	Tranexamic acid
WFH	World Federation of Hemophilia
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:Ac	von Willebrand factor activity
VWF:Ag	von Willebrand factor antigen
VWF:CB	von Willebrand factor collagen binding activity
VWF:GPIbM	von Willebrand factor activity by glycoprotein Ib binding assay
VWF:GPIbR	von Willebrand factor activity by GPIb and ristocetin
VWF:MM	von Willebrand factor multimer analysis
VWF:RCo	von Willebrand factor ristocetin induced activity
WBA	Whole blood aggregometry
WB-RIPA	Whole blood ristocetin-induced platelet agglutination

1 Background and review of literature

1.1 Hemostasis

Hemostasis is the physiological mechanism that leads to the cessation of bleeding from a blood vessel and can be divided into four stages: blood vessel constriction, platelet plug formation, stable insoluble fibrin clot formation, and fibrin clot removal (LaPelusa & Dave, 2022). It involves multiple interlinked steps that depend on vascular response, platelet number/function, adequate VWF level/function, and adequate pro- and anti-coagulant factors' levels (LaPelusa & Dave, 2022). Dysregulation of hemostasis can result in bleeding or thrombotic disorders (LaPelusa & Dave, 2022).

Bleeding disorders are a group of disorders that share the inability to form a proper blood clot, including hemophilia A, hemophilia B, VWD, and other rare bleeding disorders (Figure 1). The most common of these is VWD, which is caused by deficiency and/or defects in the VWF (P. D. James et al., 2021).

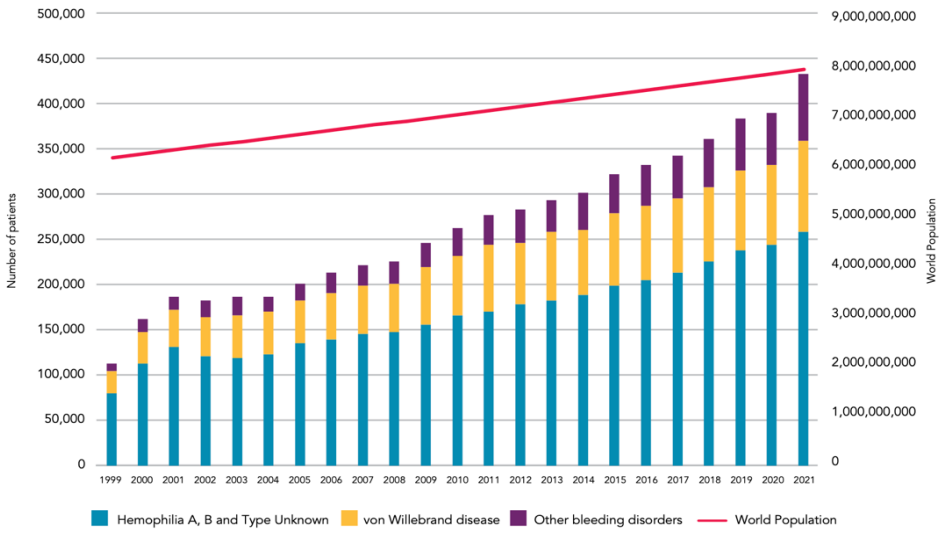


Figure 1. *Identified bleeding disorders: global representation over time (1999–2021). The report on the Annual Global Survey 2021 is published by the World Federation of Hemophilia at https://wfh.org/data-collection/#wbdr.*

1.2 von Willebrand factor

VWF is a large multimeric adhesive sialoglycoprotein (De Meyer et al., 2009; Millar & Brown, 2006) that mediates platelet adhesion to subendothelial structures and acts as a factor VIII (FVIII) carrier molecule, and thus stabilizing the procoagulant activity of FVIII in the circulation (E. J. Favaloro, 2011a; Roberts & Flood, 2015; Stockschlaeder et al., 2014). Endothelial cells and megakaryocytes are the primary sites of VWF production (Stockschlaeder et al., 2014). VWF is synthesized as a polypeptide and is composed of identical monomers (Figure 2). The VWF monomer is composed of identical repeated domains, where each domain completes a specific function, and mutations in some domains lead to the loss of specific functions (Hassan et al., 2012):

- D1–D2 – domains represent the propeptide (Lenting et al., 2015)
- D´–D3 – domains provide the binding sites for FVIII (Roberts & Flood, 2015)
- A1 – binding site is for platelet GPIb, vascular collagen, ristocetin, heparin (Reininger, 2008; Roberts & Flood, 2015)
- A2 – ADAMTS-13 cleavage site (Reininger, 2008)
- A3 – binding site for vascular collagen (Roberts & Flood, 2015)
- C4 – binding site for GPIIb/IIIa (Lillicrap, 2013)
- CK – dimerisation site (Zhou et al., 2012)

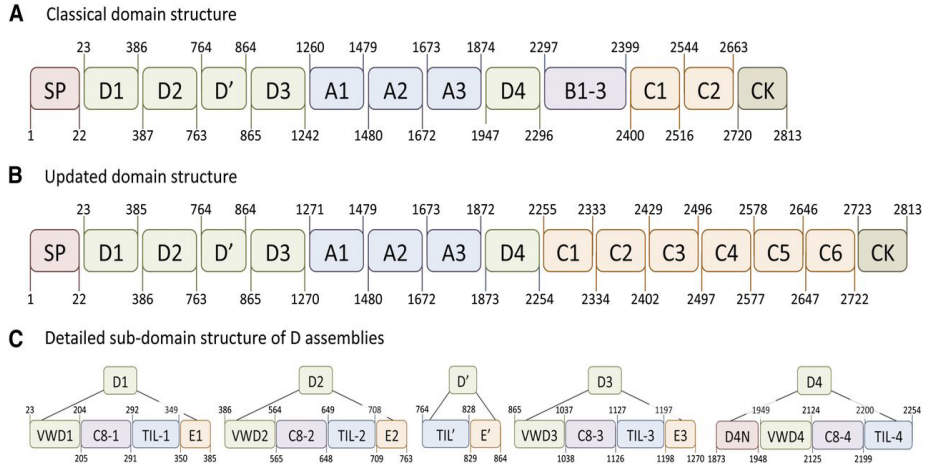


Figure 2. *Schematic illustration of the domain arrangement of VWF (Lenting et al., 2015).*

The VWF monomers assemble into a series of multimers, and multimer organization is critical for the function of VWF (Stockschlaeder et al., 2014). The size of multimers may range from 500 kDa to > 20,000 kDa, and are usually classified into categories according to the number of multimers (dimers) and size: low-molecular-weight (LMW, 1–5 dimers, 500–2,500 kDa); intermediate-molecular-weight (IMW, 6–10 dimers, 3,000–5,000 kDa); high-molecular-weight (large) (HMW, 11–20 dimers, 5,500–10,000 kDa); and ultra-high-molecular-weight (ultra-large) (UHMW, > 20 dimers, up to 20,000 kDa) forms (E. J. Favaloro et al., 2016; Stockschlaeder et al., 2014). UHMW multimers do not typically circulate in blood because of rapid proteolysis by the disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13), which cleaves UHMW forms into smaller multimers soon after secretion (Stockschlaeder et al., 2014). HMW multimers being most functionally effective (Schneppenheim, 2011). The function of VWF correlates with its multimer structure (Budde & Schneppenheim, 2014).

VWF levels are regulated by both genetic and nongenetic factors. The most important genetic factor is ABO blood group. Individuals with blood group O have approximately 25% lower levels of VWF than those with non-O blood group, and there is also an increase in VWF levels in African ancestry (E. J. Favaloro et al., 2005). Increased VWF levels can be associated with age, sex hormone changes during pregnancy, oral contraception, nicotine and caffeine exposure, previous or current thromboembolic events, inflammation, and physical exercise; thus, contributing to the high biological variability of VWF (Colonne et al., 2021).

1.3 von Willebrand disease

VWD is considered the most common autosomal inherited bleeding disorder in humans and is caused by a deficiency or functional abnormality in VWF (P. D. James et al., 2021).

Symptoms usually include excessive mucocutaneous bleeding, epistaxis, heavy menstrual bleeding in women, prolonged bleeding from minor wounds, gastrointestinal bleeding, easy bruising, bleeding after surgery, childbirth, and in the most severe cases, musculoskeletal bleeding (P. D. James et al., 2021).

1.3.1 Epidemiology

The estimated prevalence of VWD appears to be between 0.01% and 1% (E. J. Favaloro, 2011b). Most patients are asymptomatic or have mild type 1 VWD, which may be difficult to distinguish from healthy individuals (E. J. Favaloro, 2011b). The estimated numbers of VWD cases in the Nordic hemophilia centers were type 1 (n = 725), type 2 (n = 409), and type 3 (n = 73). Data on type 2 VWD subtypes were available only from three centers: type 2A (n = 163), 2 B (n = 68), 2M (n = 27), and 2N (n = 5) (Szanto et al., 2018).

Estonia is a northeastern European country with approximately 1.3 million inhabitants. Unfortunately, accurate data on the prevalence of VWD in Estonia are lacking.

1.3.2 Classification of VWD

VWD is classified into partial or total quantitative deficiencies of the VWF (VWD types 1 and 3) and qualitative variants (VWD types 2A, 2B, 2M, and 2N), (P. D. James et al., 2021) as summarized in Table 1. The National Heart, Lung and Blood Institute Expert Panel report (Nichols et al., 2008) suggested that VWD type 1 can be diagnosed when VWF antigen (VWF:Ag) or VWF activity is < 30%, and levels of VWF:Ag between 30% and 50% should be classified as low VWF.

***Table 1**. Classification of von Willebrand disease (Itzhar-Baikian et al., 2019; P. D. James et al., 2021).*

Type of VWD	Pathophysiology of VWD
1	Partial deficiency of functionally normal VWF with plasma VWF levels usually between 5 and 30%. “Low VWF” (mild/moderate VWD) – partial deficiency of functionally normal VWF with plasma VWF levels between 30 and 50%.
2A	Decreased GPIb binding due to a deficiency of high molecular weight multimers of VWF. Enhanced proteolysis by ADAMTS-13, defective secretion, defective dimerization or defective multimerization of VWF.
2B	Enhanced, spontaneous GPIb binding due to gain-of-function mutations.
2M	Decreased GPIb binding and/or decreased collagen-binding due to loss-of-function mutations.
2N	Decreased factor VIII binding due to loss-of-function mutations.
3	Total deficiency of VWF.

Correct classification of the type and subtype of VWD is important for patient management and therapeutic approaches (E. J. Favaloro, 2011a). Figure 3 shows the locations of VWF mutations resulting in various VWD subtypes.

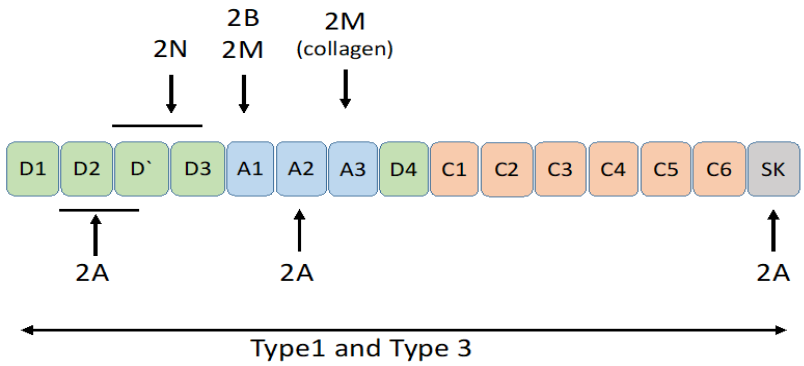


Figure 3. *Schematic illustration of the locations of VWF mutations in various VWD subtypes (Lillicrap, 2013).*

1.3.3 Acquired von Willebrand syndrome

AVWS was first defined by the VWF subcommittee in 2000 (Federici et al., 2000). A diagnosis of AVWS can be made based on the following criteria: lack of previous lifelong bleeding incidents, relevant family history, clinical picture, and laboratory investigation results (A. H. James et al., 2016). AVWS is a rare but frequently underdiagnosed bleeding disorder with an unknown prevalence that may develop in various underlying disorders (Ichinose et al., 2022). The mechanisms VWF abnormalities depend on the type of underlying disorder (Michiels et al., 2001). The pathogenesis of AVWS (Federici et al., 2000; Mital, Prejzner, Bieniaszewska, et al., 2015; Tiede, 2012) is as follows:

- Reduced VWF synthesis: severe hypothyroidism, drugs (e.g., valproic acid);
- Increased shear stress and proteolysis of VWF: aortic valve stenosis, artificial heart valves, left ventricular assist devices, other heart defects with disturbed flow (mostly congenital), and extracorporeal membrane oxygenation (ECMO);
- Inhibition/clearance by paraproteins or autoimmune inhibitors: autoimmune disorders, multiple myeloma, Waldenström macroglobulinemia, monoclonal gammopathy of undetermined significance (MGUS), B-cell lymphomas;
- Adsorption of VWF high-molecular weight multimers: myeloproliferative disorders (e.g., polycythemia vera, essential thrombocythemia), Wilms' tumors, and solid tumors.

1.4 Diagnosis

The diagnosis of VWD is based on the presence of bleeding symptoms and/or family history, and laboratory evidence of abnormal VWF levels or function. In AVWS, there were no previous lifelong bleeding episodes or relevant family history.

1.4.1 Bleeding Assessment Tool

It is recommended that a Bleeding Assessment Tool (BAT) should be used before laboratory testing to evaluate a patient's personal and family bleeding history (P. D. James et al., 2021). The BAT is a questionnaire used for the quantification of bleeding symptoms based on their severity and to generate an overall quantitative bleeding score (Tosetto et al., 2006). In 2010, the International Society on Thrombosis and Haemostasis (ISTH)

proposed a single BAT to standardize the reporting of bleeding symptoms for use in adult and pediatric populations (Rodeghiero et al., 2010). The normal bleeding score values are presented in Table 2.

Table 2. *ISTH BAT normal ranges (Elbatarny et al., 2014).*

	Normal	**Abnormal**
Children	0–2	3 or greater
Adult female	0–5	6 or greater
Adult male	0–3	4 or greater

1.4.2 Laboratory methods

Laboratory testing for the diagnosis or exclusion of VWD and AVWS is based on a complex of different diagnostic assays (Baronciani & Peyvandi, 2020; Roberts & Flood, 2015): platelet count (PLT), bleeding time (BT) performed on the skin of the patient or closure time (CT) on platelet function analyser (PFA), prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, VWF:Ag, VWF activity, coagulation factor VIII (FVIII:C), VWF multimer analysis (VWF:MM), VWF collagen binding assay (VWF:CB), VWF-FVIII binding assay (VWF:FVIIIB), propeptide of VWF (VWFpp), ristocetin-induced platelet agglutination assay (RIPA) and molecular analysis of VWF gene. PT and fibrinogen concentration are both normal in the VWD. Activated partial thromboplastin time (APTT) is usually normal but can be prolonged depending on the FVIII level and sensitivity of the APTT reagent. Currently, standardized diagnostic algorithms for the laboratory evaluation of VWD are unavailable because of variable technical and analytical availability in different laboratories and countries (Berntorp et al., 2022).

To reduce errors caused by preanalytical issues and variability in assay performance, all measurements of VWF and its functions should be repeated (at least twice) on a separate (new) sample to confirm or negate the initial investigation results (E. J. Favaloro et al., 2018).

PLT count

Fully automated hematology analyzers are widely used for platelet reliable counting.

Screening tests for primary hemostasis

The BT test is designed to evaluate *in vivo* primary hemostasis and assess the ability of platelets to develop a hemostatic plug by recording the time taken by platelets to occlude the skin wound to stop bleeding (Quick, 1975). The technique is quick and simple but is poorly standardized and dependent on many variables (i.e., operator's skills, skin thickness, temperature) (Paniccia et al., 2015).

Platelet function analyzers (PFA-100 and PFA-200; Siemens Healthineers, Germany) were introduced as a screening tool to detect problems with primary hemostasis. These analyzers, in part, replaced the BT because of their superior standardization and demonstration of a high negative predictive value (Paniccia et al., 2015). Nevertheless, PFA results may be affected by various factors (e.g., hematocrit, PLT count, conditions associated with thrombocytopathies) (Paniccia et al., 2015).

Ristocetin-induced platelet aggregation (RIPA)

Optical density-based light transmission aggregometry (LTA), designed by Born in the 1960s, is the historical "gold standard" for assessing platelet-to-platelet clump formation via *in vitro* aggregation (Paniccia et al., 2015). It is the most widely used method for evaluating platelet function disorders (Kaiser et al., 2022). However, this assay has some drawbacks: it is sensitive to preanalytical conditions (i.e., low PLT, lipids in plasma, hemolysis, plasma preparation technique), may be affected by the type of reagents and final concentration of agonists, time-consuming, and requires large sample volume (Paniccia et al., 2015). A multicenter study (Kaiser et al., 2022), which included five coagulation centers and performed Born-based LTA platelet function assays reported that despite the international and national guidelines on LTA, the method continues to be handled differently. The differences were found to be associated with the preanalytical conditions, types of reagents and their final concentrations, and the analyzers, which differ in the wavelengths used in their detection systems (Kaiser et al., 2022).

Multiplate® (Roche, Germany) is another widely used platelet aggregometry analyzer for screening platelet function disorders (PFDs) (Moenen et al., 2019). It incorporates impedance whole-blood aggregometry (WBA) technology, which allows for the assessment of platelet function in anticoagulated whole-blood samples. Sample processing is not required and the test is run under physiological conditions, given the fact that other blood elements contribute to platelet function (Paniccia et al., 2015). Moreover, WBA has potential diagnostic value for VWD by performing ristocetin-induced platelet aggregation in whole blood (WB-RIPA) (Schmidt et al., 2017). The diagnostic accuracy of WB-RIPA has been proven in patients with previously diagnosed VWD and is in agreement with the Born aggregometry results (Valarche et al., 2011). In a study of 30 patients with VWD, WB-RIPA was as sensitive as LTA in detecting VWD, with a 76% correlation between the two methods (Valarche et al., 2011).

VWF antigen

The VWF antigen (VWF:Ag) represents the quantitative level of VWF. VWF:Ag assays are based on enzyme-linked immunosorbent assays (ELISA), which are predominantly used in research laboratories. Latex-enhanced immunoassays (LIA) dominate clinical diagnostic laboratories (E. J. Favaloro et al., 2017). Both methods demonstrated high sensitivity and low imprecision (Colonne et al., 2021).

VWF functions and their measurements

The approved nomenclature of VWF activity assays, suggested by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis) characterizes the differences between the available methodologies (Bodó et al., 2015):

Platelet-dependent VWF activity

Numerous VWF activity assays assess the ability of VWF to bind platelets.

- VWF:RCo (Ristocetin cofactor assay) – historically "gold standard" assay that assesses the capacity of VWF to agglutinate platelets in the presence of ristocetin. However, its clinical utility is compromised by an extremely high limit of detection (LOD) for VWF, suboptimal sensitivity, and large assay imprecision (E. J. Favaloro et al., 2008). LOD of 10–20% aggravates the diagnosis of severe VWD cases. A high coefficient of variation may lead to a false diagnosis at slightly reduced or borderline

VWF levels (Bodó et al., 2015), and poor precision at low VWF values may lead to uncertainty in the diagnosis of all VWD types (E. J. Favaloro et al., 2016).

The relatively new VWF activity assays overcome these disadvantages as they are independent of lyophilized platelets and/or ristocetin (E. J. Favaloro & Mohammed, 2014):

- VWF:GPIbR assays – based on ristocetin-induced binding of VWF to a recombinant WT GPIb fragment. Currently, two fully automated methods (one immunoturbidimetric and the other based on chemiluminescence) are available for coagulation instruments, HemosIL AcuStar and ACL TOP (Instrumentation Laboratory, Bedford, MA, USA), which demonstrate impressive sensitivity to low VWF levels and are highly precise (E. J. Favaloro & Mohammed, 2016).
- The newest automated VWF-GPIbM assay is based on a gain-of-function mutation in glycoprotein GPIb, which does not require ristocetin to bind to VWF. The mutated GPIb fragments were captured onto latex particles using a monoclonal mouse antibody. VWF from the test plasma-agglutinated latex particles, and the turbidity was measured against a calibration curve (Higgins & Goodwin, 2019). Recent studies have shown acceptable comparability of this method with the VWF:RCo assay, with high sensitivity and low variability (Patzke et al., 2014). However, the analytical performance was most extensively evaluated only for Sysmex coagulation instruments (Patzke et al., 2014).
- The VWF-FVIII binding activity assay is useful for identifying or excluding defective VWF:FVIII binding in patients with type 2N VWD (E. J. Favaloro, 2020; E. J. Favaloro et al., 2016).
- VWF collagen-binding activity (VWF:CB) is usually determined using ELISA or chemiluminescent immunoassay (CLIA) (E. J. Favaloro et al., 2016).

Nordic centers collaborate because some of the tests, such as the VWF:FVIII binding assay and VWF:CB binding assay, are not available at all laboratories (Szanto et al., 2018).

Coagulant activity of FVIII

A one-stage APTT-dependent assay of 'coagulant' FVIII activity (FVIII:C) is simple and commonly used by laboratories to assess FVIII levels on automated analyzers. The Factor VIII chromogenic assay is less commonly performed and is generally only available in hemophilia centers (E. J. Favaloro et al., 2016).

VWF multimers

Historically, multimer analysis has been performed only by a limited number of expert laboratories because it is technically difficult, laborious, non-standardized, and time-consuming (Torres et al., 2012). The main methods for visualizing VWF multimers in clinical practice are electrophoresis techniques developed "in-house". Typically, electrophoresis is performed on agarose gels (alternatively, nitrocellulose or polyvinylidene difluoride) at concentrations ranging from 1–3% overnight, followed by different options of immunological detection and multimer visualization (e.g., radioactive, colorimetric, luminographic, or fluorometric methods) (Laffan et al., 2014; Ledford-Kraemer, 2010; Torres et al., 2012). Radioactive techniques set special requirements for working environment, and conventional non-radioactive methods lack sensitivity and optimal resolution power (Budde et al., 2006), potentially leading clinicians to misclassify VWD

subtypes (Pruthi et al., 2010). Luminographic methods are remarkably safer and allow the visualization of multimers with confidence and high sensitivity (Budde et al., 2006). In-house techniques are considerably difficult to standardize, and a relatively high proportion of laboratories may generate unreliable VWF multimer results (E. J. Favaloro et al., 2014).

To overcome technical difficulties and attempt to standardize the method, Sebia (Lisses, France) introduced a simplified*, same-day-results* semi-automated assay (Hydragel 5 and 11 von Willebrand multimers) that provides visualization of VWF multimers on a gel and allows quantification of multimer band patterns based on densitometric data (Figure 4). The multimer methodology developed by Sebia has been previously described in detail elsewhere (Oliver et al., 2017).

Genetic analysis

Genetic testing is recommended only in selected cases as confirmatory testing of types 2A, 2B, 2M, 2N, and PT-VWD, given their diagnostic difficulty and selectively as an investigation of type 3 VWD (E. Favaloro, 2014).

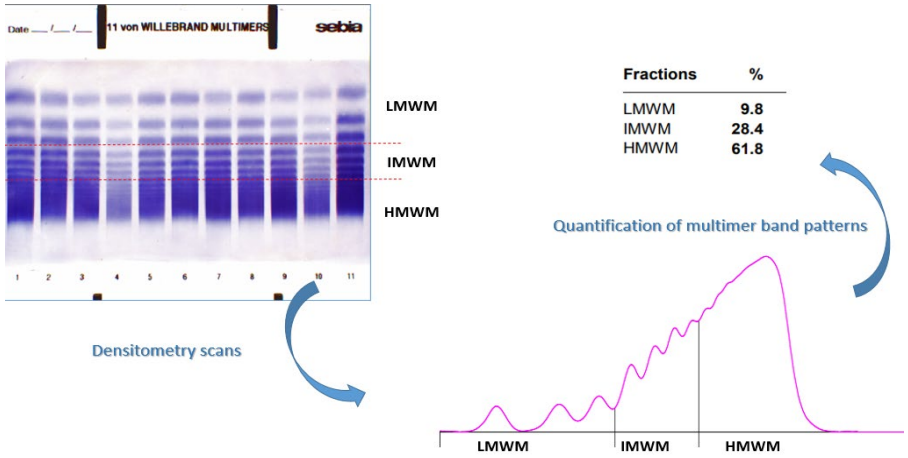


Figure 4. *Schematic representation of multimer profiles: gel and quantification of multimer band patterns based on densitometric data. One band on the gel and one peak in the densitogram correspond to one multimer band. Densitometry curves shown from left to right, peaks representing LMWM, IMWM and HMWM, corresponding to bands at the top, middle and bottom of the electrophoresis gels. LMWM, low molecular weight multimers; IMWM, intermediate molecular weight multimers; HMWM, high molecular weight multimers.*

Due to the known variability and limited availability of appropriate VWF testing in different countries, diagnosis of VWD and AVWS remain challenging, and new laboratory assays to support a simple, accurate, timely diagnosis and management are necessary (Berntorp et al., 2022). It is important for laboratories, as mentioned in recent guidelines (P. D. James et al., 2021), to focus on implementation of assays that have the best quality performance characteristics (the lowest limits of detection, lowest CV, method-specific calibration, standardization, accurate VWF assays cut-offs and ratios that are crucial in the VWD diagnostic workup).

1.5 Treatment

Treatment of VWD bleeding involves the use of desmopressin (DDAVP), tranexamic acid (TA), and plasma-derived recombinant VWF concentrates (Fogarty et al., 2020). A DDAVP test is recommended to evaluate individual responses, which depend on various factors (e.g., phenotype and genotype) (Castaman & Linari, 2017). Replacement therapy with VWF concentrates is used for DDAVP non-responders or patients with type 2B disease for whom DDAVP is contraindicated (Castaman & Linari,2017).

In AVWS cases, treatment of the patient's underlying condition is important and includes immunosuppressants, chemo- or radiotherapy, and surgery, which can lead to AVWS remission but is not always feasible and successful (Tiede et al., 2011).

2 Aims of the study

This study aimed to improve the diagnostic possibilities of VWD and AVWS, gain insight into the value of innovative techniques, and evaluate the applicability of relatively new assays for the diagnosis of VWD and AVWS.

Specifically, this study aims to:

- Evaluate the analytical performance characteristics of selected VWF activity assay;
- Evaluate the usefulness of the VWF activity/antigen ratio in VWD and AVWS screening;
- Evaluate the analytical performance characteristics of the new VWF multimers assay;
- Determine the reference intervals for LMW, IMW, and HMW multimers; and
- Develop a diagnostic workflow and evaluate the usefulness and fitness for clinical purpose of the aforementioned techniques for diagnosing VWD and AVWS.

3 Methods

3.1 General study design

This study consisted of two parts: preclinical and clinical evaluations. An outline of this thesis is presented in Figure 5.

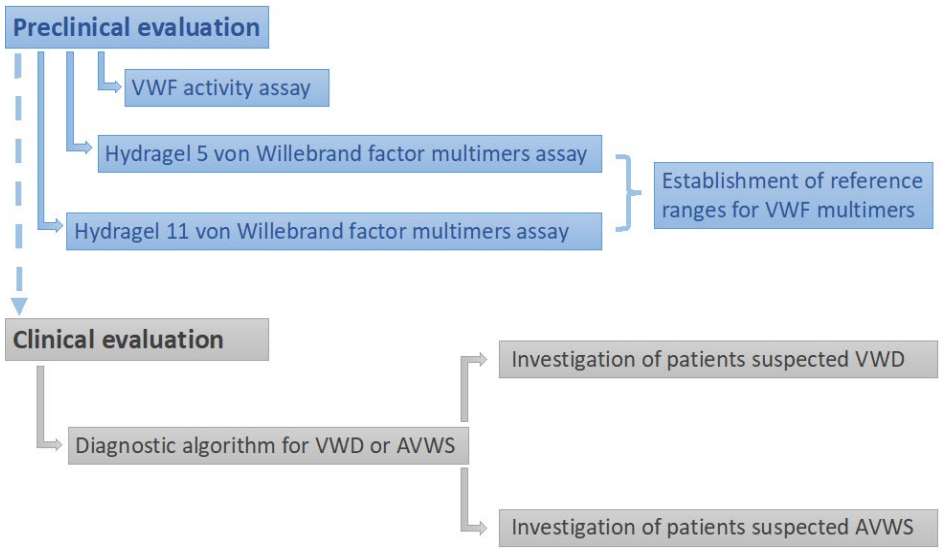


Figure 5. *General study design. VWF, von Willebrand factor; VWD, von Willebrand disease; AVWS, acquired von Willebrand syndrome.*

3.1.1 Preclinical evaluation (Publications I, II, III and VI)

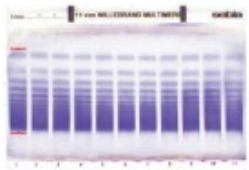
We evaluated the analytical performance of the selected relatively new VWF activity and VWF multimer assays (Figure 6). To establish reference intervals for the VWF multimer fractions, international cooperation was initiated between four countries (Estonia, Latvia, France, and the USA) to collect a larger sample size. Data and samples were collected from 131 healthy volunteers (80 nonpregnant females and 51 males). We used densitometry to determine the reference intervals for the LMWM, IMWM, and HMWM fractions.

Preclinical evaluation

VWF activity assay

- Inter- and intra-assay precision
- External quality assessment
- Stability
- Linearity
- Threshold of the method
- Difference between three VWF:Ac assays:
  - INNOVANCE® VWF Ac (Siemens Healthcare Diagnostics, Germany)
  - HemosIL VWF Activity (Instrumentation Laboratory, USA)
  - HemosIL VWF:RCo (Instrumentation Laboratory, USA)

von Willebrand factor multimers assay



- Samples from patients with known VWD type
- Internal quality assurance
- External quality assessment
- Sensitivity
- Repeatability
- Reproducibility
- Differences between H5VW and H11VW

Establishment of reference ranges for VWF multimers

Proposed reference intervals

- Gender related difference

- Geographical locations related difference

- Age related difference

Figure 6. *Preclinical evaluation study design.*

3.1.2 Clinical evaluation (Publications IV and V)

We retrospectively selected 138 individuals from the laboratory information system (LIS). The selection was based on an existing request for VWF profile tests between May 2016 and December 2020. Samples were obtained from patients visiting the outpatient clinic and hospitalized patients. Clinical information of the patients was provided by clinicians and was available in the LIS. The adult and pediatric ISTH-BAT was primarily translated into Estonian and has already been incorporated into routine practice to identify individuals with clinically relevant bleeding tendencies and symptoms. The diagnostic criteria for VWD were based on the revised VWD classification (P. D. James et al., 2021).

All patients met the criteria for VWD or AVWS based on laboratory findings, bleeding symptoms, and presence or absence of a history of a bleeding disorder.

3.2. Participants

In Publications II to VI, the study volunteers were relatively healthy individuals without a history of hemorrhagic episodes who were not taking any medication for at least 10 days before blood collection, had a normal coagulation screen profile and normal VWF screening assays results, and provided written consent. The exclusion criteria consisted of a positive personal and/or family history of bleeding, inflammation, pregnancy, and oral contraceptive use. Blood donor plasmas were not used because the questionnaire for blood donors did not include information regarding family bleeding history, individual mild bleeding episodes, and that donors were not routinely screened for VWD.

In Publication II, 10 plasma samples were obtained from patients with known VWD type 1, type 2 (subtypes 2A, 2 B, 2N), or type 3. Of whom, nine patients were from Finland, and one from Estonia; one patient with hemophilia A was from Estonia. This study was performed in collaboration with Helsinki University Hospital, HUSLAB Laboratory Services, Coagulation Disorders Unit, Finland.

In Publications IV and V, patients from North Estonia Medical Centre and Tallinn Children's Hospital referred for bleeding tendency evaluation were recruited.

3.3 Samples

All samples were collected in 3.2 % sodium citrate tubes (BD Vacutainer; BD Diagnostics, Plymouth, UK) for coagulation assays and hirudin blood tubes (Roche Diagnostics, Basel, Switzerland) or hirudin tubes (Sarstedt, Nümbrecht, Germany) for platelet aggregation evaluation.

Samples for reference interval studies were collected according to locally approved venous blood sampling procedures of the participating institutions. Samples were aliquoted and stored frozen (at least –20 °C) until testing, which was performed within 30 days. Aliquots were thawed in a water bath (+37 °C) for 5 minutes and mixed well before testing.

3.4 Laboratory investigations

All parameters were analyzed using standard methodology in an ISO 15189 accredited laboratory (Table 3).

Table 3. *Analytical platforms used in the study.*

Parameter	Reagents	Analyzer
PLT	Component of complete blood count	Sysmex XE-5000 (Roche Diagnostics, Germany)
PT	Neoplastine Cl Plus (Diagnostica Stago, France)	STA-R Evolution (Diagnostica Stago, France)
APTT	PTT-A (Diagnostica Stago, France)	
FVIII:C	STA Deficient VIII, STA PTT-A (Diagnostica Stago, France)	
FVIII:C	STA-ImmunoDef VIII, STA-C.K.Prest (Diagnostica Stago, France)	
VWF:Ag	STA Liatest VWF:Ag (Diagnostica Stago, France)	
VWF:Ac (VWF:GPIbM)	INNOVANCE VWF Ac (Siemens Healthineers, Germany)	
RISTOhigh	Ristocetin (final concentration 0.77 mg/mL) (RISTOtest, Roche, Germany, or Hart Biologicals, USA)	Multiplate (Roche Diagnostics, Germany)
RISTOlow	Ristocetin (final concentration 0.2 mg/mL) (RISTOtest, Roche, Germany, or Hart Biologicals, USA)	
VWF:MM	H5VW, H11VW (Sebia, France)	Hydrasys 2 Scan (Sebia, France)

3.5 Statistical analysis

In Publication **I**, descriptive statistics was used to analyze the mean, median, standard deviation (SD), and coefficient of variation (CV, %) of the different VWF activity assays. Differences between the VWF activity assays were tested using the non-parametric Mann–Whitney U test. Statistical analyses were performed using IBM SPSS software version 20 (Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

In Publication **III** the results are reported as the mean % of the corresponding molecular weight fraction of multimers ± SD and CV. The difference between the two commercial kits (H5WV vs H11VW) was evaluated using the nonparametric Mann–Whitney U test in IBM SPSS software, version 21.0 (Chicago, IL, USA). Values were considered statistically significant at $P < 0.05$.

In Publication **V**, baseline patients' characteristics are presented as median and interquartile range (IQR) or number of cases (in percentage, counting data). Spearman's correlation coefficient was calculated to test the association between HMWM and the ratio of VWF:GPIbM to VWF:Ag, and RISTOhigh vs. VWF:GPIbM. Differences between

variables were tested using the Mann–Whitney U test. Statistical analysis was performed using IBM SPSS software, version 23 (Chicago, IL, USA), and statistical significance was set at $p < 0.05$.

In Publication **VI** statistical analyses were performed using MedCalc® software version 18.11.6. (MedCalc Software, Belgium), and IBM SPSS Statistics version 23 (Chicago, IL, USA). Descriptive statistics were used to analyze demographic data and laboratory characteristics. Data were analyzed according to age, gender, and geographical location. Results were expressed as median (interquartile range [IQR]). Differences between variables were tested using the Mann-Whitney U test. Reference intervals were established according to a robust method following CLSI C28-A3 standard to calculate the 2.5th and 97.5th percentiles, and associated 90% confidence intervals (CI) for each VWF multimeric fraction. Data distributions were tested for normality using the Shapiro–Wilk test. Outlier detection was performed using Grubs double-sided and Tukey methods. Statistical significance was set at $P < 0.05$.

3.6 Ethical considerations

The study was performed in accordance with the Declaration of Helsinki and was approved by the appropriate local or national ethical committees or the local Institutional Review Board of each participating institution.

4 Results and discussion

4.1 Preclinical evaluation (Publications I, II, III and VI)

The analytical performance evaluation of relatively new VWF:GPIbM and H5VW / H11VW multimers for routine application was done in line with current laboratory practices and guidelines of Clinical and Laboratory Standards Institute (CLSI: H57-P, EP5-A2, and EP28-A3c).

4.1.1 VWF activity assay, VWF:GPIbM (Publication I)

In recent years, numerous VWF activity assays have been developed to evaluate the functions of VWF (Higgins & Goodwin, 2019). One of which is the Innovance VWF Ac (Siemens Healthineers, Germany) reagent kit intended for automated Siemens or Sysmex coagulation instruments (Higgins & Goodwin, 2019). It fits well with the current nomenclature of VWF activity methodologies (Bodó et al., 2015):

- Assays based on platelets and ristocetin (VWF:RCo);
- Assays based on antibody directed against the GPIb-binding site on VWF (VWF:Ab);
- Assays based on the GPIb containing gain-of-function mutations (**VWF:GPIbM)** (used in this study);
- Assays based on wild type GPIb and ristocetin (VWF:GPIbR).

In May 2016, an adapted protocol for measuring VWF activity using Siemens reagents was implemented on STA-R Evolution (Diagnostica Stago, France) analyzer.

In our study, we used Standard Human Plasma (Siemens Healthineers, Germany), in which VWF activity was calibrated against the WHO standard. The linearity between the assigned values of the original and newly adapted methods demonstrated an excellent correlation ($y = 0.9951x + 0.4266$; $r^2 = 0.9999$).

Imprecision was found to be acceptable (coefficient of variation (CV) < 10%). Intra-assay precision (representing repeatability) showed CV 4.00% for Control Plasma N and 5.02% for Control Plasma P. Inter-assay precision (representing reproducibility) CV was 4.69% for Control Plasma N and 5.00% for Control Plasma P. Similar results for intra- and inter-assay imprecision (CV < 3.0% for normal control and CV < 3.5% for pathological control) were reported in study (Lawrie et al., 2013) using reagents from Siemens but on a Sysmex CS-2000i analyzer (Sysmex UK Ltd). Boender et al. previously demonstrated that the VWF:GPIbM assay had the lowest coefficient of variation compared to the VWF:RCo and VWF:GPIbR assays, making it the most precise assay in their study (Boender et al., 2018).

Evaluation of stability demonstrated that in fresh and frozen thawed plasma VWF activity results were comparable: the correlation was excellent (Spearman's $r = 0.952$, $p < 0.001$; $y = 1.0892x - 14.626$; $r^2 = 0.9386$), and the difference was statistically insignificant (114.70 ±50.77% vs 110.30 ±57.08%, $p = 0.201$).

As an individual performance indicator used in this study, the Z-scores obtained from the external quality assurance (EQA) survey reports for VWF assays (ECAT Foundation, The Netherlands) were acceptable (Z-score <±2), and confirmed that the results were reliable and accurate.

By diluting Standard Human Plasma (Siemens Healthineers, Germany), we found that threshold for VWF activity was similar to manufacturer's declared value of 4%. Similar

results for limit of detection (LOD) were found by the WiN study group (Boender et al., 2018). We additionally examined 20 consecutive runs of 0.9M NaCl solution that revealed undetectable results or results below 4% of VWF activity.

Historically, assays based on platelets and ristocetin reagents (particularly termed VWF:RCo) were considered as "gold standard" assay for evaluation of VWF activity; however, the technical limitations of most VWF:RCo assays used in laboratories worldwide make the ratio of VWF:RCo to VWF:Ag unreliable, especially at levels of VWF:Ag < 15–20 IU/dL (%) (Sadler et al., 2006). The LOD for VWF:RCo > 10 IU/dL (%) is unacceptable, makes the assay be imprecise (E. J. Favaloro et al., 2008). Furthermore, VWF:RCo is technically challenging. Consequently, the diagnosis of VWD in some countries is often difficult or delayed (Lawrie et al., 2013). Therefore, the superior precision of the VWF:GPIbM assay and better LOD allow improved discrimination of the activity-to-antigen ratio compared to VWF:RCo (Graf et al., 2014).

We performed a VWF:GPIbM comparison study using two alternative VWF:RCo activity assays (both reagents and an analyzer from the Instrumentation Laboratory, USA). The correlation of the implemented VWF:GPIbM activity assay with the two HemosIL assays showed to be perfect: Spearman's correlation coefficients were 0.986 and 0.982 (HemosIL VWF:RCo and HemosIL VWF:Ac, respectively; both $p < 0.001$). Previously, a good correlation was reported (Patzke et al., 2014) between VWF:GPIbM and VWF:RCo, and the mean difference between methods was 6 IU/dL (%) to 7 IU/dL (%) lower by VWF Ac Innovance. In addition, the WiN study group reported an excellent correlation between VWF activity assays: VWF:RCo, VWF:GPIbR, and VWF:GPIbM (Boender et al., 2018). The clinical utility of VWF:GPIbM for the diagnosis and subtyping of VWD has been demonstrated in several independent studies (Graf et al., 2014; Patzke et al., 2014); however, the analytical performance characteristics of VWF:GPIbM have been extensively evaluated only for Sysmex coagulation instruments (Patzke et al., 2014).

In conclusion, the analytical performance of the new VWF:GPIbM assay (a combination of Siemens reagents and a Diagnostica Stago analyzer) was found to be acceptable. The simplicity of implementation of the adapted protocol, together with its improved sensitivity and precision, make this new VWF:GPIbM assay suitable for the diagnosis of VWD, especially in clinical laboratories that are unable to utilize Sysmex coagulation instruments.

4.1.2 VWF multimers (Publications II and III)

The measurement of VWF multimers has become a part of the laboratory workflow for the identification and classification of VWD (E. J. Favaloro, 2011a).

In this study, we analyzed the following analytical performance characteristics of VWF multimers: repeatability (intra-assay variability/in-gel between-track variation), reproducibility (inter-assay variability/between-gel variation), sensitivity, and differences between two commercially available VWF multimer kits (H5VW and H11VW). We used samples from patients with previously confirmed VWD diagnoses and from EQA organizers.

Internal quality control (IQC) materials were not provided by the manufacturer. Therefore, the plasma pool of healthy volunteers, the so-called in-house reference plasma (IRP) samples, and commercial lyophilized pooled normal plasma (CNP) samples (Diagnostica Stago, France) were compared and evaluated. We found a relative loss of HMW multimers in the CNP samples, probably due to lyophilization during the preparation of commercial plasma (Meijer & Haverkate, 2006), as demonstrated by

visual and quantitative evaluations. Our study findings were confirmed by researchers from Australia (Oliver et al., 2019) who identified the loss of HMWM in Diagnostica Stago control plasmas. Therefore, IRP was preferred as the control (reference) plasma in further studies.

In this study, the H11VW kit was used for intra-assay variability (repeatability/in-gel between-track variations) analysis, and 11 measurements were performed, each for a single healthy individual's plasma, which was applied to 11 tracks of the gel. We have demonstrated intra-assay variability performance equivalent to previously published H5VW kit repeatability values (Bowyer et al., 2018): CVs were 6.9% for LMWM, 10.3% for IMWM, and 4.8% for HMWM. To evaluate inter-assay variability (reproducibility/ between-gel variation), the VWF multimer fractions' results of the same IRP from 55 different gel runs were analyzed. Calculated CV values were 16.6 % for LMWM, 6.2% for IMWM, and 8.1% for HMWM.

Moreover, we assessed sensitivity of new gel diluting the single volunteer's plasma with known VWF:Ag values in dilution series (1:2, 1:4, 1:6, 1:8, 1:16, and 1:32), which resulted in final VWF antigen values of 52%, 19%, 13%, 9%, 5%, and 2%, respectively. Sensitivity analysis with serial dilutions revealed a cut-off (VWF:Ag value of 9%). Our study results were confirmed in another study (Crist et al., 2018) wherein researchers found that VWF protein concentrations of approximately 5–10% of normal are required to adequately visualize multimers.

We evaluated the differences between two commercial kits (H5WV and H11VW). There was no statistically significant difference detected between H5VW and H11VW kits for different fractions of multimers: LMWM 17.95 ±2.94 vs 18.31 ±3.32, $p = 0.807$; IMWM 33.24 ±1.98 vs 32.47 ±2.48, $p = 0.183$; HMWM 48.82 ±3.65 vs 49.22 ±3.57, $p = 0.774$.

In collaboration with the European Hemophilia Comprehensive Care Center (EHCCC) from Helsinki, we evaluated 11 samples: nine samples from Finland (VWD types 1, 2A, 2B, 2N, or 3), one VWD type 2N sample from Estonia, and one hemophilia A sample from Estonia. All measurements were performed without prior knowledge of previous measurements or diagnoses. We demonstrated that VWF multimer electrophoresis interpretations were successfully reproduced and were in agreement with a previously confirmed diagnosis. The VWF multimer electrophoresis results for healthy individuals and different VWD types are depicted in Figure 7. Healthy volunteers' and three patients' with type 1 VWD plasma showed a normal VWF multimer pattern, although a relative decrease in the intensity of the multimer bands was observed in patients with type 1 VWD (Figure 7B). As expected, loss of HMWM was identified in both patients with type 2A (Figure 7C) and type 2B (Figure 7D) VWD. Patients with type 2N VWD showed normal multimeric patterns (Figure 7E). No signal was detected in the two patients with type 3 VWD (Figure 7F), leading to the interpretation of undetectable VWF multimers. Hemophilia A results were completely normal.

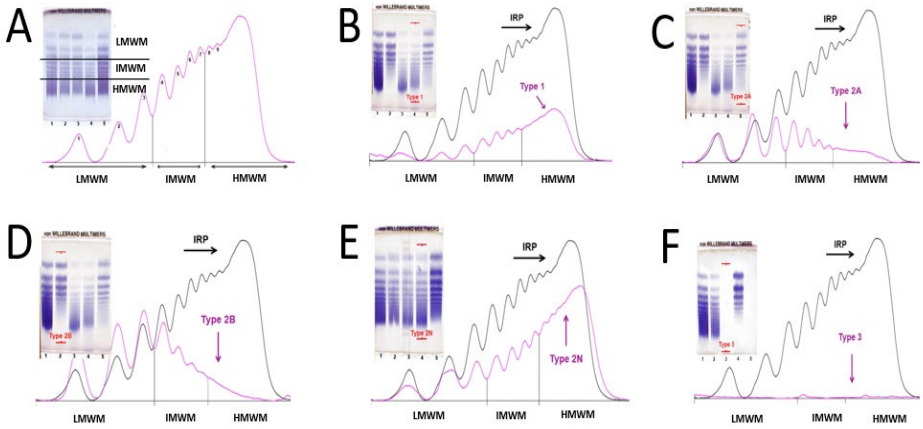


Figure 7. *VWF multimer electrophoresis results of healthy individuals and different VWD types: A – electrophoresis gel of plasma samples from 4 different healthy individuals (tracks 1-4) and an IRP sample (track 5) and densitogram of IRP; B – electrophoresis gel of IRP (track 1) vs type 1 VWD patient plasma (track 4) and densitograms: IRP vs type 1 VWD patient plasma; C – electrophoresis gel of IRP (track 1) vs type 2A VWD patient plasma (track 5) and densitograms: IRP vs type 2A VWD patient plasma; D – electrophoresis gel of IRP (track 1) vs type 2B VWD patient plasma (track 2) and densitograms: IRP vs type 2B VWD patient plasma; E – electrophoresis gel of IRP (track 1) vs type 2N VWD patient plasma (track 4) and densitograms: IRP vs type 2N VWD patient plasma; F – electrophoresis gel of IRP (track 1) vs type 3 VWD patient plasma (track 3) and densitograms: IRP vs type 3 VWD patient plasma. IRP, in-house reference plasma; LMWM, low molecular weight multimers; IMWM, intermediate molecular weight multimers; HMWM, high molecular weight multimers.*

Throughout the study period, our laboratory successfully participated in 13 external quality assessment (EQA) schemes of VWF modules, including VWF:Ag, VWF:GPIbM, FVIII:C, VWF multimers, and the final conclusion (interpretation), provided by the ECAT Foundation (Netherlands). We demonstrated that the VWF multimer electrophoresis interpretation and final interpretation of the VWD type were in agreement with expert opinions. In our study, all EQA samples (including those from patients with type 1 and type 2A VWD) were correctly visualized and interpreted using a new commercial VWF multimer assay (H5VW and/or H11VW), demonstrating its reliability.

Historically, VWF multimer analysis has been performed by only a limited number of expert- or reference laboratories mainly because the assay is technically laborious, complex, requires specially trained personnel, is time-consuming, and is non-standardized (Torres et al., 2012). Previously, it was noted that only 16–18% of ECAT Foundation EQA participating laboratories were registered for VWF multimer evaluation (Meijer & Haverkate, 2006). In addition, a certain proportion of interpretative errors arose because the test panels lacked a VWF multimer assay (E. J. Favaloro et al., 2014). ECAT Foundation collected data revealed substantial error rates for VWF multimer evaluation ranging from 10 to 52% (Meijer & Haverkate, 2006). Furthermore, the North American Specialized Coagulation Laboratory Association (NASCOLA) has shown an overall 14.7% (7–22%) erroneous survey response rate from laboratories performing in-house VWF multimer analyses (Chandler et al., 2011). Because high precision is needed for VWF multimer quantification, standardization of its measurements is crucial for accurate diagnosis, and the role of quality assurance in hemostasis laboratories is considerably important (E. J. Favaloro, 2019). Other researchers have evaluated the accuracy of the new Sebia

VWF multimer assay using different approaches. They compared plasma samples from patients presenting with different types of VWD with those from healthy volunteers (Vangenechten & Gadisseur, 2020), commercial Standard Human Plasma (E. J. Favaloro et al., 2020), donors, and commercial frozen normal donor plasmas (Bowyer et al., 2018). Our results not only support previously published data (Oliver et al., 2019) but also provide additional insights into analytical performance characteristics.

Another important issue is the turnaround time of VWF multimer analysis. The abovementioned in-house methods are time-consuming, and although several of them have been somewhat optimized, they still require several hours or even 3–4 days to complete (Budde et al., 2006). In our case, the Sebia Hydragel H5VW/H11VW VWF multimer electrophoresis assay produced same-day results within 6 h and 40 min. A significantly shorter turnaround time could encourage clinical laboratories to select this method instead of traditional, time-intensive procedures. Furthermore, in the case of analytical failure, same-day multimer analysis is more attractive because laboratories can repeat testing and release results relatively quickly, although confirmation of the VWD type is not an urgent analysis.

Recently, the Sebia method was extensively evaluated by other authors who provided positive comments on the fundamental consistency of our data (Bowyer et al., 2018; Oliver et al., 2017; Crist et al., 2018).

In conclusion, the new commercial VWF multimer assay (Sebia, France) represents a good alternative to traditional in-house assays. We conclude that the analytical performance of the Hydragel 5/11 von Willebrand multimer assay is acceptable and provides a perspective to standardization of the VWF multimer assay (Sebia, France), which can help reduce interlaboratory variability and the variability between different measurement runs. Both visual and densitometry-based investigations make interpretation easier, allow the overlay of patient curves with normal controls, and enable the estimation of the relative quantification of each multimer subset, providing useful information for the diagnosis of VWD.

Following the success at the EQA schemes and with the above performance data, the VWF:GPIbM and VWF:MM assays with H5VW/H11VW kits were accredited in the North Estonia Medical Centre laboratory according to ISO 15189.

4.1.3 Reference intervals for VWF multimers (Publication VI)

The current classification of VWD does not consider the magnitude of HMWM loss (Pruthi et al., 2010). However, quantitative results can provide objective measures of VWF structure to better define subtle changes in VWD subtypes (Michiels et al., 2017). The Sebia method provides quantitative VWF multimer results and allows, if desired, the splitting of curves into multimer subsets, as shown in Figure 4. The reference intervals were not originally defined by the manufacturer. Owing to the lack of reference values for VWF multimer fractions, the interpretation of results can be challenging in some cases. However, quantitative multimer analyses may be required to detect subtle abnormalities and changes following therapeutic interventions (Torres et al., 2012).

An important goal of this study was to determine the reference intervals for LMW, IMW, and HMW multimers.

International cooperation provided an opportunity to collect higher numbers of samples. In total, 131 samples of relatively healthy individuals were analyzed for VWF multimers (51 males and 80 non-pregnant females aged 17–69 years). The institutions that participated in this study are as follows:

- Coded L1 and represented two institutions from Baltic countries: L1A – Laboratory of North Estonia Medical Center, Tallinn Estonia; and L1B – Riga East University Hospital, Riga, Latvia; both Estonian and Latvian samples were analyzed in the Laboratory of North Estonia Medical Centre, and thus accounted for one group L1;
- Coded L2 and representing Department of Biology, Foch Hospital, Suresnes, France;
- Coded L3 and representing University of Utah / ARUP Laboratories, Salt Lake City, Utah, United States.

In this study, we found that the values of the three testing locations for LMWM, IMWM, and HMWM were normally distributed; thus, the reference values were calculated based on a normal distribution. The main result of this study was the proposal of reference intervals for VWF:MM fractions: LMWM 10.4–22.5%, IMWM 22.6–37.6%, HMWM 45.6–66.6%.

In 2018, other researchers (Bowyer et al., 2018), investigated multimeric patterns in 51 samples collected from healthy volunteers and using commercial frozen normal donor plasma (Cryocheck; Precision Biologic, Halifax, NS, Canada). They suggested variable ranges for HMWM (35–58.5%) but noted that Gaussian distribution was not observed. Importantly, the storage conditions for their commercial Cryocheck Normal Donor Set were as low as –40 to –80 °C. Any storage and/or transport issues, due to which the plasma could have reached temperatures outside the acceptable range, could have potentially affected the establishment of the lower limit of the reference HMWM interval.

A group of researchers from Belgium (Vangenechten & Gadisseur, 2020) calculated the normal reference intervals for VWF multimer fractions using samples from 40 healthy volunteers. They have reported 40.8–63.2% interval for HMWM.

The reference intervals determined in these two previous studies were similar to our results but were calculated using a relatively low-powered sample size. According to the CLSI guidelines C28-A3, a sample size > 120 can be considered representative. In our study, we collected and established reference intervals for the LMWM, IMWM, and HMWM fractions using acceptable amounts (n = 131) of samples from relatively healthy adults, thereby obtaining more accurate results.

It should be noted, that multimer fraction separation and their percentage values calculation is based on the scanned gel and are not directly measured quantitatively, and thus an interpretation of “gray zone” should be considered in future studies evaluating clinical decision making possibilities.

To assess possible differences in VWF multimer fractions from the three participating regions, we additionally compared the results, which were reported for the first time:

- We found that geographical locations related differences in VWF multimer fractions existed only in LMWM results, which were higher in group L2 (16.1%, CI 14.5–19.1%) than in groups L1 (15%, CI 12.7–17.2%) and group L3 (14%, CI 12.4–16.0%). The differences between L2 vs. L1 and L2 vs. L3 were statistically significant ($p < 0.05$) but clinically irrelevant, and the difference between L1 and L3 was insignificant ($p = 0.260$). There were no significant differences in IMWM and HMWM between geographical locations.
- Moreover, we demonstrated that there was no gender-related difference in the VWF multimer fractions: LMWM, $p = 0.067$; IMWM, $p = 0.507$; and HMWM $p = 0.060$.

- An interesting finding was the relationship between certain multimer fractions and age of the studied individuals. The tendency of LMWM scores to increase and HMWM scores to decrease with increasing age was observed in our data. Meanwhile, the IMWM values vary during adulthood. Nevertheless, definitive conclusions could not be drawn because of the small sample size. The discovered tendencies, especially that of HMWM to decrease with increasing age, could potentially be analyzed in detail in future larger studies. It is well known that VWF levels increase with age among healthy adults (E. Favaloro et al., 2014). A thorough search of the literature did not reveal any studies on the variation in HMWM depending on age. Several authors (Abou-Ismail et al., 2023) have noted that it remains unclear whether the normalization of VWF levels in older patients results in decreased bleeding symptoms, and this could potentially be evaluated in future larger studies.

In conclusion, the quantification of VWF multimer fractions is an additional valuable tool to supplement the qualitative visual assessment of VWF multimer patterns. It has the potential to aid in the differential diagnosis of the VWD and AVWS subtypes. The reference values calculated in this study can be used in future studies to establish clinical decision limits.

4.2 Clinical evaluation (Publications IV and V)

4.2.1 von Willebrand disease (Publication IV)

We retrospectively evaluated the data of 131 patients who were referred for the evaluation of bleeding tendency between 2016 and 2020. The frequency of complaints indicating VWD testing are demonstrated in Figure 8. Diagnostic criteria for VWD were based on the latest revised classification (P. D. James et al., 2021).

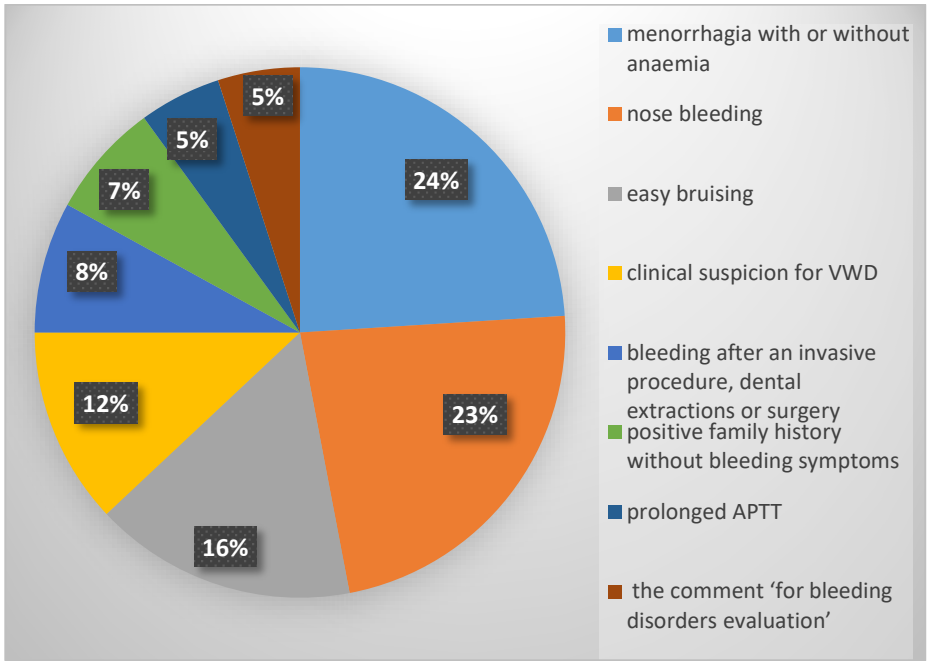


Figure 8. *Reasons (indications) for VWD testing and their frequencies.*

Currently, there is no standardized diagnostic algorithm for laboratory evaluation of VWD due to the technical/analytical availability and variability of VWF assays in different laboratories and countries. (Berntorp et al., 2022). Therefore, based on available international recommendations, we created a complex diagnostic algorithm for VWD and AVWS (Figure 9), which was widely discussed at the World Hemophilia Federation Congress in 2018.

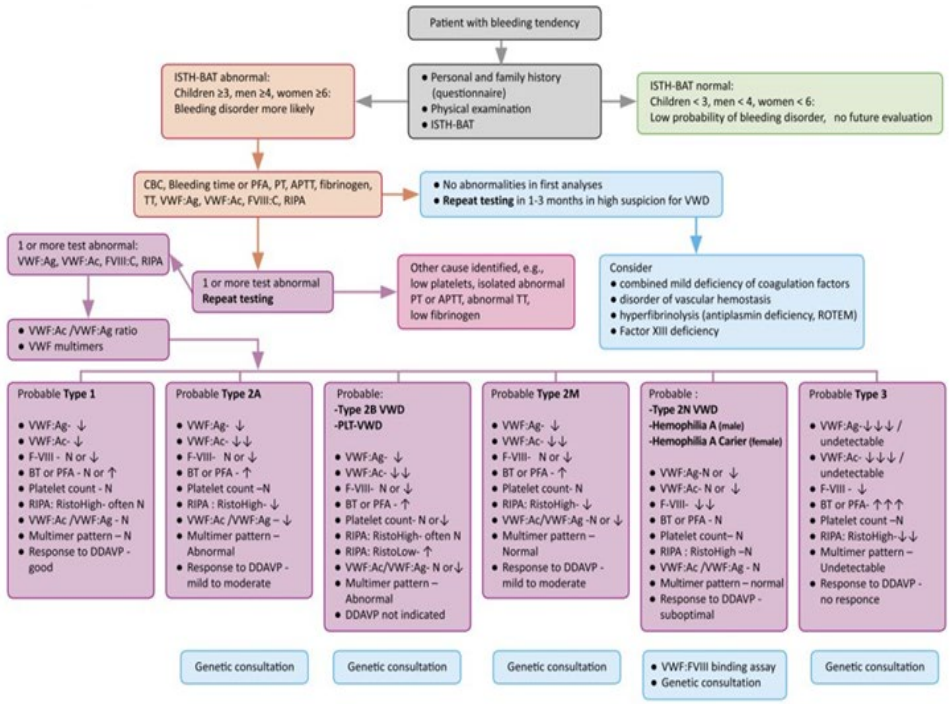


Figure 9. *Diagnostic algorithm for VWD and AVWS. Source: Pikta et al., 2018: Development of diagnostic algorithm for von Willebrand disease within WFH the Twinning Tallinn-Helsinki program. WFH 2018 World Congress, Glasgow, Scotland, May 20-24, 2018. Available at: www.postersessiononline.eu/173580348_eu/congresos/WFH2018/aula/-M-P_139_WFH2018.pdf*

In our study, all measurements of VWF:Ag, VWF:GPIbM, FVIII:C, and whole blood ristocetin-induced platelet agglutination (WB-RIPA), were repeated at least twice on a separate (newly collected) sample to confirm or negate initial investigation results. Based on the laboratory investigations (results are presented in Publication **IV** (Appendix)), all study participants were divided into different groups (Figure 10 and Table 4). Patients in Groups 4–7 were designated as suspected cases. Their results in relation to genetic testing will be analyzed in greater detail in the future.

In Figure 10, more than half of the study participants are as suspected to have type 1 VWD (including "low VWF" cases), with suspected VWD types 2A or 2M, representing the second largest group. Similar frequencies have been reported in population-based studies conducted in other countries (Nummi et al., 2018; Szanto et al., 2018).

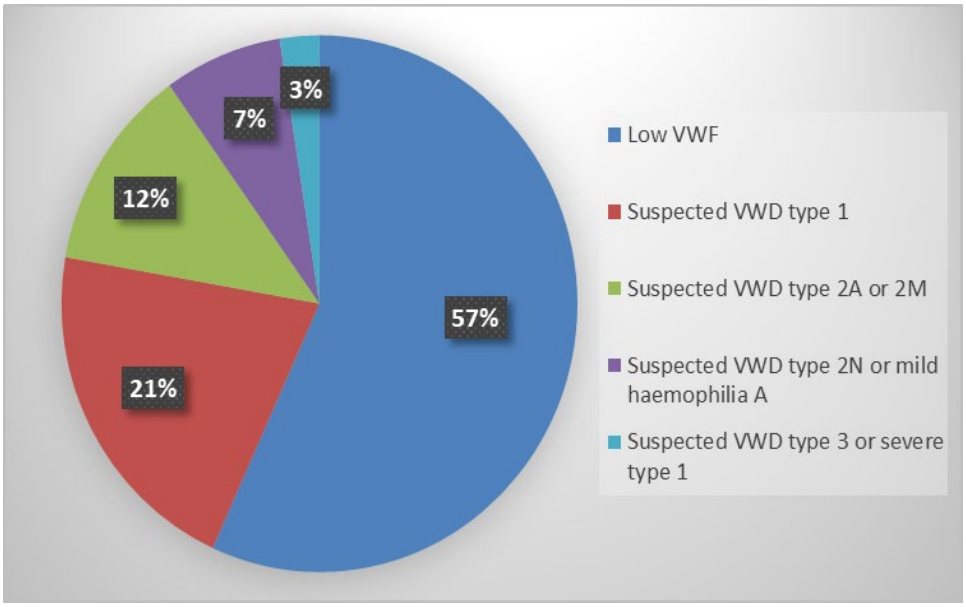


Figure 10. *Distribution of VWD types.*

In publication IV, we illustrated the platelet aggregation (RISTOhigh) test results in different study groups. However, WB-RIPA was not performed in the control group. Patients in groups 2, 3, and 6 had partly overlapping results with the reference intervals provided by the manufacturer. Platelet aggregation was reduced at a ristocetin concentration of 0.77 mg/mL in the group with suspected type 1 VWD and progressively reduced in the group with suspected type 2A or 2M VWD. Furthermore, the patients in these groups had low VWF and GPIbM values. No response to ristocetin was observed in group with suspected type 3 or severe type 1 VWD. RISTOhigh positively correlated with VWF:Ag ($r = 0.518$, $p < 0.01$) and VWF:GPIbM ($r = 0.484$, $p < 0.01$) in all participants.

Similar to our study, Swedish researchers (Schmidt et al., 2017) investigated the diagnostic accuracy of the WBA. They performed aggregation with a high ristocetin concentration, studied 100 patients with VWD, and reported that reduced WB-RIPA correlated with reduced VWF activity, making it a sensitive screening test to exclude VWD. Another group from Finland (Nummi et al., 2018) proposed the use of Multiplate WB-RIPA to rule out VWD and reported that patients with type 2A, 2M, and 2N exhibited clearly decreased RIPA with high ristocetin concentration in “low VWF” or type 1 VWD cases compared with normal controls. These findings are consistent with our data.

Table 4. *Definition of study groups and laboratory phenotype of participants.*

Groups	Laboratory phenotype
Group 1 Control group	Healthy individuals without bleeding symptoms and family history
Group 2 Non-VWD	Patients who do not currently fulfil the diagnostic criteria for VWD
Group 3 Low VWF	Patients with VWF:Ag and/or VWF:GPIbM values 30–50% and normal VWF:GPIbM/VWF:Ag ratio (> 0.7)
Group 4 Suspected VWD type 1	Patients with VWF:Ag and/or VWF:GPIbM values < 30% and normal VWF:GPIbM/VWF:Ag ratio (> 0.7)
Group 5 Suspected VWD type 2A or 2M	Patients with ratio of VWF:GPIbM/VWF:Ag < 0.7 and WB-RIPA results without enhanced response with low-dose ristocetin
Group 6 Suspected VWD type 2N or mild hemophilia A	Patients with VWF:Ag and VWF:GPIbM values within reference intervals and decreased FVIII:C results
Group 7 Suspected VWD type 3 or severe type 1	Patients with VWF:Ag values < 10%

The FVIII levels in healthy individuals were comparable to those in non-VWD patients ($p = 0.787$) and were lower in other groups ($p < 0.05$). VWF is the carrier of FVIII and therefore can be found in equal amounts in circulation as FVIII/VWF complex with FVIII/VWF ratio equal to one (Eikenboom et al., 2002) and the calculated FVIII/VWF:Ag ratio can be a useful laboratory marker, because such a ratio is > 1 in VWD type 1 and < 1 in VWD type 2N. We found that this ratio in healthy individuals and non-VWD patients was 1.17 and 1.42, respectively. Moreover, we confirmed that the FVIII/VWF: Ag ratio increased to ≥ 2 in patients with suspected type 1 VWD, demonstrating that a defect in VWF secretion from endothelial cells was the main cause of quantitative deficiency. Similar results were reported in other studies (Eikenboom et al., 2002; Michiels et al., 2017). Concomitantly, the ratio of FVIII/VWF:Ag was < 1 in patients with suspected type 2N VWD or mild hemophilia A, supporting the hypothesis of defective binding affinity of VWF to FVIII or FVIII deficiency in patients with hemophilia A. In our study, we found that the ratio of FVIII/VWF:Ag has the lowest results (0.33; 0.12–0.52) in patients with suspected type 2N VWD or mild hemophilia A and was statistically different compared with the other groups ($p < 0.05$). Our results are in agreement with other studies (Eikenboom et al., 2002) that demonstrated reduced FVIII/VWF:Ag ratio (0.24) in homozygotes for type 2N mutations.

Multimeric analysis of VWF was conducted for all patient samples. Of all the VWF multimer fractions, HMWM played the greatest role in terms of hemostasis (binding capacity for collagen and platelet receptor glycoproteins Ib and IIb/IIIa, and platelet aggregation) (Stockschlaeder et al., 2014). Qualitative and/or quantitative abnormalities in multimers result in defective hemostasis because the hemostatic activity of multimers is directly proportional to their size (Vangenechten & Gadisseur, 2020).

The reference ranges for HMWM (45.6–66.6%), as established in preclinical evaluation were used to interpret and decide whether HMWM fraction is decreased or not. Currently, no consensus exists regarding decision making. In this study, based on the

results from EQA schemes, we used 40% and 38% as cut-offs for HMWM in patients' and in lyophilized samples, respectively. HMWM values 40–45% were regarded as gray zone results in our study. However, our suggested cut-offs need to be clarified in larger studies.

VWF antigen and activity values in the control group were similar to those in group 2 ($p = 0.68$) and in the group with suspected type 2N VWD or mild haemophilia A ($p = 0.621$), and were statistically different from those in other groups. A normal VWF multimeric pattern was observed in the control group, and the multimer fractions were within the established reference intervals. Group 2 (50 of 131 samples; 38%) did not fulfil the diagnostic criteria for VWD, and therefore were defined as non-VWD cases. Their multimeric pattern was normal with normal VWD phenotypic profile, except HMWM was 36% (lower than the cutoff of 40%) in one patient. One international research group (Bowyer et al., 2018) evaluated the Sebia VWF multimer assay in comparison to in-house assays. This group demonstrated that a quantitatively normal pattern was observed with both multimer methods in 51 of 55 healthy normal donors (HMWM ranged 35–58.5%). Additionally, they found a loss of HMWM using the H5VW assay in patients classified as having normal VWF or type 1 VWD with genetic mutations in VWF linked to type 2 VWD. Bowyer et al. noted that VWF multimer assays should be performed in all patients with reduced levels of VWF activity and VWF:Ag and that testing should also be considered in individuals with normal VWF activity and VWF:Ag who experience significant bleeding of unknown cause (Bowyer et al., 2018).

In group 3, 46 of 131 (35%) suspected patients were identified to have low VWF (ranged 30–50%). Of which, 43 samples had a normal multimeric distribution. However, VWF multimer interpretation was difficult or impossible in three cases (interestingly, single-family members) because a smear-like appearance was visible on the gel. HMWM ranged from 25–37% by densitometry, and these family members had a normal ratio of VWF activity to antigen and normal platelet aggregation results. One group of researchers (Michiels et al., 2017) reported normal VWF multimer patterns with minor smearing in type 1 VWD (due to mutations in the D4–B1-3–C1-3 domain).

Group 4 included 17 patients of 131 (13%) categorized as suspected type 1 VWD (VWF antigen or activity value < 30%). All 17 samples showed normal multimeric pattern, but HMWM was 36% in one patient. The cut-off value of 30% (VWF antigen or activity level) used in this study for VWD type 1 diagnosis was based on expert consensus and previous guidelines (Nichols et al., 2008). In our study, VWF:Ag levels were < 50% and < 30% in 52% and 18% of the patients, respectively. Moreover, the values of VWF:GPIbM were < 50 % and < 30 % in 36% and 17% of the cases, respectively. In different studies, patients with VWF levels of 30–50% had VWF mutations detected 44–60% of cases (Lavin et al., 2017; Flood et al., 2016). Recently updated guidelines (P. D. James et al., 2021) recommend using VWF cutoffs of < 30% or < 50% in the presence of abnormal bleeding phenotypes.

Another important aspect is whether the VWF multimer assay is required in the VWD testing panel if the VWF:RCo (alternatively, VWF activity) to VWF:Ag ratios are successfully used as surrogate markers for the loss of HMW multimers (E. J. Favaloro et al., 2016). The VWF activity/VWF:Ag ratio can be used to aid in the diagnosis of type 2 VWD, with a ratio < 0.7, indicating the presence of dysfunctional VWF (Lawrie et al., 2013; P. D. James et al., 2021). In this study, we demonstrated a visible HMWM decrease (ranged 1.3–39 by densitometry) in all patients with suspected type 2A or 2M VWD. One of them showed a visual loss of IMWM and HMWM on the gel and quantitatively (IMWM, 7.1%; HMWM, 1.3%). Several authors (E. J. Favaloro et al., 2021) have also demonstrated

a loss of HMWM in patients with types 2A and 2B VWD (in most cases), and type 2A VWD is sometimes associated with IMWM loss. However, the recognition of the 2A and 2M subtypes based on multimer patterns is sometimes ambiguous because detecting the 2M phenotype with non-proteolyzed multimers is not possible using low-resolution gels (Budde et al., 2008). Group of researchers (Bowyer et al., 2018) found normal multimer patterns by in-house method in 28 patients classified as type 2M VWD, however, six of them had reduced HMWM (20.2–33.4%) on H5VW densitometry, exhibiting a flattened appearance of the HMWM peak.

Furthermore, we found that the ratio of VWF:GPIbM to VWF:Ag was lower in this group, suspected type 2A or 2M VWD, (0.51; 0.39–0.59), and the difference with the other groups was statistically significant ($p < 0.05$) demonstrating a dysfunctionality of VWF. However, the technical and analytical limitations of most VWF:RCo assays used in laboratories worldwide make the VWF:RCo/VWF:Ag ratio unreliable, especially at the levels of VWF:Ag < 15–20% (Sadler et al., 2006). In such cases, the VWF multimer assay is remarkably helpful in confirming or neglecting evidence of the loss of HMW multimers (Lillicrap, 2013). Moreover, we found that decreased levels of the VWF activity-to-antigen ratio were related to a reduction in HMWM. VWF:GPIbM/VWF:Ag positively correlated with HMWM ($r = 0.35$, $p < 0.01$) in all participants. Recently, a good correlation was found between various VWF activity/Ag ratios and HMWM (E. J. Favaloro et al., 2020).

Group 6 comprised of 5% of all patients who were classified as suspected VWD type 2N or mild hemophilia A. All samples showed normal multimeric pattern, but HMWM was 38% (lower than the cutoff of 40%) in one patient. In one study (Bowyer et al., 2018), 11 samples from patients with type 2N VWD demonstrated a normal multimer distribution evaluated by an in-house method, and 10 of them were normal according to the H5VW assay. Researchers found some loss of HMWM in one patient (31.7%) who was homozygous for p.Q1053H.

In group 7 two patients were categorized as having suspected VWD type 3 or severe VWD type 1. Consequently, VWF multimers were undetectable in both cases. According to VWF multimer assay sensitivity (9%), which was illustrated in preclinical study, it was not possible to perform densitometric analysis of the patients (VWF antigen levels were 2% and 8%). Similarly, the absence of multimers using both methods (in-house and H5VW) has been demonstrated in type 3 VWD samples (Bowyer et al., 2018).

Genetic testing has limited utility in the routine evaluation of VWD, given the highly polymorphic structure of the VWF gene; however, it should be available for specific, more challenging cases (Abou-Ismail et al., 2023). Genetic analyses have not been routinely performed for type 1 VWD. However, this procedure is often performed for VWD types 2 and 3 (Roberts & Flood, 2015b). In the current study, genetic testing was performed to differentiate between type 2N VWD and HA in four patients. Two of these patients were previously diagnosed with HA. Differential diagnosis between VWD and HA is important because HA therapy is monospecific (e.g., recombinant FVIII) and the management of VWD may be less effective if DDAVP or VWF replacement therapy is not provided (Michiels et al., 2017).

Generally, there are several issues in the diagnosis of VWD, which are mainly attributable to the heterogeneity of the disorder, limitations of laboratory assays, and the significant impact of various physiological processes on VWF (Abou-Ismail et al., 2023). Currently, there are no Food and Drug Administration (FDA)-cleared VWF:GPIbR, VWF:GPIbM, or VWF:CB assays; however, in Australia and Europe, all such assays are available (E. J. Favaloro, 2022). Medical information initially provided by clinicians is

required for appropriate laboratory evaluation of patients with bleeding disorders (E. J. Favaloro & Lippi, 2018). Moreover, non-specific results are considerably difficult to interpret and patient-related preanalytical issues should be taken into consideration (E. J. Favaloro & Lippi, 2018) especially in terms of perceived "diagnosis/exclusion" where values are close to important cut-off values (e.g., 30 or 50% for VWF activity or antigen). It is well understood that:

- FVIII and VWF are acute-phase proteins, and their levels are elevated after exercise and during inflammatory disorders; thus, repeat testing is needed after the resolution of these conditions;
- High estrogen levels are associated with increased VWF levels and can lead to a confounding diagnosis of VWD during pregnancy or hormonal replacement therapy in postmenopausal women (Abou-Ismail et al., 2023);
- Oral contraception and menstrual-related variations (estrogen level increases in the mid-follicular phase of menstrual cycles may be taken into consideration) can affect VWF levels.

In conclusion, this is the first report of a VWD laboratory evaluation in Estonia, which provides insights into the potential clinical significance of utilizing new VWF activity (VWF:GPIbM) and VWF multimer assays. The interpretation of VWF multimers must be complemented by quantification of multimer fractions by densitometry and visual gel examination.

4.2.2 Acquired von Willebrand syndrome (Publication V)

This retrospective study was performed in collaboration with North Estonia Medical Centre and Helsinki University Hospital, HUSLAB laboratory services, Coagulation Disorders Unit, in partnership with The Twinning Program of the World Federation of Hemophilia (WFH). All cases were discussed in interdisciplinary meetings between laboratory and clinical staff.

In contrast to inherited VWD, AVWS is not inherited and is associated with an increased risk of bleeding (Tiede et al., 2011). The diagnosis of AVWS is challenging, because no single automated VWF assay is sufficient to exclude or confirm AVWS (Janjetovic et al., 2022). In the absence of a family history of bleeding, AVWS is diagnosed based on laboratory assays used to diagnose inherited VWD (Tiede, 2012). The diagnostic algorithm presented in Figure 9 was used in this study.

All patients in our study met the criteria for AVWS diagnosis based on laboratory findings and bleeding symptoms, together with the absence of any previous history of a bleeding disorder. The most common clinical symptoms were easy bruising, epistaxis, menorrhagia, and bleeding complications after tooth extraction. The demographic and laboratory characteristics, and case series of the study participants are described in detail in Publication V (Appendix).

In the current study, we present the clinical and laboratory data of seven patients with AVWS and various underlying diseases, including non-Hodgkin's lymphoma (NHL), essential thrombocythemia (ET), polycythemia vera (PV), secondary polycythemia due to cardiovascular diseases with obstructive sleep apnea syndrome, monoclonal gammopathy of undetermined significance (MGUS), and autoimmune thyroiditis (AT). Previous studies (Michiels et al., 2001) have shown that MGUS, NHL, ET, and AT are associated with AVWS. The mechanisms behind VWF abnormalities depend on the type of underlying disorder and may include increased clearance, inhibition of VWF functions, adsorption to the

platelet surface, enhanced shear stress, and subsequent proteolysis, or rarely, decreased synthesis (Michiels et al., 2001). As reported in a registry of the ISTH, collected data showed that in 211 AVWS cases, lymphoproliferative disorders were the most frequent underlying disorder (48% of cases), whereas solid tumors and myeloproliferative neoplasms (e.g., ET, PV) accounted for 15% and 5% of cases, respectively (Federici et al., 2013).

We described the clinical-laboratory data of two patients with diagnosis of NHL and MGUS who had the laboratory phenotype characterized by significantly reduced levels of FVIII (42% and 21%), reduced VWF (25% and 29%), diminished VWF function (14% and 11%), associated with decreased levels of HMWM (10.8% and 31.8%). In line with a previous study (Valarche et al., 2011), our results revealed that AVWS was associated with a decreased response to higher ristocetin concentrations (RISTOhigh 12 U and 38 U), demonstrating a defect in primary hemostasis. In lymphoproliferative disorders and monoclonal gammopathies, the common mechanism is the increased clearance or inhibition of VWF by paraproteins or autoantibodies (Kumar et al., 2002). Mechanisms involving the selective adsorption of HMW multimers on tumor cells, leading to enhanced plasma clearance, have been described in lymphoproliferative diseases (multiple myeloma, Waldenström's macroglobulinemia, NHL, MGUS, and hairy cell leukemia), and solid cancers (Franchini & Mannucci, 2020). Antibodies that neutralize platelet-related VWF activity (inhibitors) have seldom been reported. However, in most cases, they are difficult to detect (Sadler et al., 2006) because standardized assays are not yet available (Federici et al., 2013).

According to the available data, AVWS may develop in every fifth patient with ET (Mital, Prejzner, Bieniaszewska, et al., 2015). Furthermore, from 5–30% of patients with ET may develop bleeding disorders of various severities (Rupa-Matysek et al., 2015). We present two cases of AVWS associated with an ET-positive *JAK2* (V617F) mutation. In both patients normal VWF:Ag levels (61% and 83%) with low VWF:GPIbM levels (34% and 29%) and decreased activity to antigen ratio (0.55 and 0.35) were observed, demonstraing the VWF dysfunctionality. VWF multimeric analysis revealed a decrease in HMW multimers, supporting AVWS in both cases. Similar results were reported in a study (Lancellotti et al., 2015), consists of 69 ET patients; laboratory findings were associated with high VWF:Ag levels, with a parallel reduction in VWF activity, a major decrease in VWF:Ac/VWF:Ag ratios, and atypical cleavage bands of VWF multimers. In a retrospective analysis of 170 patients the adsorption of VWF onto cell membranes and subsequent clearance were also involved in AVWS associated with ET (Mital, Prejzner, Bieniaszewska, et al., 2015). Moreover, adsorption on platelets is the mechanism of ET, with an inverse relationship between platelet count and defects in HMW multimers, and may cause increased VWF proteolysis (Mital, Prejzner, Bieniaszewska, et al., 2015). This mechanism has been reported in ET and PV, as well as in chronic myeloid leukemia, primary myelofibrosis, and sometimes in acute leukemia (Tiede et al., 2011).

The prevalence of AVWS in patients with ET and PV has not yet been established, as the available data are based on small case series and case reports. In retrospective study involving 142 consecutive patients, AVWS was observed in 17 patients with PV (12%) (Mital, Prejzner, Świątkowska-Stodulska, et al., 2015). Features that correlated with AVWS in this group included younger age, higher red blood cell, white blood cell, and platelet counts, bleeding symptoms, and lack of PV remission. In our study, we described clinical-laboratory profile of a patient with PV and demonstrated that RIPA, FVIII, VWF:Ag, and VWF:GPIbM levels were normal; however, the VWF activity to antigen ratio

(0.61), and HMW multimers were decreased (28.4%), confirming diagnosis of AVWS. These findings are similar to the study, which included 142 patients with PV (Mital, Prejzner, Świątkowska-Stodulska, et al., 2015), and reported a decrease in VWF activity to antigen ratio in 17 (12%) patients. Defects in the composition of VWF multimers have been reported less frequently in patients with PV and thrombocytosis (Franchini & Mannucci, 2020).

Cardiovascular conditions associated with AVWS include aortic stenosis, congenital cardiac defects, angiodysplasia, atrial septal defects, and mitral valve prolapse (Federici et al., 2013). Under these conditions, the synthesis of VWF and its release into circulation are normal, and decreased levels of HMW multimers are the result of increased shear stress and subsequent proteolysis (Tiede, 2012). Moreover, one review reported that the association of cardiovascular diseases with AVWS increased during the past 20 years: from 12% in the early published literature (31/288) to 41% in the reports of the German registry (344/840) (Federici et al., 2013). It was previously noted (Federici et al., 2000) that 77% of these patients were classified as bleeders. In our study, we evaluated a patient with cardiovascular disease and obstructive sleep apnea syndrome. The levels of VWF:Ag, VWF:GPIbM, and FVIII:C increased, whereas the VWF function/antigen ratio decreased (0.65). In this patient, we did not detect any abnormalities in the VWF pattern during the visual investigation of gel, yet densitometric data provided additional information about the VWF multimeric structure (HMW multimers decreased to 29%). Our results are consistent with the literature (Tiede et al., 2008) showing that in most cases, VWF:Ag and VWF:RCo are normal or even increased, and the VWF:RCo/Ag ratio is often, but not always, reduced. Another study have illustrated that in some cases, a loss or decrease in HMW multimers is the only laboratory abnormality indicative of AVWS (Tiede et al., 2011).

In patients with hypothyroidism (caused by autoimmune thyroiditis), AVWS is induced by decreased synthesis of an otherwise qualitatively normal VWF (Franchini & Mannucci, 2020). The earliest reports (Michiels et al., 2001; Federici et al., 2013) demonstrated a link between thyroid disorders and coagulation abnormalities. In the current study, we present a patient with autoimmune thyroiditis with decreased levels of VWF:Ag and VWF:GPIbM, and a normal VWF function/antigen ratio. Platelet aggregation was normal and multimeric analysis revealed a normal distribution pattern. Similar results were reported in a cohort study of 90 hypothyroid patients, 33% of whom had AVWS (Ordookhani & Burman, 2017).

The main treatment goals for patients with AVWS are prevention of acute bleeding in high-risk situations and achievement of stable remission or cure of AVWS (Tiede et al., 2011). Treatment of the patient's underlying condition with immunosuppressants, chemo- or radiotherapy, and surgery can lead to remission of AVWS, but is not always feasible and successful (Tiede et al., 2011). In our study, we showed that patient (case 7) who was treated with l-thyroxine and a year later had normal thyroid-stimulating hormone (TSH) values and normalized VWF levels, indicating that AVWS was reversed. It was previously reported that bleeding symptoms in patients with hypothyroidism were negatively correlated with VWF levels, and that after the restoration of hypothyroidism, VWF levels significantly increased (Ordookhani & Burman, 2017).

Differential diagnosis of VWD and AVWS is important because treatment approaches may differ (Tiede, 2012). Important hallmarks for the differential diagnosis are summarized in Table 5.

Table 5. *Differential diagnosis between VWD and AVWS (Tiede et al., 2011).*

Aspect	VWD	AVWS
Personal history	Early onset of bleeding No uneventful surgery or no previous high-risk situations	Late onset of bleeding Uneventful surgery before onset of bleeding
Family history	Positive	Negative
Pathogenesis	VWF gene mutation	a variety of pathogenic mechanisms
Laboratory evaluation	structural or functional disturbances of VWF	structural or functional disturbances of VWF
AVWS-associated disorder	Absent	Present
Treatment	Normal recovery and half-life of VWF-containing concentrate Sustained response to desmopressin	Remission after treatment of underlying disorder Response to IVIG (in IgG-MGUS-associated AVWS) Short-lived response to VWF-containing concentrates or desmopressin

In clinical practice there is one intriguing question: should we routinely order *VWF screening panel* to all patients with AVWS-associated disorders? Consistent with previous studies (Federici et al., 2000; Tiede et al., 2011), we implemented a strategy for routine *VWF screening panel* use, as illustrated in Figure 11.

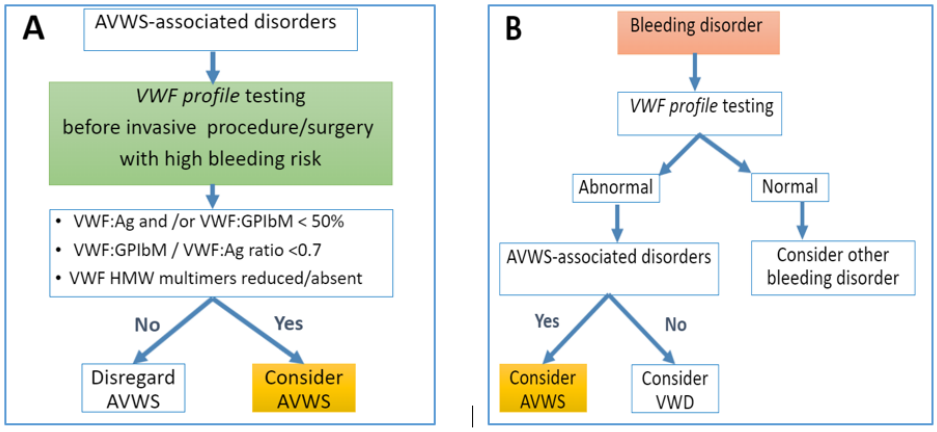


Figure 11. *Reason for testing in patients with AVWS-associated disorders: A – patients with AVWS-associated disorders before invasive procedure/surgery with high bleeding risk; B – every new patient with bleeding tendency/symptoms who does not have another obvious etiology (Tiede et al., 2011).*

As reported previously, AVWS often correlates with a reduced ratio of VWF:RCo to VWF:Ag (Federici et al., 2013). The same findings were observed in our study in all cases, except in a case where the patient's clinical presentation was caused by decreased synthesis of VWF. In our case series, we found that decreases in the levels of VWF:GPIbM and the ratio of VWF:GPIbM to VWF:Ag were associated with the selective loss or decrease in HMW multimers. Our results are in line with those of other clinical study

(Tiede et al., 2008), which suggested that a reduced VWF:RCo/Ag ratio in AVWS indicates inhibitory antibodies and selective loss or decrease in HMW multimers.

Notably, we found that patients with lower HMW multimers by densitometric evaluation presented with more severe bleeding complications.

In conclusion, the current study demonstrates that the diagnosis of AVWS is complex and requires extensive laboratory evaluation. Our results confirm that the new VWF:GPIbM assay and VWF multimer analysis are important tools for the diagnosis of AVWS. Complex laboratory evaluations and interdisciplinary collaboration are of paramount importance for the early recognition of AVWS and the selection of appropriate clinical management protocols.

5 Conclusions

Current study findings allow to draw several conclusions.

First, the Innovance VWF Ac assay (Siemens Healthineers, Germany) has proven to be reliable and precise, can be easily implemented on an STA-R Evolution analyzer (Diagnostica Stago, France) by a simple adapted protocol, and can be utilized as a component of a screening panel for the detection, classification, and monitoring therapy of VWD and/or AVWS.

Second, we confirmed that a VWF activity/antigen ratio (where VWF activity is measured by a newly implemented assay) cut-off < 0.7 may serve as a simple screening tool for VWD and AVWS.

Third, the new semiautomated VWF multimer assay (Sebia, France) may represent a good alternative to traditional in-house assays and can be successfully implemented for the second-stage evaluation of VWD and/or AVWS. Visualization of the multimer distribution and densitometric analysis, together with the applied LMW, IMW, and HMW multimer classifications, provided adequate resolution to correctly classify type 1, 2A, 2B, 2N, and 3 VWD cases.

Fourth, the established reference intervals for VWF multimers is a useful tool for results interpretation.

Last, these results prove that new laboratory assays, standardized diagnostic approaches followed by the three Estonian hemophilia treatment centres and their association with clinical information provide the possibility of a more accurate diagnosis and management of VWD and AVWS (the most common bleeding disorder) in Estonia.

Future perspectives

This work is envisioned to support the improvement of VWD and AVWS diagnoses in Estonia, and it is suggested that the actual VWD prevalence should be evaluated in the future.

It is of great interest that the results of this study will be analyzed in relation to the correlation between laboratory phenotypes and genotypes. This plan is in the project preparation phase at our center, in collaboration with geneticists.

The reference values calculated in this study can be used in future studies to establish clinical decision limits.

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Abstract

Implementation of innovative techniques in the diagnostic work-up of von Willebrand disease

Background

Von Willebrand disease (VWD) is the most common autosomal inherited bleeding disorder in humans and is caused by a deficiency or functional abnormality in VWF. Acquired von Willebrand syndrome **(**AVWS) is a rare but frequently underdiagnosed bleeding disorder with unknown prevalence and develops in various underlying disorders. Accessible and accurate diagnosis is considerably important for the correct management of VWD or AVWS, as treatment options and prognoses may differ. However, these diagnostic assays are often available only at a few specialized centers and can be time-consuming and costly. Prior to 2016, it was not possible to confirm the diagnosis of VWD or AVWS in Estonia because of the limited availability of VWF assays.

This thesis aimed to provide a preclinical and clinical evaluations of new VWF activity and VWF multimer assays for the diagnosis of VWD and AVWS.

Methods

In the first part of the study, the analytical performance characteristics of the new VWF activity and VWF multimer assays were investigated, and data for VWF multimer reference intervals were collected. The second part of the study focused on validating the clinical laboratory approach for the diagnosis of VWD and AVWS. The main objective was to analyze the value of incorporating new VWF activity and VWF multimer assays into the initial approach for the diagnosis of VWD and AVWS.

Results

Data from preclinical and clinical investigations demonstrated acceptable precision and accuracy of the new VWF activity and new VWF multimer assays, which were also accredited in the North Estonia Medical Center laboratory, according to ISO15189:2012. The proposed reference intervals for VWF:MM were calculated for LMWM 10.4–22.5%, IMWM 22.6–37.6%, HMWM 45.6–66.6%.

Conclusion

The new techniques evaluated in this study have been shown to be precise and reliable, and can be used as components of VWD and AVWS diagnostic algorithms.

Lühikokkuvõte

Innovaatiliste meetodite juurutamine von Willebrandi tõve diagnostikas

Taust

Von Willebrandi tõbi (VWD) on kõige sagedasem autosomaalne kaasasündinud veritsushäire. Selle põhjuseks on kas kvantitatiivne või kvalitatiivne von Willebrandi faktori puudulikkus. Omandatud von Willebrandi sündroom (AVWS) on aladiagnoositud ja harvaesinev (esinemissagedus ei ole teada) veritsushäire, mis on seotud mitmete allasetsevate haigustega. Kättesaadavad ja täpsed VWD ja AVWS diagnoosimise meetodid on vajalikud antud haiguste korrektseks käsitluseks, raviks ja haiguskulu prognoosimiseks. Samas on spetsiifilised diagnostilised meetodid sageli kättesaadavad ainult vähestes spetsialiseerunud keskustes, samuti võivad need olla aeganõudvad ja kallid. Eestis ei olnud enne 2016. aastat võimalik kinnitada VWD ja AWVS diagnoosi, sest puudusid vajalikud laboratoorsed meetodid.

Selle väitekirja eesmärgiks on prekliiniliselt ja kliiniliselt hinnata VWD ja AVWS diagnostikaks kasutatavaid uusi laboratoorseid meetodeid VWF aktiivsuse ja VWF multimeeride (MM) määramiseks.

Meetodid

Me hindasime uuringu esimeses faasis uute VWF aktiivsuse ja VWF multimeeride määramise meetodite analüütilisi parameetreid ja kogusime andmeid VWF multimeeride referentsväärtuste kindlakstegemiseks. Uuringu teine faas keskendus VWD ja AVWS kliinilis-laboratoorse diagnostilise algoritmi valideerimisele. Uuringu peamine eesmärk oli analüüsida uute VWF aktiivsuse ja VWF multimeeride määramise meetodite poolt antavat lisaväärtust VWD ja AVWS varasemale diagnostikale.

Tulemused

Prekliiniliste ning kliiniliste uuringute andmed näitavad uute VWF aktiivsuse ja VWF multimeeride määramise meetodite piisavat diagnostilist täpsust. Need uued meetodid on ka akrediteeritud Põhja-Eesti Regionaalhaigla laboris ISO15189:2012 alusel. Meie poolt pakutavad VWF:MM referentsväärtused on arvutuslikult järgnevad: LMWM 10.4–22.5%, IMWM 22.6–37.6% ja HMWM 45.6–66.6%.

Kokkuvõte

Käesolevas uuringus leidsime, et ülalkirjeldatud uued metoodikad on täpsed ja usaldusväärsed, mistõttu saab neid kasutada VWD ja AVWS diagnostilises algoritmis.

Appendix

Publication I

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Implementation and Verification of New VWF:Ac Assay System with Components from Different Manufacturers

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Summary

Background. The von Willebrand factor activity (VWF:Ac) assay is important in the diagnosis of von Willebrand disease (VWD), but the availability of this test is limited, because manufacturers of analyzers may not offer a possibility to measure VWF:Ac. Thus fully automated VWF:Ac system comprising reagents and analyzer by different manufacturers was implemented and verification of this ristocetin independent assay was done.

Methods and materials. The INNOVANCE® VWF Ac (Siemens Healthcare Diagnostics, Germany) kit was adapted on STA-R Evolution (Diagnostica Stago, France). Calibration was done with Standard Human Plasma, inter- and intra-assay precision was estimated with 2 levels of commercial QC material – Control Plasma N and P (all from Siemens Healthcare Diagnostics, Germany). Randomly selected residual patient plasmas were analyzed in parallel by 2 commercial kits (HemosIL vWF:RCo and HemosIL vWF:Ac assays on automated coagulometer ACL TOP500 (both reagents and analyzer by Instrumentation Laboratory, USA)) and results compared. Storage conditions of -20 °C were evaluated.

Results. Intra- and inter-assay variability ranged 4.00–5.02% and 4.69–5.00% respectively. Fresh and frozen thawed plasma VWF:Ac results correlated (r^2=0.952) and difference was not significant (p=0.201). VWF:Ac results of implemented assay and 2 HemosIL assays were comparable (vWF:Ac r^2=0.984; vWF:RCo r^2=0.978) and difference was not significant (p=0.222 and p=0.835 respectively).

Conclusions. The analytical performance of implemented VWF:Ac assay was shown to be acceptable. Thus VWF:Ac assay can be easily obtained by implementing it on different manufacturer's analyzer by adapted protocol and can be used for the diagnosis, classification of VWD and monitoring VWD therapy.

Keywords: von Willebrand disease, von Willebrand factor, activity assay, analytical performance.

INTRODUCTION

The diagnosis of von Willebrand disease (VWD) is not complete without von Willebrand factor (VWF) activity assessment. However, routine laboratories face challenges while selecting the suitable VWF activity assay (VWF:Ac). First, not all manufacturers of analyzers offer a possibility to measure VWF:Ac. Secondly, recently several assays have been developed as an alternative to imprecise and insensitive ristocetin cofactor activity

Table 1. **Calibration curve data of implemented VWF:Ac assay**

Assigned value, %	98	74	59	49	29	10
Measured value, %	98	74	59	49	30	10
Measured value, DOD	0.090	0.072	0.057	0.045	0.020	-0.002

DOD – the change in optical density (OD) as delta absorbance.

Table 2. **Intra- and inter-assay variability of implemented VWF:Ac assay**

Material	Assigned value (range), %	Intra-assay variability (repeatability), CV%	Inter-assay variability (reproducibility), CV%
Control Plasma N	92 (74–110)	4.00	4.69
Control Plasma P	28 (22–34)	5.02	5.00

(VWF:RCo) assay, which traditionally is considered as a "gold standard" assay for evaluating VWF function. The approved nomenclature of VWF activity assays, suggested by SSC of the ISTH (Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis), characterizes differences between available methodologies [1]. One of commercially available automated assays (INNOVANCE® VWF Ac by Siemens Healthcare Diagnostics, Germany) is based on the spontaneous binding of VWF to a gain-of-function mutant GPIb fragment and does not require ristocetin. Recently published studies showed a good comparability of latter method with the VWF:RCo assay [2, 3]. To overcome challenges in selecting VWF:Ac method fully automated assay application for determination of VWF activity was implemented on STA-R Evolution analyzer (Diagnostica Stago, France) using different manufacturer's reagents (INNOVANCE® VWF Ac by Siemens Healthcare Diagnostics, Germany). An international standard EN ISO 15189:2012 (Medical laboratories – Requirements for quality and competence) states the requirement for laboratories to validate the performance of non-standard, laboratory designed methods (5.5.1.3 Validation of examination procedures) [4]. The aim of this study was to evaluate the analytical performance of the adapted ristocetin independent VWF:Ac assay by the standard methodology described in laboratory of North Estonia Medical Centre (accredited according to EN ISO 15189:2012).

MATERIALS AND METHODS

The INNOVANCE® VWF Ac (Siemens Healthcare Diagnostics, Germany) kit with three ready to use reagents was equipped to create an alternative VWF activity test protocol on different manufacturer analyzer STA-R Evolution (Diagnostica Stago, France). Standard Human Plasma (Siemens Healthcare Diagnostics, Germany), in which VWF activity is calibrated against WHO standard, was used for the new method calibration. 3rd order polynomial calibration mode was selected.

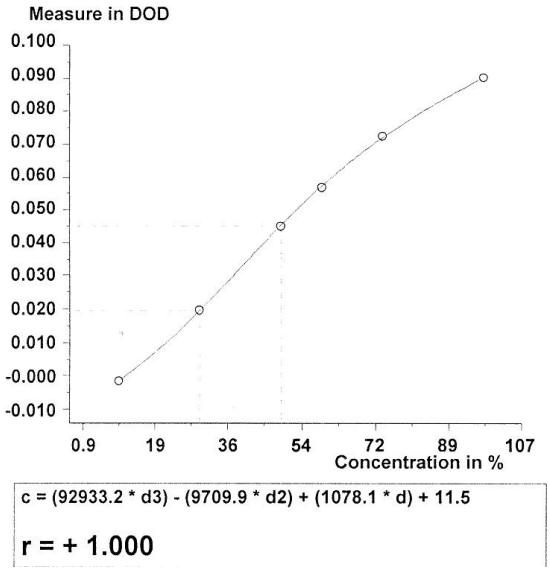

Fig. 1. **Calibration curve of implemented VWF:Ac assay**

Table 3. **Descriptive statistics and Passing-Bablok agreement of three VWF:Ac assays**

Parameter	Implemented VWF:Ac	HemosIL VWF:Ac	HemosIL vWF:RCo
n	15	15	15
Mean	74.27	78.96	73.73
Median	60.00	61.00	54.00
Std. deviation	55.65	67.61	60.27
Min	18	17	18
Max	228	278	242
25th percentile	36.00	37.00	30.00
75th percentile	106.00	106.80	94.00
Slope*	N/A	1.117	0.977
Slope 0.95 CI low*	N/A	1.016	0.818
Slope 0.95 CI high*	N/A	1.236	1.142
Intercept*	N/A	-3.27	-0.17
Intercept 0.95 CI low*	N/A	-8.15	-9.87
Intercept 0.95 CI high*	N/A	0.42	7.57

* – parameter compared to an implemented VWF:Ac assay.
CI – confidence interval. N/A – not applicable

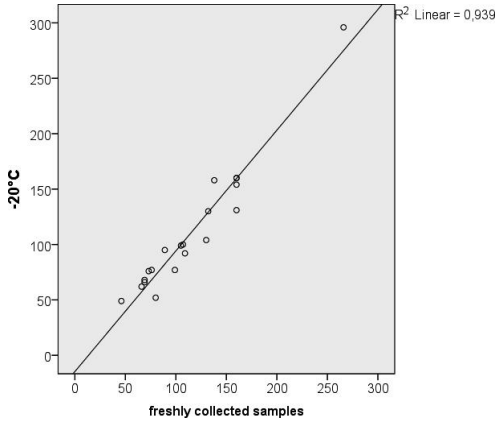

Fig. 2. **Linearity plot of VWF:Ac results obtained from freshly collected plasma samples versus –20 °C stored plasma samples**

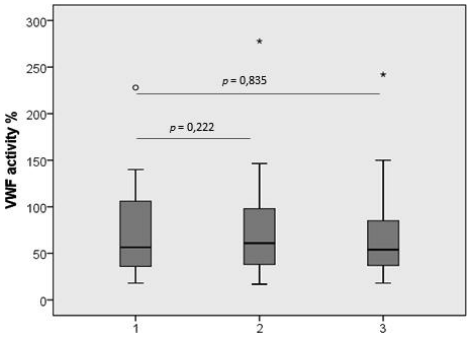

Fig. 3. **Difference between three VWF:Ac assays: 1 – INNOVANCE® VWF Ac (Siemens Healthcare Diagnostics, Germany); 2 – HemosIL VWF Activity (Instrumentation Laboratory, USA); 3 – HemosIL vWF:RCo (Instrumentation Laboratory, USA)**

According to an adapted protocol with standard dilution 1/3, range of valid calibration curve was 10 to 80%. To correctly test samples higher than 80% or lower 10%, dependent assay protocols were set up with dilution conditions, respectively 1/6 dilution for the measuring range 80 to 160%, and 1/1 dilution for the measuring range 3.33 to 10%. Inter- and intra-assay precision was estimated with 2 levels of commercially available quality control material – Control Plasma N and Control Plasma P (Siemens Healthcare Diagnostics, Germany). Precision study was carried out 5 times on each of 5 consecutive days. The coefficient of variation less than 10% was selected as an acceptable imprecision [5]. To test the difference between sample storage conditions (freshly collected plasmas versus frozen and once thawed plasma), 20 random plasmas were frozen -20 °C and retested after 2 weeks of storage. Linearity and threshold of the method was evaluated. Values of implemented VWF:Ac were measured in 15 randomly selected patient samples, which have been analyzed in parallel by two commercial kits: HemosIL vWF:RCo and HemosIL vWF:Ac assays on automated coagulometer ACL TOP500 (both reagents and analyzer by Instrumentation Laboratory, USA). Latter two methods have been compared previously and data published [6]. Statistical analysis was performed with SPSS software (version 20, IBM). Statistical significance was considered if $p<0.05$. To assure that results are reliable and accurate laboratory participated in the external quality assurance (EQA) survey for von Willebrand Factor assays (ECAT Foundation, The Netherlands). An individual performance was evaluated by Z-score, acceptance criteria: Z-score ±2. All experiments were carried out on commercially available material and residual material remaining after the completion of any diagnostic tests in accordance with ethical requirements.

RESULTS

Successful calibration verification is represented by 3rd order polynomial calibration curve (Figure 1) and calibration data (Table 1). Linearity between original method assigned values and new method measured values (table 1) demonstrates excellent correlation ($y=0.9951x+0.4266$; r squared = 0.9999). Intra-assay preci sion (representing repeatability) and inter-assay precision (representing reproducibility) are summarized in Table 2. Diluting Standard Human Plasma (Siemens Healthcare Diagnostics, Germany) threshold of newly implemented VWF:Ac assay was found to be similar to manufacturer's declared value – 4%. 20 consecutive runs of 0.9M NaCl solution revealed undetectable results or results below 4% of VWF activity. Fresh and frozen thawed plasma VWF activity results provide comparable data: correlation is excellent (Spearman's r=0.952, $p<0.001$; $y=1.0892x-14.626$; r squared = 0.9386) and difference is statistically insignificant (114.70±50.77% vs 110.30±57.08%; p=0.201). Correlation of implemented VWF activity assay with two HemosIL assays showed to be perfect: Spearman's correlation coefficients were 0.982 and 0.986 (both $p<0.001$) and for HemosIL vWF:Ac and HemosIL vWF:RCo respectively. Linearity study results were as follows: implemented VWF:Ac versus HemosIL vWF:Ac – $y=1.2058x-10.612$, r squared = 0.9844; implemented VWF:Ac versus HemosIL vWF:RCo – $y=1.0699x-5.5963$, r squared=0.9781. Paired samples test analysis did not reveal any significant difference between three assays (Figure 3). Descriptive statistics and Passing-Bablok agreement of the methods is provided in table 3. As an individual performance indicator, the Z-score obtained from EQA reports (Figure 4, Table 4) is acceptable.

DISCUSSION

The analytical performance of implemented new VWF:Ac assay was found to be acceptable. EQA results, where normal plasma and plasma of

Table 4. **Performance of implemented VWF:Ac assay in EQA program's particle ligand-receptor assay methods' group (INNOVANCE® vWF:Ac) (reproduced with permission from ECAT Foundation, The Netherlands)**

ECAT Foundation EQA program	n	Assigned value	CV%	Range	Our result	Z-score
Von Willebrand Factor parameters (2016-1)	90	105	8.9	78-146	116	1.15
Von Willebrand Factor parameters (2016-2)	91	33	6.4	25-38	36	1.46

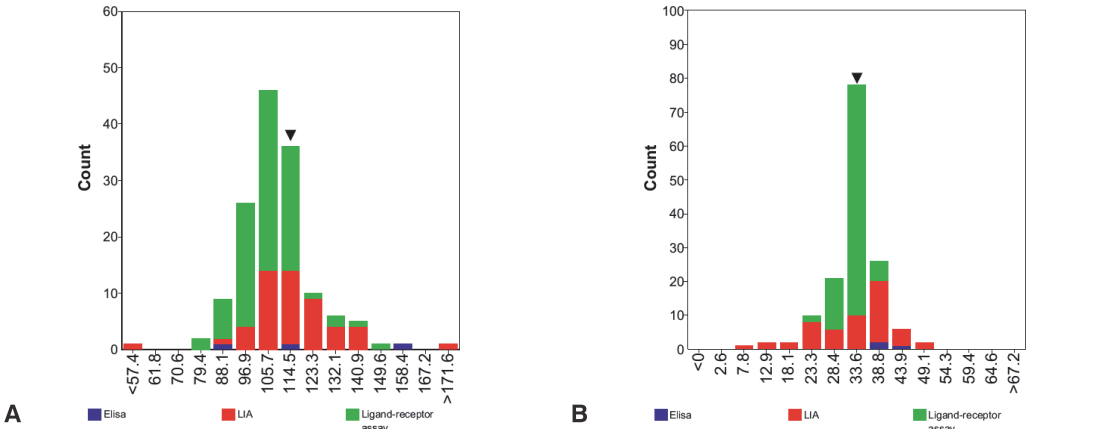

Fig. 4. **Histograms of ECAT Foundation EQA program 'Von Willebrand Factor parameters': A – survey 2016-1; B – survey 2016-2 (reproduced with permission from ECAT Foundation, The Netherlands).**
Black arrow represents laboratory's result within the distribution

a patient with type 1 VWD were provided, and vWF:Ac/vWF:Ag ratio values as well as interpretations were in accordance, also approve method's validity. Thus, despite reagent's and instrument's manufacturer is not the same the INNOVANCE® VWF Ac assay can be easily installed on a STA-R Evolution analyzer and according to our results can be used for the diagnosis, classification of VWD and monitoring VWD therapy. In respect that VWF activity assay should be used as first level laboratory tests [7], the possibility to measure VWF:Ac with other components of a VWD screen panel (i.e. VWF:Ag assay, factor VIII level) is an important advantage for routine laboratory in evaluation of patients with bleeding disorders.

Disclosures

All authors state that they have no conflict of interest. ◆

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Santrauka

NAUJOS VWF:Ac TYRIMO SISTEMOS, SUSIDEDANČIOS IŠ SKIRTINGŲ GAMINTOJŲ KOMPONENTŲ, ĮDIEGIMAS IR VERIFIKAVIMAS

Marika Pikta, Valeria Zolotareva, Jelena Tõnne, Margus Viigimaa, Valdas Banys

Darbo tikslas. Vilebrando faktoriaus aktyvumo (VWF:Ac) tyrimas yra svarbus Vilebrano ligos diagnostikai, tačiau metodo prieinamumas yra ribotas. Kai kada kraujo krešėjimo analizatorių gamintojai negali pasiūlyti priemonių VWF:Ac atlikti. Todėl buvo įdiegta visiškai automatizuota VWF:Ac sistema, kurią sudaro skirtingų gamintojų reagentai ir analizatorius. Atlikta šio nuo ristocetino nepriklausomo VWF:Ac metodo sistemos įsisavinimo procedūra.

Tyrimo medžiaga ir metodai. INNOVANCE® VWF Ac (*Siemens Healthcare Diagnostics*, Vokietija) reagentų rinkinys buvo adaptuotas *STA-R Evolution* analizatoriui (*Diagnostica Stago*, Prancūzija). Kalibravimas atliktas standartine žmogaus plazma, o pakartojamumas ir atkuriamumas įvertinti dviejų lygmenų komercinėmis kontrolinėmis medžiagomis – kontroline plazma N ir P (visi reagentai *Siemens Healthcare Diagnostics*, Vokietija). Atsitiktinai pasirinktų pacientų plazmos likučiai buvo lygiagrečiai tiriami dviem komerciniais rinkiniais – *HemosIL vWF:RCo* ir *HemosIL vWF:Ac* automatizuotu analizatoriumi *ACL TOP500* (ir reagentai, ir analizatorius *Instrumentation Laboratory*, JAV), o rezultatai palyginti. Įvertinta mėginių laikymo –20 °C temperatūroje sąlyga.

Tyrimo rezultatai. Pakartojamumo variacijos koeficientas buvo 4,00–5,02 %, o atkuriamumo – 4,69–5,00 %. Šviežios ir šaldytos plazmos VWF:Ac rezultatai tarpusavyje koreliavo (r^2=0,952), o skirtumas buvo nereikšmingas (p=0,201). Įdiegto metodo VWF:Ac ir dviejų komercinių HemosIL metodų rezultatai buvo palyginami (vWF:Ac r^2=0,984; vWF:RCo r^2=0,978), o skirtumai nereikšmingi (atitinkamai p=0,222 ir p=0,835).

Išvados. Analizinės įdiegto VWF:Ac metodo charakteristikos yra priimtinos. Metodas, susidedantis iš skirtingų gamintojų komponentų, yra lengvai įdiegiamas taikant specialiai sukurtą protokolą ir yra tinkamas Vilebrando ligos diagnostikai, klasifikacijai ir gydymo stebėsenai.

Reikšminiai žodžiai: Vilebrando liga, Vilebrando faktorius, aktyvumo tyrimas, analizinės charakteristikos.

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Publication II

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RESEARCH ARTICLE

WILEY

Preclinical evaluation of a semi-automated and rapid commercial electrophoresis assay for von Willebrand factor multimers

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Background: The von Willebrand factor (VWF) multimer test is required to correctly subtype qualitative type 2 von Willebrand disease (VWD). The current VWF multimer assays are difficult, nonstandardized, and time-consuming. The purpose of this study was to evaluate the clinical utility of the commercial VWF multimer kit by Sebia (Lisses, France), an electrophoresis technique yielding same-day results.

Methods: Ten healthy volunteer plasma samples, in-house reference plasma (IRP) and commercial normal plasma (CNP) samples, 10 plasma samples from patients with a known VWD type, 1 hemophilia A plasma sample, and 7 external quality assurance (EQA) samples were analyzed using the commercial VWF multimer kit. Additional coagulation testing included measurements of VWF antigen (VWF:Ag), VWF activity (VWF:Ac), and FVIII activity (FVIII:C).

Results: The CNP results revealed a relative loss of the highest molecular weight multimers; therefore, IRP was preferred as the reference sample. The interpretations of 10 patients with a known VWD type could be successfully reproduced and agreed with previous VWF multimer results. In all EQA surveys, the multimer results and final VWD diagnosis agreed with expert opinion.

Conclusions: The VWF multimer assay by Sebia is easy to perform and can be successfully implemented in any clinical laboratory for second-stage evaluation of VWD. The resolution power of multimer distribution is adequate to correctly classify VWD types 1, 2A, 2B, and 3.

KEYWORDS

electrophoresis, subtyping, von Willebrand disease, von Willebrand factor, von Willebrand factor multimer

1 | INTRODUCTION

von Willebrand factor (VWF) is a large multimeric adhesive sialoglycoprotein[1,2] that mediates platelet adhesion to sub-endothelium structures and acts as a factor VIII (FVIII) carrier molecule, thus stabilizing the procoagulant activity of FVIII in the circulation.[1-5] The protein is synthesized by endothelial cells and megakaryocytes[4,6-8] as a polypeptide and is composed of identical monomers that assemble into a series of multimers. The multimer organization is critical for the function of VWF.[4] Multimers may range in size from 500 kDa to >20 000 kDa[7] and are usually classified into categories according to the number of multimers (dimers) and size: low-molecular-weight (LMW, 1-5 dimers, 500-2500 kDa), intermediate-molecular-weight (IMW, 6-10 dimers, 3000-5000 kDa), high-molecular-weight (large) (HMW, 11-20 dimers, 5500-10 000 kDa), and ultra-high-molecular-weight (ultra-large) (UHMW, >20 dimers, up to 20 000 kDa) forms.[4,7]

UHMW multimers do not typically circulate in blood because of rapid proteolysis by the disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (known as ADAMTS13) that cleaves UHMW forms into smaller multimers soon after secretion.[4]

von Willebrand disease (VWD) is the most common congenital bleeding disorder, with a worldwide prevalence of 1%.[3,7,8] The current classification of VWD variants by the International Society on Thrombosis and Haemostasis (ISTH)[9] recognizes six different types, reviewed with updates elsewhere.[1,4,5,7] The diagnosis of VWD is aided by good correlation between the clinical picture and traditional (screening) assays, such as FVIII, VWF antigen (VWF:Ag), VWF activity (VWF:RCo or alternatively VWF:Ac),[3] and, in some cases, the collagen binding capacity of VWF (VWF:CB).[10,11] Classical screening assays are highly heterogeneous in terms of methodology and diagnostic efficacy[10]; thus, they may lead to over-, under- or misdiagnosis[7] and inadequate or inappropriate treatment of affected patients.[12] Therefore, additional confirmatory VWD tests, such as VWF multimers, are needed to distinguish type 2A and 2B VWD from type 2M (or type 1) VWD[12,13] and diagnose acquired VWD. However, multimer analysis is currently performed only by a limited number of expert laboratories because it is technically difficult, laborious, nonstandardized, and time-consuming.[14] Indeed, a high proportion of laboratories generate unreliable VWF multimer results[15-17] using in-house assays. To overcome technical difficulties and help in the standardization of the method, Sebia (Lisses, France) developed a simplified, same-day results semi-automated assay (Hydragel 5 von Willebrand multimers) to visualize VWF multimers. The purpose of our study was to evaluate the usefulness and fitness for clinical purpose of this newly available commercial agarose gel electrophoresis technique.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Four targets for the evaluation of the VWF multimer pattern were selected as follows: (i) 10 healthy volunteer plasma samples; (ii) in-house reference plasma (IRP) samples and commercial lyophilized pooled normal plasma (CNP) samples (Diagnostica Stago S.A.S., Asnières sur Seine, France); (iii) 10 plasma samples from patients with known VWD type 1, type 2 (subtypes 2A, 2B, 2N), or type 3, among whom 9 patients were from Finland and 1 was from Estonia, plus one hemophilia A patient was selected; and (iv) 7 external quality assurance (EQA) samples from the "von Willebrand Factor parameters" survey provided by the ECAT Foundation (Voorschoten, the Netherlands).

The study was carried out at North Estonia Medical Centre in collaboration with Helsinki University Hospital, HUSLAB Laboratory, Coagulation Disorders Unit (in partnership with The Twinning Program of the World Federation of Hemophilia (WFH)). The study was performed according to the Declaration of Helsinki and was approved by two national ethical committees (both the Tallinn and Helsinki Ethical Committees on Medical Research).

2.2 | Coagulation assays

All plasma samples in question were analyzed for FVIII:C, VWF:Ag, and VWF:Ac using the STA-R Evolution analyzer (Diagnostica Stago S.A.S., Asnières sur Seine, France). The FVIII:C (STA Deficient VIII, STA PTT-A) and VWF:Ag (STA Liatest VWF:Ag) reagents were purchased from Diagnostica Stago S.A.S. (Asnières sur Seine, France). VWF:Ac was measured using the INNOVANCE VWF Ac reagent (Siemens Healthcare Diagnostics, Marburg, Germany) according to a previously described method.[18]

2.3 | Preparation of IRP

IRP was prepared from 10 healthy volunteer plasma samples. Blood specimens were collected into 3.2% sodium citrate tubes (BD Vacutainer, BD Diagnostics, Plymouth, UK), centrifuged at 1500 *g* for 10 minutes at room temperature to generate platelet-free plasma, pooled, aliquoted into Eppendorf type tubes (composed of a nonactivating plastic), and frozen at −70°C. Prior to testing, samples were thawed in a 37°C water bath (for approximately 5 minutes) and mixed thoroughly. The volunteers were healthy laboratory employees without history of hemorrhagic episodes, who were not taking any medication for at least 10 days before blood collection, had a normal coagulation screen profile and normal VWF screening assays results, and who provided written consent. Exclusion criteria consisted of a positive personal and/or family bleeding history, inflammation, pregnancy, and oral contraceptive use.

2.4 | VWF multimer method developed by Sebia

All constituents of the assay (reagents, instruments, and software) were provided by Sebia (Lisses, France). Plasma samples were treated with sample diluent (pH 5.0 ± 0.5) and were pre-incubated for 20 minutes at 45°C. The dilution ratio was adapted based on the VWF:Ag result according to the manufacturer's instructions. The treated plasma samples were grouped into quintuplets, loaded onto Hydragel 5 von Willebrand multimer gels, and then subjected to a migration step using a Hydrasys 2 system with the following parameters: under 1 W constant, 10°C, controlled by the Peltier effect, until 170 Vh has accumulated, and a duration of approximately 115 minutes. The multimers were fixed on the gel using rabbit origin anti-VWF antibodies and then were probed with a second-step immunofixation by horseradish peroxidase (HRP)-conjugated anti-mammalian IgG. VWF multimers were evaluated by visualization after coloring the gels with commercially available reagents (commercial abbreviation TTF1/TTF2), and densitometric gel scan/graphical curves were produced and visualized with Sebia Phoresis CORE software.

2.5 | Previous studies confirming the VWD diagnosis

Nine patients with VWD from Finland with a complex evaluation and follow-up at the European Haemophilia Comprehensive Care Center (EHCCC) in Helsinki were included. VWF multimers from 8 patients were

previously analyzed by SDS agarose electrophoresis, western blotting, and luminescent visualization recorded by photoimaging in 1 of 4 reference laboratories with long-standing expertise in VWD diagnostics: type 1 and type 2 VWD patients were analyzed at Lund University (Malmö, Sweden), Karolinska Institute (Stockholm, Sweden), or Finnish Red Cross (Helsinki, Finland), and type 3 patients were assessed in collaboration with Dr. R. Schneppenheim (Hamburg, Germany).[19] Genetic testing was performed (i) in type 2B and 2N to differentiate between platelet-type VWD and hemophilia A and (ii) in type 3 in connection with genetic counseling, and the results were consistent with the VWD types. The genotypes of all type 3 VWD patients have been previously reported.[19]

3 | RESULTS

3.1 | VWF multimers in normal samples

The Hydragel 5 von Willebrand multiyear agarose gels were used to run 4 test samples and one control (reference) sample simultaneously. Comparative analysis of the size spectrum and banding pattern of VWF multimers in IRP and CNP samples on gels and the quantitative results of IRP (LMW-M 15.9%, IMW-M 33.2% and HMW-M 50.9%) and CNP (LMW-M 24.6%, IMW-M 34.7% and HMW-M 40.7%) densitometric curves revealed a relative loss of the highest HMW multimers in CNP samples, probably due to the lyophilization process while preparing commercial plasma (Figure 1).[17,20] Thus, IRP was preferred as control (reference) plasma in further studies.

The qualitative visual assessment of the size and distribution of VWF multimers in the plasma samples of four healthy subjects resulted in the pattern depicted in Figure 2, where one band on the gel and one peak in the densitogram correspond to one multimer band. LMW multimers are located on the top of the gel and at the left side of the *x*-axis of the densitogram. Correspondingly, HMW multimers are located on the bottom of the gel and at the right side of the *x*-axis of the densitogram, and IMW multimers are located in-between. Although there is no consensus on the definition of the areas comprising LMW, IMW, and HMW multimers, for convenience in interpreting the results, the multimer bands of this quartet of healthy subjects were classified as follows: 1-3 left to right peaks in the densitogram would represent LMW multimers, peaks 4-7 would represent IMW multimers, and peaks 8 and onwards would represent the group of HMW multimers (Figure 2). The applied classification is specific to the Sebia method and differs from

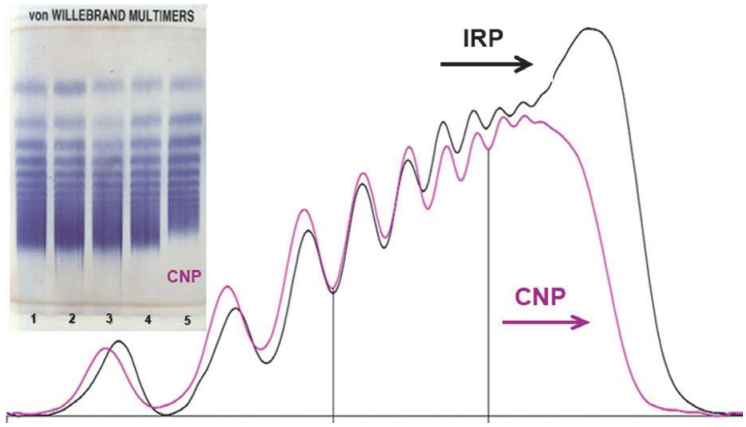

FIGURE 1 Electrophoresis gel of IRP samples (tracks 1-2), plasma samples from 2 randomly selected healthy individuals (tracks 3-4), and CNP samples (track 5). Densitograms of CNP and IRP

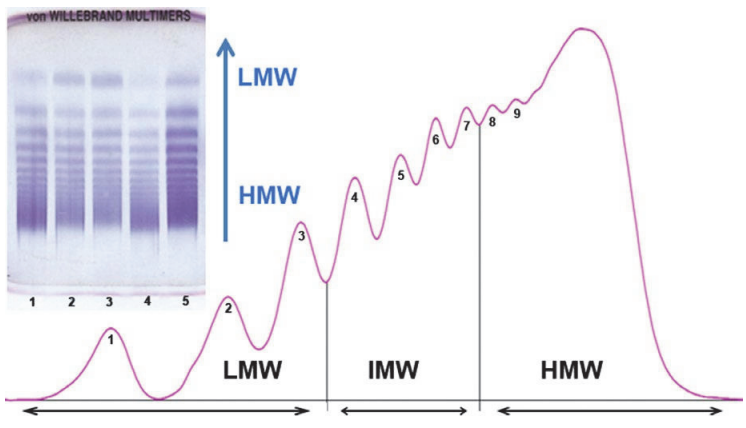

FIGURE 2 Electrophoresis gel of plasma samples from 4 different healthy individuals (tracks 1-4) and an IRP sample (track 5). Densitogram of IRP

reports in the literature (cited in the introduction), thus highlighting methodological differences between previously reported in-house and Sebia assays.

3.2 | VWF multimers in patient samples

VWF multimer electrophoresis interpretations of 9 VWD type (1, 2A, 2B, 2N, or 3) samples from HUSLAB, one type 2N VWD sample from Estonia, and one hemophilia A sample from Estonia could be successfully reproduced and were in agreement. The VWF:Ag, VWF:Ac, Ac/Ag ratio, FVIII:C results and VWF multimer pattern interpretations are summarized in Table 1. Several examples of the VWF multimer electrophoresis results of different VWD types are depicted in Figures 3-7. Three patients with type 1 VWD showed a normal VWF multimer pattern, although a relative decrease in the intensity of the multimer bands could be seen (Figure 3). As expected, the loss of HMW multimers was seen in both type 2A (Figure 4) and type 2B (Figure 5) VWD samples. Type 2N VWD patients exhibited normal multimeric patterns (Figure 6), and no signal was detected in two type 3 VWD patients (Figure 7), consequently leading to undetectable VWF multimer interpretation. The hemophilia A results were completely normal.

3.3 | VWF multimers in EQA samples

Throughout 2016 and 2017, the North Estonia Medical Centre Laboratory participated in seven EQA schemes of VWF modules, including VWF:Ag, VWF:Ac, FVIII:C, VWF multimers, and final conclusion (interpretation) (The ECAT Foundation, the Netherlands). In all 7 surveys, VWF multimer electrophoresis interpretation and final interpretation of the VWD type were in agreement with expert opinion (Table 2).

4 | DISCUSSION

VWF multimers should not be used as a standalone test to diagnose VWD.[21] The critical clinical utility of VWF multimers is in differentiating type 2A and 2B VWD from type 2M (or type 1) VWD,[12] as the correct classification of VWD is very important for the final diagnosis and treatment management.[22]

Unfortunately, until now, VWF multimer analysis has been performed only by a limited number of expert or reference laboratories (only 16%-18% of the participants of The ECAT Foundation EQA surveys[17]), mainly because the assay is technically complex, laborious, requires specially trained personnel, and is nonstandardized and time-consuming.[14,17,23] In addition, a certain proportion of interpretative errors arise due to test panels lacking the VWF multimer assay.[5,16]

The main methods in clinical use for the visualization of VWF multimers remain in-house-developed electrophoresis methods with typical overnight electrophoretic runs in agarose gels (alternatively, nitrocellulose or polyvinylidene difluoride) at concentrations ranging from 1% to 3%[14,21,23] with different options for immunologically detected multimer visualization (either radioactive, colorimetric, luminographic, or fluorometric methods).[23] Radioactive techniques are potentially hazardous, but conventional nonradioactive methods lack sensitivity and optimal resolution power,[13] potentially leading clinicians to misclassification of the VWD subtype.[20] Luminographic methods are much safer and are reported to allow visualization of multimers with confidence and high sensitivity.[13]

A very important issue is the turnaround time of VWF multimer analysis. The above-mentioned in-house methods are time-consuming, and although several of them have been somewhat optimized, they still require dozens of hours or even 3-4 days to complete.[13,20] In our case, the evaluated Sebia Hydragel 5 von Willebrand multimer electrophoresis assay produced same-day results in only 6 hours and 40 minutes. A significantly shorter turnaround time could encourage clinical laboratories to select such a method instead of the traditional, time-intensive procedures. Furthermore, in the case of analytical failure, same-day multimer analysis is more attractive because laboratories could repeat testing and release results quickly, although confirmation of the VWD type is not an urgent analysis.

Another disadvantage that many in-house electrophoresis methods possess is their inability to carry out quantitative analysis of VWF multimers.[13,20] Quantitative results can provide objective measures of the VWF structure to better define subtle changes in the VWD subtypes, such as dominant VWD type 1/2E (IIE) due to mutations in the D3 domain with aberrant triplet structure or the lack of outer bands or pronounced inner bands together with a relative decrease in LMW multimers.[24,25] However, the current classification for VWD does not consider the quantity of loss of the HMW multimers.[20] The Sebia method provides quantitative VWF multimer results and allows, if desired, splitting curves into multimer subsets. Laboratories may be able to establish normal ranges for different multimer sizes (LMW, IMW, and HMW) and quantify the percentage of loss in abnormal samples. The quantitative performance of the Sebia VWF multimer assay (reference ranges, clinical decision limits) should be assessed in future studies. Unfortunately, the Sebia method does not allow the visualization of VWF multimer triplets. Therefore, the main difference of it, compared with noncommercial assays, is the "quantification" itself, although this can be equally addressed by the in-house methods equipped with densitometers and associated software. Whichever method is in use, difficult cases, when increased subbands or abnormal triplet structures are observed, should undergo consultation with expert laboratories.

Other assays have been proposed in the literature—for example, direct biophysical fluorescence correlation spectroscopy—suggesting the quantitative nature of the method, short analysis time, and potentially low cost per sample.[14] However, such a method is not widely available for clinical laboratories, reinforcing the need for a rapid and commercially available VWF multimer method.

Currently, VWF multimer analysis demonstrates a relatively high error rate,[6] mainly reflected by the rather complex nature of the available methodologies. The ECAT Foundation collected data

TABLE 1 Summary of the VWF multimer and other coagulation results from patients with a known VWD type, previously examined by in-house VWF multimer assays or confirmed genetically

Patient No.	VWD type	VWF:Ag, %	VWF:Ac, %	VWF:Ac/VWF:Ag	FVIII:C, %	In-house VWF multimer interpretation[a]	Sebia VWF multimer			
							Interpretation	Quantitative results, %		
								LMW-M	IMW-M	HMW-M
1	1	32	34	1.06	52	Normal pattern	Normal pattern	9.0	15.7	75.3
2	1	23	22	0.96	59	Normal pattern	Normal pattern	14.3	28.9	56.8
3	1	51	42	0.82	57	Normal pattern	Normal pattern	12.5	32.0	55.5
4	2A	21	10	0.48	36	Loss of HMW multimer, abnormal triplet structure	Loss of HMW multimers	49.5	32.9	17.6
5	2B	126	51	0.40	70	Loss of HMW multimer, abnormal triplet structure	Loss of HMW multimers	74.8	21.6	3.6
6	2B	24	26	1.08	42	No data: type confirmed genetically	Loss of HMW multimers	55.8	35.2	9.0
7	2N	29	17	0.59	19	Normal pattern, decreased level of bands	Normal pattern	32.1	29.6	38.3
8	2N	58	71	1.22	33	No data: type confirmed genetically (see section 2.5)	Normal pattern	16.7	25.2	58.1
9	3	<5	<4	N/A	2	Undetectable	Undetectable	Quantitative results unavailable		
10	3	<5	<4	N/A	2	Undetectable	Undetectable	Quantitative results unavailable		
11	Not VWD (hemophilia A)	87	77	0.89	2	No data: type confirmed genetically	Normal pattern	18.3	30.6	51.1

[a]Results of former analysis of VWF multimer in patient samples—results were not known until analyzed by Sebia method.

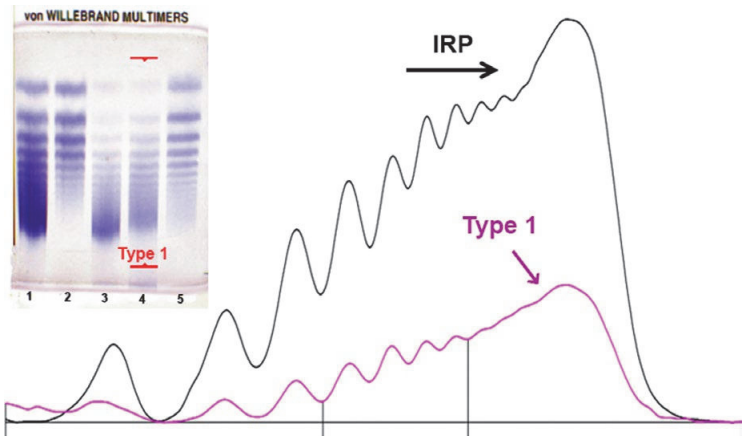

FIGURE 3 Electrophoresis gel of IRP (track 1) and type 1 VWD patient plasma (track 4). Densitograms: IRP vs type 1 VWD patient plasma

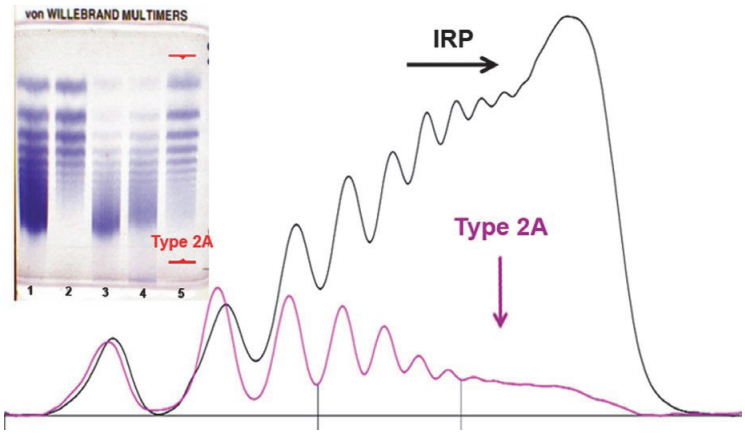

FIGURE 4 Electrophoresis gel of IRP (track 1) and type 2A VWD patient plasma (track 5). Densitograms: IRP vs type 2A VWD patient plasma

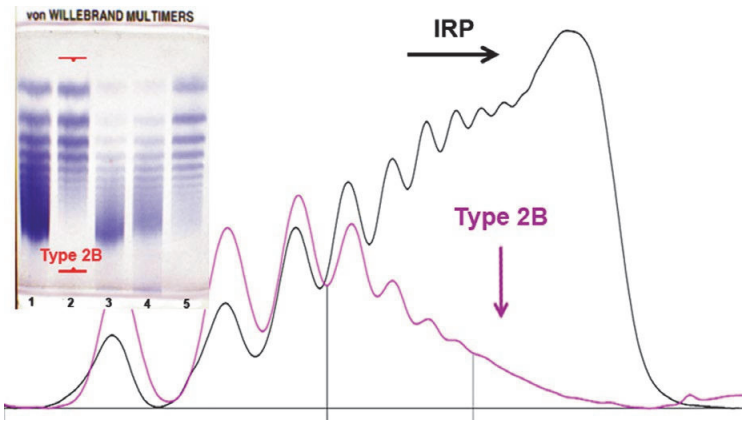

FIGURE 5 Electrophoresis gel of IRP (track 1) and type 2B VWD patient plasma (track 2). Densitograms: IRP vs type 2B VWD patient plasma

showing substantial error rates ranging from 10% to 52%.[17] The North American Specialized Coagulation Laboratory Association (NASCOLA) showed an overall 14.7% (7-22%) erroneous survey response rate from laboratories performing in-house VWF multimer analysis.[15] In our case, all EQA samples (including type 1 and type 2A VWD patients) were correctly visualized and interpreted by the new commercial VWF multimer assay, demonstrating its reliability.

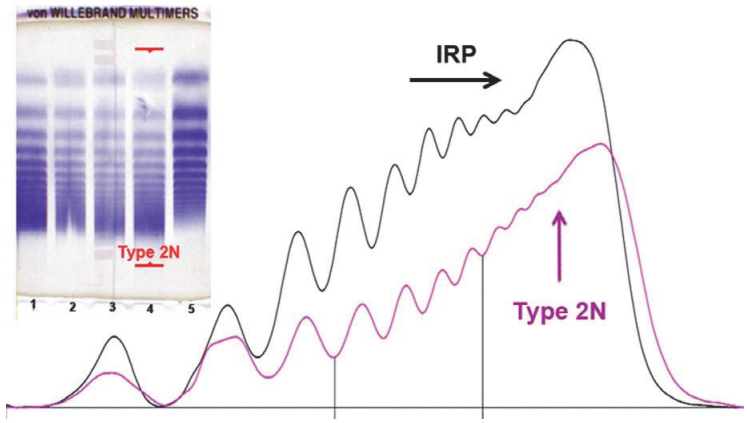

FIGURE 6 Electrophoresis gel of IRP (track 1) and type 2N VWD patient plasma (track 4). Densitograms: IRP vs type 2N VWD patient plasma

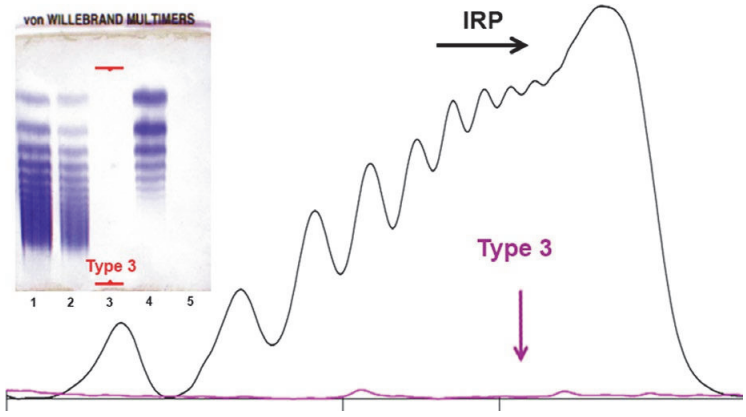

FIGURE 7 Electrophoresis gel of IRP (track 1) and type 3 VWD patient plasma (track 3). Densitograms: IRP vs type 3 VWD patient plasma

TABLE 2 Summary of VWF multimer analysis in EQA samples. Reproduced with permission from The ECAT Foundation (the Netherlands)

		Sebia VWF multimer				
			Quantitative results, %			
EQA survey No.	EQA sample	Interpretation	LMW-M	IMW-M	HMW-M	Conclusion on VWD type
2016-1	Normal control plasma	Normal distribution	23.3	33.1	43.6	Not VWD
2016-2	Type 1 VWD patient	Normal distribution	18.3	30.0	51.7	Type 1 VWD
2016-3	Type 2 VWD patient	Lack of IMW-M and HMW-M	73.8	11.5	14.7	Type 2A VWD
2016-4	Type 1 VWD patient	Normal distribution	10.7	26.8	62.5	Type 1 VWD
2017-1	Normal control plasma	Normal distribution	26.9	31.9	41.2	Not VWD
2017-2	Type 2 VWD patient	Lack of IMW-M and HMW-M	84.0	11.3	4.7	Type 2A VWD
2017-3	Type 1 VWD patient	Normal distribution	24.7	33.0	42.3	Type 1 VWD

Another question is whether the VWF multimer assay is needed in the VWD testing panel if VWF:RCo (alternatively VWF:Ac) and VWF:CB to VWF:Ag ratios are used as surrogate markers for the loss of HMW multimers.[7,10,11,16] The UK Haemophilia Center Doctors Organization guideline approved by the British Committee for Standards in Haematology recommends that such ratios be used to distinguish between types 2A and 2M (evidence level 1B).[21] A VWF:RCo/VWF:Ag ratio <0.7 should lead clinicians to look for type 2 VWD with a qualitative VWF defect and not type 1 VWD. However, the technical limitations of most VWF:RCo assays used

in laboratories worldwide make the VWF:RCo/VWF:Ag ratio unreliable, especially at the levels of VWF:Ag less than 15-20 IU/dL (%).[9] In such cases, the VWF multimer assay is very helpful to confirm or neglect the evidence of the loss of HMW multimers.[26]

Recently, the Sebia method was extensively evaluated by other authors, who provided positive comments on the fundamental consistency of the obtained data and presented reports at international meetings.[27] The same group of scientists additionally published a chapter on VWF multimers in the book available from Springer Science + Business Media (Hemostasis and Thrombosis: Methods and Protocols, Methods in Molecular Biology, vol. 1646). This paper describes the multimer methodology developed by Sebia in detail.[28]

5 | CONCLUSIONS

The new commercial VWF multimer assay (Hydragel 5 von Willebrand multimers; Sebia, Lisses, France) may represent a good alternative to traditional in-house assays. The Sebia method is easy to perform and can be successfully implemented in any clinical laboratory for second-stage evaluation of VWD. This method is a semi-automated agarose gel electrophoresis assay with ready-to-use gel and reagents, simple to carry out, and rapid (same-day results) compared with other (mainly in-house) methods. Visualization of the multimer distribution and densitometric analysis, together with the applied LMW, IMW, and HMW multimer classification, provide adequate resolution to correctly classify types 1, 2A, 2B, and 3 VWD cases. This new assay can be processed in routine use on a classical Sebia Hydrasys 2 multiparameter instrument. Furthermore, there is no need for additional training of laboratory technicians, and all of the main steps and instrument software are easily understandable and operated in a similar manner as other Sebia electrophoresis techniques (ie, serum/urine protein electrophoresis, immunofixation). Only the interpretation of the results should be carried out by or in consultation with experts. Nevertheless, the utility and value of this commercial method as an alternative for in-house assays must still be confirmed in future analyses. Evidence should be collected by the EQA organizers who have a substantial amount of data on the available methods. Likewise, larger-scale methods and comparison studies should be carried out because the small number of patients in our study was a major limitation.

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CONFLICT OF INTERESTS

HB and GN are employees of Sebia (Research and Developments Department). MP, GZ, TS, MV, and VB have nothing to declare.

AUTHOR'S CONTRIBUTION

Marika Pikta (MP), Valdas Banys (VB), Margus Viigimaa (MV), and Galina Zemtsovskaja (GZ) designed the study. MP was responsible for recruiting the healthy volunteers and supervised the study. Timea Szanto (TS) provided samples for comparison. MP and GZ supervised the measurements. MP, VB, TS, and GZ interpreted the data. Hector Bautista (HB) and Georges Nouadje (GN) consulted authors in the process of writing the methods section of the report. MP, VB, and TS were the main contributors to the writing of the manuscript. All authors have accepted responsibility for the entire content of this submitted manuscript and have approved its submission.

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Publication III

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EVALUATION OF A NEW SEMI-AUTOMATED HYDRAGEL 11 VON WILLEBRAND FACTOR MULTIMERS ASSAY KIT FOR ROUTINE USE

PROCENA NOVOG POLUAUTOMATSKOG SETA ZA TESTIRANJE 11 FON VILDENBRANDOVOG FAKTORA ZA RUTINSKU UPOTREBU

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Summary

Background: Accurate diagnosis and classification of von Willebrand disease (VWD) are essential for optimal management. The von Willebrand factor multimers analysis (VWF:MM) is an integral part of the diagnostic process in the phenotypic classification, especially in discrepant cases. The aim of this study was to evaluate the performance of a new Hydragel 11VWF multimer assay (H11VW).
Methods: Analytical performance characteristics such as repeatability (intra-assay variability, in gel between track variation), reproducibility (inter-assay variability, between gel variation), sensitivity, EQA performance and differences between two commercially available VWF:MM kits (H5VW and H11VW) were analysed in healthy volunteers' plasmas using in-house prepared reference plasma.
Results: Repeatability and reproducibility results of H11VW demonstrated acceptable and equivalent performance with previously verified H5VW. Participation in EQA was successful. No statistically significant difference was detected

Kratak sadržaj

Uvod: Tačna dijagnoza i klasifikacija Fon Vilebrandove bolesti (VVD) su neophodni za optimalni rad. Multimerska analiza Fon Vilebrandovog faktora (VVF:MM) je u fenotipskoj klasifikaciji, posebno u slučajevima neslaganja, sastavni deo dijagnostičkog procesa. Cilj ove studije je bio da proceni performanse novog Hidragel 11VVF multimer testa (H11VV).
Metode: Karakteristike analitičkih performansi, kao što su ponovljivost (varijabilnost unutar testa, u gelu između varijacije traga), obnovljivost (varijabilnost među testovima, između varijacije gela), osetljivost, EQA performanse i razlike između dva komercijalno dostupna VVF:MM seta (H5VV i H11VV) su analizirane na plazmama zdravih dobrovoljaca i interno pripremljenoj referentnoj plazmi.
Rezultati: Rezultati ponovljivosti i obnovljivosti H11VV su pokazali prihvatljive i ekvivalentne performanse sa prethodno verifikovanim H5VV. Učešće u EQA je bilo uspešno. Nije utvrđena statistički značajna razlika između H5VV i

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List of abbreviations: VWF, von Willebrand factor; VWD, von Willebrand disease; VWF:MM, von Willebrand factor multimer assay; LMWM, low molecular weight multimers; IMWM, intermediate molecular weight multimers; HMWM, high molecular weight multimers; IRP, in-house reference plasma; H5VW, Hydragel 5VWF multimer assay kit; H11VW, Hydragel 11VWF multimer assay kit.

between H5VW and H11VW kits for different fractions of multimers: LMWM p=0.807; IMWM p=0.183; HMWM p=0.774.
Conclusions: H11VW demonstrated acceptable analytical performance characteristics. H11VW kit conveniently offers a more significant number of samples on a single gel. H5VW and H11VW kits can be used in daily practice interchangeably

Keywords: electrophoresis, multimers, von Willebrand factor, von Willebrand disease

H11VV setova za različite frakcije multimera: LMVM p = 0,807; IMVM p = 0,183; HMVM p = 0,774.
Zaključak: H11VV je pokazao prihvatljive karakteristike analitičkih performansi. Korisno je što H11VV komplet nudi veći broj uzoraka na jednom gelu. Kompleti H5VV i H11VV se mogu koristiti naizmenično u svakodnevnoj praksi.

Ključne reči: elektroforeza, multimeri, Fon Vilebrandov faktor, Fon Vilebrandova bolest

Introduction

Deficiency or/and abnormality of von Willebrand factor (VWF) leads to von Willebrand disease (VWD), which is the most common inherited bleeding disorder (1). Bleeding features are commonly characterised by mucocutaneous hemorrhage (e.g. epistaxis, menorrhagia), but hematomas and hemarthrosis may also occur in severe forms. The diagnosis of VWD presents many challenges: 1) there is a great overlap of clinical phenotypes and laboratory parameters between healthy individuals and those with type 1 VWD, and 2) a variety of increasingly specific laboratory tests are necessary for an accurate diagnosis of VWD (1). The choice of the validated test panel is essential for the correct typing (type 1, 2 and 3 VWD) and subtyping of type 2 VWD. Due to the steadily increasing interest of VWD reclassification of prediagnosed VWD, the number of publications regarding VWD diagnosis has increased in recent years (2, 3). In addition to first-line tests, such as factor VIII, VWF antigen and VWF activity assays, assessment of VWF multimers testing (VWF:MM) is important for the correct classification of VWD subtypes (1, 4). However, the availability of VWF:MM is limited due to technical difficulties, variable results and long turnaround time of conventional VWF multimer techniques (5–7).

A novel semi-automated Hydragel 5VWF multimers assay kit (H5VW) has been already evaluated for use on the Hydrasys 2 Scan instrument (Sebia, Lisses, France) by several authors (8–12). In May 2019, the VWF:MM analysis with 5VWF kit was accredited in the North Estonia Medical Centre laboratory according to ISO15189:2012. Recently, a new Hydragel 11VWF multimers assay kit (H11VW), which allows more significant sample size determinations, has become commercially available.

The aim of this study was to evaluate the performance characteristics of H11VW.

Materials and Methods

Study objects

Two types of normal citrated plasma samples were used in the H11VW performance evaluation: in-house reference plasma (IRP) and plasma from 10 healthy individuals recruited voluntarily. IRP has been used in North Estonia Medical Centre laboratory for a couple of years, and detailed procedure on the preparation of IRP was published previously elsewhere (11). For healthy individuals, a well-structured questionnaire was used to obtain information about age, gender, individual/family bleeding history, medication. Information provided enabled us to classify them preliminary as non VWD individuals. Venous blood samples were collected into 3.8% NC Buffered Citrate (Vacutest KIMA s.r.l., Arzergrande, Italy) tubes, which were centrifuged at 1500 g for 15 minutes at room temperature to generate platelet-free plasma (residual platelet count $< 10\times10^9$/L), aliquoted and stored frozen at -70 °C until further analysis. Aliquots were thawed in a water bath (+37 °C) for 5 minutes and mixed well before testing. All participating volunteers gave their informed consent. The study was performed according to the Declaration of Helsinki and was approved by the national ethics committee.

VWF profile (first-line tests) in healthy individuals

The VWF antigen (VWF:Ag, Liatest-VWF:Ag, Diagnostica Stago, France), factor VIII coagulant activity (FVIII:C) by a one-stage, clot-based assay (STA-ImmunoDef VIII, STA-C.K.Prest, Diagnostica Stago, France) and VWF activity (13) measured as VWF binding to the glycoprotein Ib (GPIb) receptor on the platelet surface (Innovance® VWF:Ac, Siemens Healthcare Diagnostics, ISTH nomenclature VWF:GPIbM) were measured on STA-R Evolution analyser (Diagnostica Stago, France) using commercial kits.

VWF:MM

VWF multimers evaluation was performed on Hydrasys 2 Scan instrument (Sebia, Lisses, France), using 2.0% SDS agarose gel, direct immunofixation, visualisation with peroxidase-labelled antibody and followed by densitometry, according to manufacturer recommendations. VWF multimers were classified as low, intermediate and high molecular weight multimers (LMWM, IMWM and HMWM respectively). Densitometry data was obtained using the Phoresis

software originating from Sebia. Principle of VWF:MM methodology for H5WV and separation of multimers fractions was previously described in detail (9–12). Technical steps for both kits (H5WV and H11WV) are very similar. The important differences are the metal weight holding mechanism for the blotting steps (1.8 kg for H5WV and 2.3 kg for H11WV) and a number of sample positions (5 tracks gel for H5WV and 11 tracks gel for H11WV).

Analytical performance characteristics

We have chosen the following analytical performance characteristics of H11VW to analyse: repeatability (intra-assay variability, in gel between track variation), reproducibility (inter-assay variability, between gel variation), sensitivity, EQA performance and differences between two commercially available VWF:MM kits (H5VW and H11VW). For repeatability analysis, 11 measurements were done, each for a single non VWD volunteer individual's plasma, which was applied to 11 tracks of the gel. Consequently, intra-assay coefficient of variation (CV) % was calculated. For reproducibility analysis, VWF:MM results of the same IRP from 55 different gels runs were collected, and inter-assay CVa% was calculated. For sensitivity analysis, the single volunteer's plasma with respectively known VWF antigen value was diluted in series (1:2, 1:4, 1:6; 1:8; 1:16 and 1:32). For of the dilution series VWF:MM assay was performed on single H11VW gel together with IRP for comparison reasons. The external quality assessment (EQA) program for VWF:MM was issued by the ECAT (External quality Control of diagnostic Assays and Tests with a focus on Thrombosis and Haemostasis). In total, North Estonia Medical Centre laboratory participated in 6 cycles of EQA using H11VW. Finally, applying the results of previously performed H5VW kit performance verification (n=26) (11), corresponding results of IRP on H11VW (n=29) were collected, and statistical comparison was carried out.

Statistical analysis

Results were reported as the mean % of the respective molecular weight fraction of multimers ± standard deviation (SD) and the coefficient of variation (CV, %). The difference between the two commercial kits (H5WV versus H11VW) was evaluated by nonparametric Man-Whitney U test on the IBM SPSS software, version 21.0 (Chicago, IL, USA). Values were considered statistically significant at $p<0.05$.

Results

Intra-assay and Inter-assay variability

Example of repeatability analysis is depicted in *Figure 1A*. Visually, 11 tracks of one single plasma on

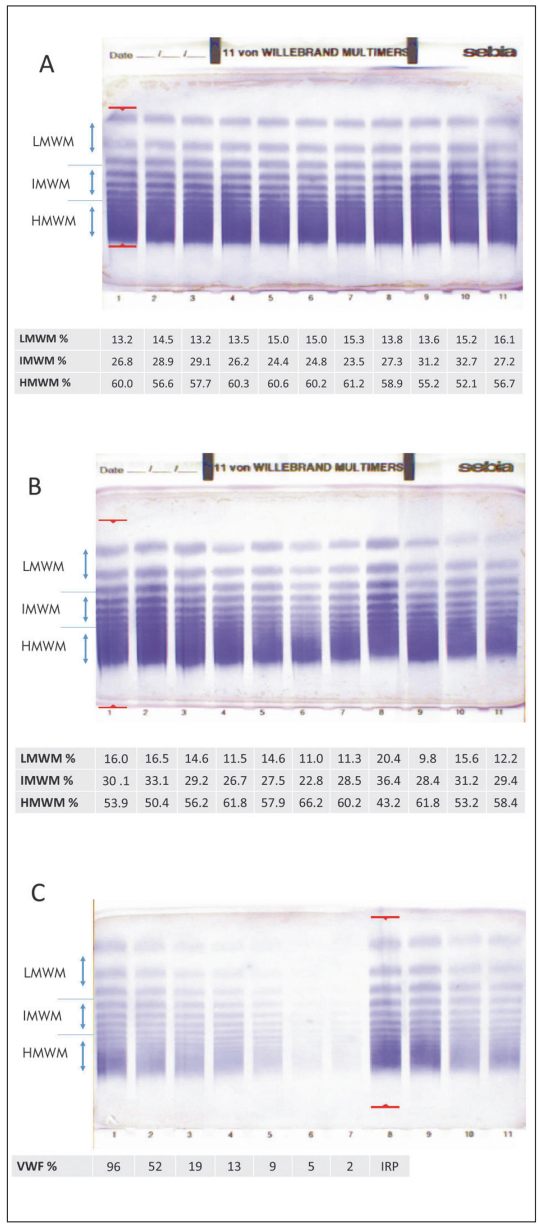

Figure 1 Examples of analysed H11VW gels and their semi-quantitative results of VWF:MM band patterns of LMWM, IMWM and HMWM: A – repeatability analysis of a single healthy individual's plasma in one gel (tracks 1–11); B – 10 healthy individuals' plasmas each on a separate track (tracks 2–11), IRP (track 1); C – sensitivity analysis by serial dilution of a single healthy individual's plasma (tracks 1–7), IRP (track 8), not relevant to the study samples (tracks 9–11).

VWF, von Willebrand factor; VWF:MM, von Willebrand factor multimer assay; LMWM, low molecular weight multimers; IMWM, intermediate molecular weight multimers; HMWM, high molecular weight multimers; IRP, in-house reference plasma; H11VW, Hydragel 11VWF multimer assay kit.

the same H11VW gel look pretty much the same, but a visual inspection is too subjective. Densitometric analysis of the gels, and consequently calculated % of different fractions of multimers (LMWM mean value 14.4%, SD 1.0; IMWM mean value 27.5%, SD 2.8; and HMWM mean value 58.1%, SD 2.8) demonstrate intra-assay variability performance equivalent to previously published H5VW kit repeatability values (12): CV were 6.9% for LMWM, 10.3% for IMWM, and 4.8% for HMWM.

Statistical data in reproducibility analysis for different multimers fractions were as follows: LMWM mean value 18.1%, SD 3.0; IMWM mean value 33.1%, SD 2.0; HMWM mean value 48.9%, SD 3.9. These results yielded higher but more or less acceptable coefficients of variation for LMWH and HMWM when compared to repeatability data, but the variability of IMWM was lower. Inter-assay CV values were 16.6% for LMWM, 6.2% for IMWM, and 8.1% for HMWM, respectively.

Healthy individuals' results

All 10 healthy individuals' plasma samples demonstrated normal FVIII:C, VWF activity and antigen levels with normal activity to antigen ratio (>0.7): FVIII:C mean value 108% (range 69–134%), VWF antigen mean value 96% (range 65–141%) and VWF activity mean value 105% (range 78–154%). Also, normal multimer patterns were detected, which resembled the normal pattern of IRP (*Figure 1B*). The means (ranges) for VWF:MM of different sizes were as follows: LMWM mean value 13.8% (9.8–20.4%), IMWM mean value 29.3% (22.8–36.4%) and HMWM mean value 56.9% (43.2–66.2%).

Sensitivity analysis

Sensitivity analysis with serial dilutions revealed a cut-off (VWF antigen values 9 % and below), which aggravates visual inspection of gels, worsens densitometric analysis by Phoresis software (Sebia, France). An example of sensitivity analysis is given in *Figure 1C*.

Plasma of a healthy individual (VWF antigen of 96% and VWF activity of 105%) was diluted according to protocol and resulted in final VWF antigen values of 52%, 19%, 13%, 9%, 5%, and 2%. As shown in *Figure 1C*, at the level of 9% multimer, bands are still clearly recognisable, densitometric distribution of different fractions is substantially lower when compared to IRP graph, but proportions of LMWM, IMWM and HMWM values resembled the normal values.

EQA survey

The performance of the North Estonia Medical Centre laboratory in the ECAT Foundation EQA programs was considered successful because results of VWF:MM of all 6 cycles were in agreement with the corresponding goals of the ECAT Foundation. Summary of EQA results is provided in *Table I*.

Comparison between the two commercial kits, H5WV versus H11VW

There was no statistically significant difference detected between H5VW and H11VW kits for different fractions of multimers: LMWM 17.95±2.94 vs 18.31±3.32, p=0.807; IMWM 33.24±1.98 vs 32.47±2.48, p=0.183; HMWM 48.82±3.65 vs 49.22±3.57, p=0.774 (*Figure 2*).

Following the success of the EQA and above provided performance data, in May 2020 VWF:MM assay with H11VW kit was accredited in the North Estonia Medical Centre laboratory according to ISO15189:2012.

Table I Summary of VWF:MM analysis in EQA samples, reproduced with permission from the ECAT Foundation (the Netherlands).

EQA survey Nr.	EQA sample	Quantitative results, %			Interpretation	Conclusion on VWD type
		LMWM	IMWM	HMWM		
2018-M3	Type 2 VWD plasma	37.1	26.1	36.8	Relative decrease of HMWM	Type 2 VWD
2018-M4	Type 1 VWD plasma	25.0	32.3	42.5	Normal distribution	Type 1 VWD
2019-M1	Normal Coagulation Control Plasma	19.8	37.6	42.6	Normal distribution	Not VWD
2019-M2	Type 1 VWD plasma	18.0	36.5	45.5	Normal distribution	Type 1 VWD
2019-M3	Type 1 VWD plasma	19.1	28.0	52.9	Normal distribution	Type 1 VWD
2019-M4	Type 2 VWD plasma	36.5	31.0	32.5	Relative decrease of HMWM	Type 2 VWD

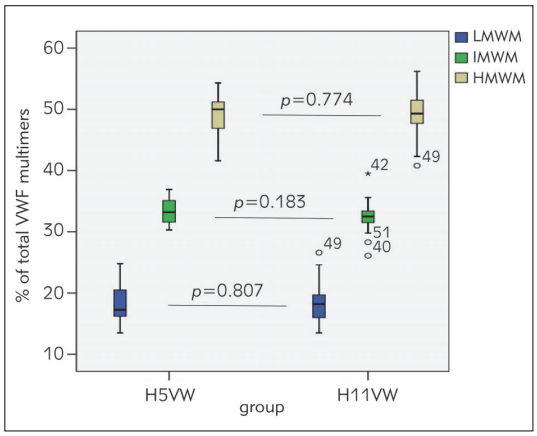

Figure 2 The difference between the two commercial kits (H5WV versus H11VW) represented as box plot results. LMWM, IMWM and HMWM bands are separated.

VWF, von Willebrand factor; LMWM, low molecular weight multimers; IMWM, intermediate molecular weight multimers; HMWM, high molecular weight multimers; H5VW, Hydragel 5VWF multimer assay kit; H11VW, Hydragel 11VWF multimer assay kit.

Discussion

The measurement of VWF multimers has become a part of the laboratory workflow for the identification and classification of VWD (1, 4). Several home-made methods have been developed in the past decades for evaluating the VWF multimeric structure in expert-level laboratories, characterised by varying analytical performances (5–7), occasional differences in interpreting the results (12).

Both visual and densitometry-based investigation makes interpretation easier, allows the overlay patients curves with normal control and enables estimating the relative quantification of each multimer subset, providing useful information for the diagnosis of VWD. Implementation of VWF multimers assay for routine use is important for the classification of VWD, leading to the improvement of VWD diagnosis and monitoring of treatment response in Baltic countries.

The role of quality assurance in a hemostasis laboratory is very important (14). Because high precision is needed in VWF multimers quantification, standardisation of its measurements is crucial for an accurate diagnosis. One important aspect is the type of plasma sample used for internal quality control (IQC) (14). It is known that present type 2A VWD-like controls are not provided by VWF test manufacturers; thus, laboratories may be able to use previously diagnosed 2A VWD patients' samples. Using normal commercial plasma for IQC multimeric evaluation might end up with a relative loss of the highest HMWM, probably due to the lyophilisation process while preparing commercial plasma (6,11). To our knowledge, at least one group of researchers have verified commercial normal reference plasma (Standard Human Plasma, Siemens) as acceptable quality control for VWF multimers evaluation (15). This study was the first to report the H11VW kit validation results, including analysis of the VWD patients' samples, and has also noted the benefits and limitations of semi-automated VWF:MM assay, including the smaller sample size H5VW kit. In the present study, the results do not only support the previously published (15) but also provide additional analytical performance characteristics evaluation, especially for the larger sample size H11VW kit.

We concluded that the analytical performance of HYDRAGEL 11 VON WILLEBRAND MULTIMERS assay is acceptable and gives a perspective to standardisation of the VWF:MM assay by Sebia (France). NewH11VW kit conveniently offers a larger number of samples on a single gel, thus saves precious time. The choice of kit (H5VWvs H11VW) can be generally based on the volume of laboratory workload (the number of collected patient samples). Considering the performance data, H5VW and H11VW kits can be used in daily practice for the visual investigation of gel and quantitative estimation of VWF multimer fractions interchangeably.

Acknowledgements. Research funding: Sebia (Lisses, France) donated the Hydragel 5von Willebrand multimers kits. The authors would like to express gratitude to dr. Lenna Örd for language editing and laboratory technicians Galina Trofimova and Tatjana Tverskaja for excellent technical assistance related to sample testing.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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RESEARCH ARTICLE

ASSESSMENT OF THE VON WILLEBRAND FACTOR MULTIMERS PROFILE IN PATIENTS REFERRED FOR BLEEDING TENDENCY EVALUATION IN ESTONIA: A PRELIMINARY REPORT OF THE VON WILLEBRAND DISEASE DIAGNOSTICS PROJECT

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ABSTRACT: **Introduction:** VonWillebrand disease (VWD) is considered the most common autosomal inherited bleeding disorder. Laboratory testing for diagnosis or exclusion of VWD is based on a complex of different diagnostic assays. In the diagnostic workup of patients with suspected VWD, the von Willebrand factor (VWF) multimer assay is one of the most important indicators for VWF quality. This study aims to assess the VWF multimers profile in patients with bleeding tendency and increase knowledge and awareness of VWD laboratory diagnosis in Estonia. **Methods**: This retrospective study investigated the laboratory results of 131 individuals who were selected from the laboratory information system based on the request of VWF tests profile and 31 healthy volunteers for comparison. **Results**: Control group, non-VWD patients and patients suspected with VWD type 2N or mild haemophilia A demonstrated normal VWF multimer (VWF:MM) pattern. Patients with low VWF and suspected with VWD type 1 also showed normal VWF:MM distribution with reduced intensity. All cases suspected with VWD type 2A or 2M had a decrease of high molecular weight multimers (HMWM); one of them showed a loss of intermediate molecular weight multimers and HMWM and low VWF activity to antigen ratio (<0.7). Furthermore, multimers were undetectable in patients suspected with VWD type 3 or severe type 1. **Conclusions**: This is the first report of VWD laboratory evaluation in Estonia to provide insight into the potential clinical significance of using VWF: MM. The interpretation of VWF multimers should be necessarily complemented by the quantification of fractions of multimers by densitometry additional to visual gel's examination.

KEYWORD: von Willebrand disease, von Willebrand factor, von Willebrand factor multimers

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INTRODUCTION:

Von Willebrand disease (VWD) is considered to be the most common autosomal inherited bleeding disorder caused by a deficiency or functional abnormality of von Willebrand factor (VWF) [1]. VWD is classified into partial and total quantitative deficiencies of VWF (VWD types 1 and 3) and qualitative variants (VWD types 2A, 2B, 2M and 2N) [1].

The National Heart, Lung and Blood Institute Expert Panel report published in 2008 [2] suggested that VWD type 1 can be diagnosed when VWF antigen (VWF:Ag) or VWF activity is <30%, and levels of VWF:Ag between 30% and 50% should be classified as low VWF.

An evaluation of the patient personal and family bleeding history is recommended using a Bleeding Assessment Tool (BAT) before laboratory tests request [3]. Laboratory testing for the diagnosis or exclusion of VWD is based on a complex of different diagnostic assays: [4,5] platelet count, patient skin bleeding time or the platelet function analyser closing time, prothrombin time, activated partial thromboplastin time (APTT), VWF:Ag, VWF activity, coagulation factor VIII (FVIII:C), VWF multimer analysis (VWF:MM), VWF collagen binding assay (VWF:CB), VWF-FVIII binding assay (VWF:FVIIIB), propeptide of VWF (VWFpp), ristocetin-induced platelet agglutination assay (RIPA) and molecular analysis of VWF gene. The treatment of VWD bleeding involves the use of tranexamic acid (TA), desmopressin (DDAVP) and plasma derived and recombinant VWF concentrates [6].

The estimated prevalence of VWD appears to be between 0.01% and 1% [1]. Most of the patients are asymptomatic or with mild type 1 VWD and may be difficult to distinguish from healthy individuals [1]. According to the present knowledge, the prevalence of VWD in Estonia is unknown. Estonia is situated in north-eastern Europe with around 1.3 million inhabitants.

Furthermore, making a definite diagnosis of VWD subtypes or severe forms of haemophilia A in Estonia until 2016 was not possible because of the limited availability of laboratory-specific tests. The VWD hypothesis was based on routine coagulation screening tests, and the measurement of VWF antigen level applied reference ranges. In 2016, a new fully automated assay protocol for VWF activity measurement (INNOVANCE® VWF Ac, Siemens, Marburg, Germany) was adapted on STA-R Evolution analyser (Diagnostica Stago, Asnieres, France), and a new VWF multimer electrophoresis assay (Sebia, Lisses, France) was evaluated preclinically, and their analytical performance was evaluated [7-9].

This retrospective study aimed to assess the VWF multimers profile in patients with bleeding tendency and to increase the knowledge and awareness of VWD laboratory diagnosis in Estonia.

MATERIALS AND METHODS:

Participants/Samples

The laboratory results of 131 individuals who were selected from the laboratory information system (LIS) based on the request of VWF tests profile, were investigated between May 2016 and December 2020. The samples were from patients visiting the outpatient clinic and from hospitalised patients. Moreover, the background clinical information of the patients, provided by clinicians, was available in LIS. The basic data of patients were anonymously collected.

The median age of the patients was 17 (range, 1–77 years). The control group included 31 healthy volunteers (seven men and 24 women) without known bleeding disorders. Samples were collected into 3.2% sodium citrate tubes (BD Vacutainer; BD Diagnostics, Plymouth, UK) for coagulation assays and hirudin blood tubes (Roche Diagnostics, Basel, Switzerland)

or hirudin tubes (Sarstedt, Nümbrecht, Germany) for platelet aggregation evaluation.

The study was performed according to the Declaration of Helsinki and was approved by the Tallinn Ethical Committee on Medical Research (approval number 680).

Laboratory Investigations

The investigation performed included VWF antigen (VWF:Ag; Liatest-VWF:Ag; Diagnostica Stago, Asnieres, France), and the VWF activity was measured as VWF binding to the glycoprotein Ib receptor on the platelet surface (VWF:GPIbM; Innovance® VWF Ac kit; Siemens Healthcare Diagnostics, Marburg, Germany) and FVIII:C determined by a one-stage, clot-based assay (Diagnostica Stago), which were measured using an automated coagulometer STA-R Evolution (Diagnostica Stago).

Whole blood aggregation (WBA) was performed using the impedance Multiplate® platelet aggregometry analyser (Roche). Ristocetin-induced platelet aggregation in whole blood (WB-RIPA) was performed with two final ristocetin concentrations (high, 0.77 mg/mL; low, 0.2 mg/mL) following the standard Multiplate® RISTOtest protocol.

The measurements VWF:Ag, VWF:GPIbM, FVIII:C and whole blood ristocetin-induced platelet agglutination (WB-RIPA) were repeated (minimum twice) on a separate new sample to confirm or refute initial investigation results.

VWF:MM was measured by gel electrophoresis (Sebia) and separates VWF according to molecular size (low molecular weight multimers (LMWM), intermediate molecular weight multimers (IMWM) and high molecular weight multimers (HMWM) as previously described) [8,9]. All parameters were analysed using a standard methodology in an accredited laboratory.

Algorithm of VWD Subtype Classification

The diagnostic criteria for VWD were based on the current revised classification by the International Society on Thrombosis and Haemostasis (ISTH) [10,11]. A diagnostic algorithm for VWD was created [12] and used in this study based on available laboratory assays in Estonia.

Statistical Analysis

The baseline patients' characteristics were presented as median and interquartile range (IQR) or number of cases (in percentage, counting data). Spearman's correlation coefficient was calculated to test the association between HMWM vs. VWF:GPIbM/VWF:Ag and RistoHigh vs. VWF:GPIbM. The difference between variables was tested using the Mann–Whitney test. Statistical significance was considered if $p<0.05$. Statistical analysis was conducted with the Statistical Package for the Social Sciences, version 23 (IBM, Armonk, NY, USA).

RESULTS:

This study analysed the results of 131 patients. Table 1 presents the main characteristics of the study subjects.

Table 1. Baseline characteristics of study subjects (refer to Table 2)

	Group-1 (n = 31)	Group-2 (n = 50)	Group-3 (n = 46)	Group-4 (n = 17)	Group-5 (n = 10)	Group-6 (n = 6)	Group-7 (n = 2)
male/female	7/24	15/35	19/27	5/12	4/6	5/1	-/2
Age range, years	18–69	4–66	1–54	4–52	1–77	4–43	7–13
Laboratory findings, units, reference ranges, *p* value (in comparison with group 1)							
VWF:Ag, % **50%–160%**	86 (65–102)	69 (59–99) *p* = 0.68	43 (39–47) *p* <0.05	24 (20–28) *p* <0.05	25 (17–33) *p* <0.05	82 (68–116) *p* = 0.621	2–8 *p* <0.05
VWF:GPIbM, % **46%–146%** **(0 group)** **61%–179% (non-0)**	85 (71–105)	77 (65–120) *p* = 0.285	51 (46–57) *p* <0.05	26 (20–34) *p* <0.05	11.5(9-13.3) *p* <0.05	89 (65–138) *p* = 0.918	3–12 *p* <0.05
VWF:GPIbM / VWF:Ag **>0.7**	1.04(0.97-1.15)	1.09(1.01-1.25) *p* = 0.078	1.17(1.09-1.32) *p* <0.05	1.05(0.92–1.22) *p* = 0.931	0.51(0.39-0.59) *p* <0.05	1.04(0.95-1.16) *p* = 0.918	1.50 *p* <0.05
FVIII:C, % **60%–150%**	101 (82–124)	103(88–126) *p* = 0.787	72 (69–83) *p* <0.05	65 (45–86) *p* <0.05	37 (26–45) *p* <0.05	29 (13–35) *p* <0.05	5–31 *p* <0.05
FVIII:C/VWF:Ag **>0.7**	1.17(1.06-1.35)	1.42(1.22-1.64) *p* = 0.05	1.64(1.47-2.04) *p* <0.05	2.81(2.07-3.59) *p* <0.05	1.45(0.93-1.89) *p* = 0.430	0.33(0.12-0.52) *p* <0.05	2.50–3.88 *p* <0.05
RistoHigh, U **98–180 U**	not determined	118(97–139)	97 (87–116)	66 (25–111)	20 (9–51)	109 (74–142)	5–10
RistLow, U **0–20 U**	not determined	8 (6–10)	7 (4–11)	5 (3–9)	6 (3–11)	6 (6–8)	4–5
VWF:MM fractions							
LMWM, % **10.4%–22.5%**	15.0 (12.7–17.2)	17.9 (14.7–19.9) *p* = 0.05	16.9 (14.4–20.3) *p* <0.05	22.2 (18.5–30.0) *p* <0.05	44.1 (32.7–53.9) *p* <0.05	16.7 (14.2–21.6) *p* = 0.209	undetectable
IMWM, % **22.6%–37.6%**	29.2 (26.7–31.2)	29.9(26.3–33.3) *p* = 0.537	26.4 (22.8–30.6) *p* <0.05	25.6 (32.1–29.7) *p* <0.05	25.5 (21.0–31.1) *p* = 0.137	30.6 (24.3–33.9) *p* = 0.837	undetectable
HMWM, % **45.6%–66.6%**	55.4 (51.1–60.2)	53.7 (47.5–56.9) *p* <0.05	55.6 (50.1–60.3) *p* = 0.724	50.8 (45.5–56.1) *p* <0.05	32.0 (20.6–36.9) *p* <0.05	53.3 (44.8–58.7) *p* = 0.333	undetectable

The median age within the cohort was 17 years (range, 1–77 years) with 63.4% female patients. The analysis of the data of indications for VWD testing found the following reported reasons: nose bleeding (23%); menorrhagia with or without anaemia (24%); easy bruising (16%); bleeding after an invasive procedure, dental extractions, or surgery (8%); positive family history without bleeding symptoms (7%); prolonged APTT (5%) and request for investigations (5%) from general practitioners with the comment 'for bleeding disorders evaluation'.

As shown in Table 2, all participants were divided into different groups based on the laboratory investigation. Patients from groups 4 to 7 were designated as suspected because all results in the relationship with genetic testing will be analysed in the future.

Table 2. Definition of study groups and laboratory phenotype of participants.

Groups	Laboratory phenotype
Group 1 Control group	Healthy individuals without bleeding symptoms and family history
Group 2 Non-VWD	Patients who do not currently fulfil the diagnostic criteria for VWD
Group 3 Low VWF	Patients with VWF:Ag and/or VWF:GPIbM values 50%–30% and normal VWF:GPIbM/VWF:Ag (>0.7) ratio
Group 4 Suspected VWD type 1	Patients with VWF:Ag and/or VWF:GPIbM values <30% and normal VWF:GPIbM/VWF:Ag (>0.7) ratio
Group 5 Suspected VWD type 2A or 2M	Patients with ratio of VWF:GPIbM/VWF:Ag<0.7 and WB-RIPA results without enhanced response with low-dose ristocetine
Group 6 Suspected VWD type 2N or mild haemophilia A	Patients with VWF:Ag and VWF:GPIbM values within reference intervals and decreased FVIII:C results
Group 7 Suspected VWD type 3 or severe type 1	Patients with VWF:Ag values <10%

The VWF antigen and activity values in the control group were similar to those in groups 2 (p = 0.68) and 6 (p = 0.621) and were statistically different from those in the other groups. Furthermore, Figure. 1 shows that the ratio of VWF:GPIbM/VWF:Ag was lower in group 5 (0.51; 0.39–0.59), and the difference with the other groups was statistically significant (p < 0.05).

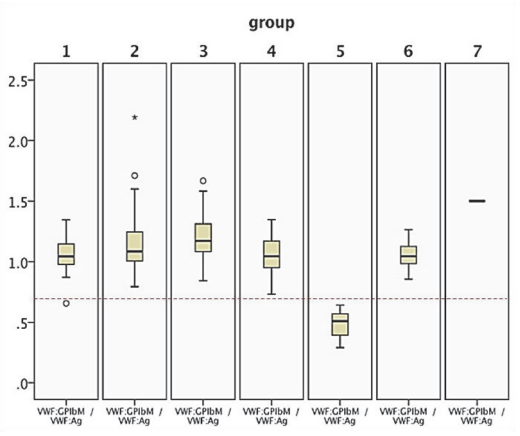

Figure 1. VWF:GPIbM/VWF:Ag in different study groups (refer to Table 2). The dashed line indicated the cut-off of 0.7. VWF:Ag: von Willebrand factor; VWF:GPIbM: VWF activity measured as VWF binding to the glycoprotein Ib (GPIb) receptoron the platelet surface.

The factor VIII level in healthy individuals was comparable with that in group 2 (p = 0.787) and was reduced in other groups (p < 0.05). In group 4, the ratio of FVIII:C/VWF:Ag has shown higher values (2.81; 2.07–3.59) compared with those in the other groups. The ratio of FVIII:C/VWF:Ag has the lowest results (0.33; 0.12–0.52) in group 6 and was statistically different compared with the other groups (p < 0.05).

Figure 2 illustrates the RistoHigh testing results in the different study groups. However, WB-RIPA was not performed in the control group. Patients in groups 2,3 and 6 had partly overlapping results with reference intervals provided by the manufacturer. Platelet aggregation was reduced at the 0.77 mg/mL ristocetin concentration in group 4 and progressively reduced in group 5. Moreover, these patients had lower VWF:GPIbM results. No response to ristocetin was demonstrated in group 7. RistoHigh positively correlated with VWF:Ag (r = 0.518, p < 0.01) and VWF:GPIbM (r = 0.484, p < 0.01) in all study populations.

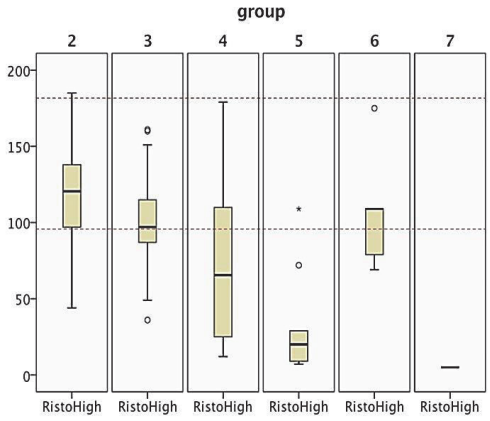

Figure 2. WB-RIPA by Multiplate at ristocetin concentration of 0.77 mg/mL in different study groups (refer to Table 2). The dashed lines indicate reference intervals. WB-RIPA: whole blood ristocetin-induced platelet agglutination; RistoHigh: ristocetin-induced platelet aggregation in whole blood with final ristocetin concentration 0.77 mg/mL.

VWF multimeric analysis was conducted in all patients. The normal ranges for HMWM were 45.6%–66.6% as previously reported [13]. However, the HMWM decrease was defined as < 40% using values 40%–45% as the grey zone. Furthermore, Fig. 3 shows the distribution of HMWM multimers in the study population.

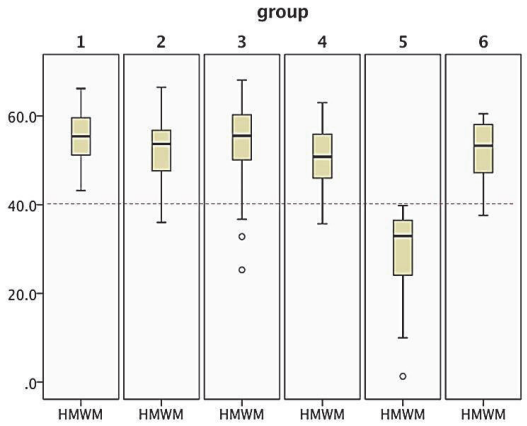

Figure 3. HMWM in the different study groups (refer to Table 2). The dashed line indicated the cut-off of 40%. HMWM: high molecular weight multimers.

The control group demonstrated a normal VWF:MM pattern, and the multimers fractions were within reference intervals. Group 2 (50 of 131 samples; 38%) showed a normal multimeric pattern with a normal VWD phenotypic profile, do not currently fulfil the diagnostic criteria for VWD and were therefore defined as non-VWD, but HMWM was 36% (lower than the cut-off of 40%) in one patient.

In group 3, 46 of 131 (35%) suspicious patients were identified to have low VWF. In this group, 43 samples had normal multimeric distribution. However, VWF:MM interpretation was difficult/impossible in three cases (single-family members) because a smeary appearance was visible with a gel, HMWM ranged from 25 to 37 by densitometry, and these family members had a normal ratio of VWF activity to antigen and normal platelet aggregation results.

Furthermore, group 4 has 17 of 131 (13%) patients categorised as suspected VWD type 1. All samples showed a normal multimeric pattern, but HMWM was 36% (lower than the cut-off of 40%) in one patient.

In group 5, 10 of 131 (8%) patients were grouped as suspected VWD type 2A or 2M. A visible HMWM decrease (range, 39–1.3 by densitometry) was found in all cases. However, one of them showed a visual loss of IMWM and HMWM on the gel as well as quantitatively (IMWM, 7.1%; HMWM, 1.3%). In this group, patients had low VWF activity to antigen ratio (<0.7).

In group 6, 5% of all patients were classified as suspected VWD type 2N or mild haemophilia A. All samples showed a normal multimeric pattern, but HMWM was 38% (lower than the cut-off of 40%) in one patient.

Furthermore, in group 7, two patients were categorised as suspected VWD type 3 or severe type 1.

Consequently, VWF:MM was undetectable in both cases.

This study found that the decreased levels of the VWF ratio activity to antigen were related to the reduction of HMWM. Moreover, VWF:GPIbM/VWF:Ag positively correlated with HMWM ($r = 0.35$, $p < 0.01$) in all study populations.

DISCUSSION:

All measurements of the VWF profile were repeated (minimum twice) on a separate new sample to confirm or refute the initial investigation results [14]. The VWF:Ag level was <50% and <30% in 52% and 18% of the patients, respectively. Moreover, the values of VWF:GPIbM were <50% and <30% in 36% and 17% of the cases, respectively.

The calculated ratio between VWF activity and antigen can aid in identifying the qualitative VWF abnormalities and help differentiate type 1 from type 2-like. In the present study, the cut-off used was <0.7 [15].

The results showed that the ratio of FVIII:C/VWF:Ag was increased to > 2 in patients suspected to have VWD type 1, demonstrating a defect in VWF secretion as the main cause of quantitative deficiency [16]. At the same time, the ratio of FVIII:C/VWF:Ag was <0.7 in patients suspected with VWD type 2N or mild haemophilia A, supporting the hypothesis of defective FVIII:C-VWF binding or FVIII:C deficiency [17].

Multiplate® platelet aggregometry analyser (Roche) is widely used for screening of platelet function disorders (PFDs) [18]. Published data about the usefulness of WB-RIPA in VWD diagnosis are controversial. Moreover, it has potential diagnostic value for VWD by performing ristocetin-induced platelet aggregation in whole blood [19]. Diagnostic accuracy has been proven for patients with previously diagnosed VWD and an agreement exists with Born aggregometry results [20]. A study with 30 previously characterised VWD patient population showed that WBA was as sensitive as Light Transmission Aggregometry (LTA) in detecting VWD with a 76% correlation between the two methods [20]. Furthermore, the clinical usefulness of Multiplate as a screening assay for PFDs is limited, and this method may represent an alternative to LTA only for Glanzmann's thrombasthenia or other severe PFDs, whereas WBA is poorly sensitive in detecting mild PFDs [21]. Moreover, researchers from Sweden [19] evaluated the diagnostic accuracy of WB-RIPA, performed at a high ristocetin concentration, in a study with 100 VWD patients and reported that reduced WB-RIPA correlated with low-VWF activity and is a sensitive screening test to exclude VWD. Nummi et al. [22] proposed the use of Multiplate-based WB-RIPA to rule out VWD. This study also found that ristocetin-induced platelet aggregation was decreased in patients suspected to have VWD type 1, 2A or 2M.

RIPA testing has been reported [19] to demonstrate no response to ristocetin in VWD type 3. Similar results were found in this study in patients assumed to have VWD type 3 or severe type 1.

In the diagnostic workup of patients suspected VWD, the VWF multimer assay is one of the most important indicators for VWF quality [23]. The HMWM interpretation using the new VWF:MM assay (Sebia) in clinical practice is based on individual decisions, and no consensus currently exists for that. The cut-offs of 40% and 38% were used for patients' samples and lyophilised samples, respectively based on the results from the External Quality Assessment [8,9]. However, these suggestions need to be clarified further.

Healthy individuals, non-VWD, low VWF and patients suspected to have VWD types 1 and 2N showed the normal distribution of VWF multimer fractions. The HMWM decrease is associated with impaired VWF function [24]. Moreover, several authors [25] have demonstrated a loss of HMWM in patients with VWD types 2A and 2B (in most cases) and also type 2A is sometimes associated with IMWM loss. Similar results were found in this study in group 6 patients. However, recognition of 2A and 2M subtypes based on multimer pattern is sometimes ambiguous because detecting the

2M phenotype with non-proteolysed multimers is not possible using low-resolution gels [26]. Thus, the VWD type 2M is misdiagnosed and under-recognised, depending on the laboratory test panel used [25]. VWF multimers were undetectable in patients suspected to have VWD type 3 or severe type 1, which corresponds to the sensitivity of the method [9]. According to the results of this study, the VWF:GPIbM/VWF:Ag ratio positively correlated with HMWM. Moreover, a recent study conducted by Favaloro et al. [27] showed that VWF activity to antigen ratios was positively related to HMWM. Their findings suggest that the highest correlation was found with the chemiluminescence method.

DDAVP, TA, or replacement VWF therapy are used for managing patients with VWD [28]. A DDAVP test–dose infusion at the time of diagnosis is recommended to evaluate the individual response, which depends on various factors (e.g., phenotype and genotype)[29]. Usually, patients with VWD type 1 demonstrate a good response to DDAVP [30]. Moreover, the replacement therapy is the treatment of choice for non-responders to DDAVP or type 2B patients for whom the DDAVP is contraindicated [29]. Previously, the response to DDAVP was assessed [31] in seven patients: six were defined as good responders, and one patient demonstrated a partial response to DDAVP.

The genetic evaluation was not yet routinely used for VWD type 1. However, it is often performed for VWD types 2 and 3 [5]. Genetic testing for VWD type 2N vs. haemophilia A was done in four patients wherein two of them were previously diagnosed with HA. Differential diagnosis between VWD and HA is important because the HA therapy is monospecific (e.g. recombinant FVIII) and management of VWD may be less effective if DDAVP or VWF replacement therapy is not provided [32].

Medical information initially provided by clinicians is required for correct laboratory evaluation for patients with bleeding disorders [32]. Moreover, non-specific results are very difficult to interpret. Additionally, patient-related preanalytical issues should be taken into consideration [33]. For selective approach in laboratory request, the adult and paediatric ISTH-BAT [34] was translated into Estonian and incorporated into routine practice to identify individuals with clinically relevant bleeding tendency/symptoms. Furthermore, regular meetings and discussions focusing on clinical cases were established between clinicians and the laboratory.

CONCLUSIONS AND FUTURE PERSPECTIVES:

This is the first report of VWD laboratory evaluation in Estonia to provide insight into the potential clinical significance of using VWF: MM. The interpretation of VWF multimers has to be complemented by the quantification of fractions of multimers by densitometry additional to visual gel's examination. It is hoped that this work supports the improvement in VWD diagnosis in Estonia, and it is suggested that the real VWD prevalence should be evaluated in the future.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Author contributions

MP: study concept and design, data acquisition, statistical analysis, analysis and interpretation of data, manuscript drafting, study supervision and critical revision of the manuscript for important intellectual content. KS, MV, MH, KI and MR: study concept and design, analysis and interpretation of data and critical

revision of the manuscript for important intellectual content. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Von Willebrand Factor Multimeric Assay in Acquired von Willebrand Disease Diagnosis: A Report of Experience from North Estonia Medical Centre

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Abstract

Objectives Acquired von Willebrand syndrome (AVWS) is a rare and frequently underdiagnosed bleeding disorder with an unknown prevalence. The diagnosis of AVWS is made based on laboratory investigations and the presence of clinical symptoms. Evaluation and management of affected patients are complex due to the need for multiple laboratory assays.

Materials and Methods Here, we describe the clinical and laboratory data of seven patients with a diagnosis of AVWS. All patients met the criteria for AVWS based on laboratory findings, bleeding symptoms, and the absence of any previous history of a bleeding disorder.

Results In all cases, the laboratory findings, lack of bleeding anamnesis, and family history suggested the presence of AVWS. Von Willebrand factor multimeric analysis showed decreased high-molecular weight (HMW) multimers in six cases. Patients with lower HMW multimers experienced more severe bleeding complications.

Conclusions The diagnosis of AVWS is complex and requires extensive laboratory evaluation. Interdisciplinary collaboration and complex laboratory evaluations are of paramount importance for the early recognition of AVWS and optimal AVWS diagnosis as well as successful clinical management.

Keywords
- acquired von Willebrand syndrome
- von Willebrand factor
- von Willebrand factor multimers

Note: The study was performed according to the Declaration of Helsinki and was approved by the Tallinn Medical Research Ethics Committee.

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Introduction

Acquired von Willebrand syndrome (AVWS) is a rare and frequently underdiagnosed bleeding disorder, mainly due to the broad spectrum of possible clinical and laboratory features affiliated with this condition.[1] The mechanisms behind von Willebrand factor (VWF) abnormalities depend upon the type of underlying disorder and may include increased clearance; enhanced shear stress and subsequent proteolysis; inhibition of VWF functions; adsorption to the platelet surface; or, rarely, decreased synthesis.[1]

The definition of AVWS was published by the VWF subcommittee in 2000.[2] A diagnosis of AVWS can be made based on the following criteria: the existence of a lack of previous lifelong bleeding incidents and relevant family history, clinical picture, and laboratory investigation results,[3] for example, VWF levels and factor VIII (FVIII) coagulant activity (FVIII:C) are sometimes decreased, a reduced VWF function/antigen ratio can indicate the existence of functional disorders, even if the absolute activity is within the normal limit, a loss or decrease in high-molecular weight (HMW) multimers may also be observable. The prevalence of AVWS remains unknown and[4] the evaluation and management of affected patients may be complex due to the need for multiple laboratory assays, especially in those in whom the underlying disease (e.g., prosthetic heart valve or essential thrombocythemia [ET]) necessitates antithrombotic therapy. The initial laboratory tests used to assess AVWS include VWF level, VWF activity, and FVIII activity assays. Further tests include VWF multimer analysis, which is a sensitive tool able to detect the structural abnormalities of VWF even in the context of normal VWF activity levels. The frequency of the detection of inhibitors, that is, antibodies against VWF, is low in AVWS. Before 2016, it was not possible to confirm a suspicion of AVWS in Estonia because of a limitation of available laboratory VWF assays, while, since 2016, all VWF-related screening assays have been available to clinicians[5] and, recently, a semiautomated VWF multimer assay has been incorporated into routine clinical practice at the North Estonia Medical Centre (NEMC).[6,7]

Here, we describe the clinical and laboratory data of seven patients diagnosed with AVWS at NEMC.

Materials and Methods

Patients

We included all consequent patients referred to and assessed at NEMC from the January 1, 2016, to December 31, 2017, who met the criteria for an AVWS diagnosis based on laboratory findings and bleeding symptoms together with the absence of any previous history of a bleeding disorder.[3]

The most common clinical symptoms were easy bruising, epistaxis, menorrhagia, and bleeding complications after tooth extraction. The mean age of the patients was 57.4 years (range: 22–80 years). The study group included five women and two men with various underlying diseases such as non-Hodgkin's lymphoma (NHL), monoclonal gammopathy of undetermined significance (MGUS), ET, polycythemia vera (PV), secondary polycythemia due to cardiovascular diseases, obstructive sleep apnea syndrome, and autoimmune thyroiditis.

All cases were discussed at interdisciplinary meetings between laboratory and clinical staff. This retrospective study was performed as a collaboration between NEMC and Helsinki University Hospital, HUSLAB laboratory services, Coagulation Disorders Unit in partnership with The Twinning Program of the World Federation of Hemophilia (WFH). The study was performed according to the Declaration of Helsinki and was approved by the Tallinn Medical Research Ethics Committee.

Blood Sampling

During this study, peripheral venous blood specimens were collected into K2-EDTA tubes (BD Vacutainer; BD Diagnostics, Plymouth, UK) for a complete blood count, 3.2% sodium citrate tubes (BD Vacutainer; BD Diagnostics) for coagulation assays, and hirudin blood tubes (Roche Diagnostics, Switzerland) for platelet aggregation evaluation.

Laboratory Investigations

Based on the laboratory assays available in Estonia, the diagnostic algorithm for von Willebrand disease (VWD)/syndrome was adopted in this study.[8] Initial laboratory evaluations included complete blood count (Sysmex XE-5000; Roche Diagnostics); prothrombin time (PT) (Neoplastine Cl Plus; Diagnostica Stago, Asnières-sur-Seine, France); partial thromboplastin time (APTT) (PTT-A; Diagnostica Stago), VWF antigen (VWF:Ag) (Liatest-VWF:Ag; Diagnostica Stago); FVIII:C determined by a one-stage, clot-based assay (Diagnostica Stago, France); and VWF activity measured as VWF binding to the glycoprotein Ib (GPIb) receptor on the platelet surface (VWF:GPIbM) (Innovance VWF Ac kit; Siemens Healthcare Diagnostics, Marburg, Germany). All parameters were measured on the STA-R Evolution analyzer (Diagnostica Stago) using commercial kits.

Mixing studies were conducted to determine the etiology of prolonged APTT; the APTT test was repeated on a mixture of the patient's plasma with normal plasma immediately and after incubation for two hours at 37°C. Depending on correction, FVIII, FIX, FXI, FXII, or lupus anticoagulant tests were performed.

Platelet aggregation was measured in whole blood by an impedance multiplate aggregometer (Roche Diagnostics) using the RISTOhigh test (final concentration of ristocetin: 0.77 mg/mL) and RISTOlow test (final concentration of ristocetin: 0.2 mg /mL). For both, the measurements were performed within 180 minutes after venipuncture.

The multimeric pattern of VWF was evaluated using the new Hydragel 5 von Willebrand multimers assay (Sebia, Lisses, France).[6,9-11] The detailed protocol has previously been described.[12] In May 2019, the VWF multimer analysis with 5VWF was accredited in the NEMC laboratory according to the ISO15189:2012 standard. Both the visual evaluation of the gels and densitometric analysis were performed. VWF multimers were classified as low-molecular weight, intermediate-molecular weight, or HMW multimers with densitometry.

Case Series

The main characteristics of the study participants are shown in ►**Table 1**. All patients had other bleeding episodes and no family history for bleeding disorders. The International Society on Thrombosis and Hemostasis–Bleeding Assessment Tool was used to score the risk of bleeding (data not presented).

Case 1. A 67-year-old female patient with a diagnosis of NHL from 2012 onward was referred for consultation with a suspected bleeding disorder. Three bleeding episodes were noted during a period of 6 months before a definite AVWS diagnosis was made. First, a severe bleeding episode had occurred related to puncture of the right maxillary sinus; then, 3 months later, she was admitted to the emergency

Table 1 Demographic and laboratory characteristics of the study participants

	Reference ranges	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Diagnosis		Non-Hodgkin's lymphoma	ET, JAK2 (V617F)	ET, JAK2 (V617F)	PV	MGUS	Secondary erythrocytosis due to cardiovascular diseases and obstructive sleep apnea syndrome	Autoimmune *thyroiditis*
Clinical symptoms		Epistaxis, bleeding complications after tooth extraction	Menorrhagia	Bleeding complications after tooth extraction	Bleeding complications after tooth extraction	Epistaxis	Epistaxis	Spontaneous hematoma
Age, gender		67 F	33 F	61 F	60 F	78 M	80 M	22 F
PT (sec)	11.5–14.5	13.3	13.2	13.0	12.6	13.0	12.9	12.6
APTT (sec)	29–38	44	48	41	48	46	34.4	33.6
APTTmix1:1 (0`, 120`)		Correction	Correction	Correction	Correction	Correction	NA	NA
VWF:Ag (%)	50–160	25	61	83	102	29	269	35
VWFGPIbM (%)	46–146 (0 group) 61–179 (non-0)	14	34	29	62	11	174	41
VWFGPIbM/Ag ratio	> 0.7	0.56	0.55	0.35	0.61	0.38	0.65	1.25
FVIII:C %	60–150	42	37	48	118	21	253	65
RISTOhigh (U)	98–180	12	ND	ND	151	38	ND	112
WBC count 10^9/L	4–10	5.6	14.9	12.5	15.2	4.2	8.1	7.7
RBC count 10^{12}/L	M 4.5–6.0; N 4.0–5.5	4.6	5.4	8.5	5.7	5.0	6.2	4.1
Hematocrit (%)	M 40–52; N 36–47	40	46	50	47	46	57	38
Platelet count 10^9/L	150–400	245	1391	1120	785	224	142	326
VWF multimers	Persons without VWD (21): Normal distribution	Loss of HMWM	Decrease of HMWM	Loss of HMWM	Decrease of HMWM	Decrease of HMWM	Decrease of HMWM	Normal distribution
LMWM (%)	15.3 (11–23)	50.9	33.1	58.3	32.5	49.1	35.1	13.8
IMWM (%)	30.2 (23.1–35.8)	38.3	39.4	33.5	39.1	19.1	35.9	25.0
HMWM (%)	54.8 (45.1–65.9)	10.8	27.5	8.3	28.4	31.8	29.0	61.2

Abbreviations: HMWM, high-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers; LMWM, low-molecular-weight multimers; NA, nonapplicable; ND, not determined; VWF: Ag, von Willebrand factor antigen; VWF: GPIbM, VWF activity assays using recombinant gain-of-function mutant GPIb fragments allowing for the spontaneous binding of VWF to the mutant GPIb without ristocetin.

department due to recurrent bleeding after tooth extraction requiring tamponade and bleeding from the right nasal cavity requiring electrocauterization. The patient was treated with tranexamic acid during all bleeding events and continues to be followed-up in the hematology clinic.

Case 2. A 33-year-old female patient with heavy menorrhagia and high platelet count was investigated. She had no antithrombotic treatment. A diagnosis of ET with a positive finding for a *JAK2* (V617F) mutation was made. Menorrhagia was caused by secondary von Willebrand syndrome, and treatment with tranexamic acid was prescribed for use during menstrual bleeding.

Case 3. A 61-year-old female patient was investigated after experiencing bleeding after tooth extraction lasting 2 days. A high blood platelet count suggested the possibility of chronic myeloproliferative disease together with secondary von Willebrand syndrome. Further investigations confirmed *JAK2* (V617F)-positive ET. Cessation of bleeding symptoms was achieved after platelet count normalization with hydroxyurea treatment.

Case 4. A 60-year-old female patient with PV from 2000 onward was referred for additional examination and consultation before planned tooth extraction. She experienced bleeding complications 2 year earlier after the tooth extraction. She was treated with hydroxyurea, blood exfusion, and low-dose aspirin. She was advised to stop aspirin 5 days before her next planned tooth extraction. Prophylactic treatment with 10 mg/kg of tranexamic acid given intravenously (IV) was prescribed three times daily on the procedure day and also one day before and after the procedure.

Case 5. A 78-year-old male patient was consulted because of recurrent epistaxis, with a need for cauterization throughout 2 previous years. His complete blood count was normal. Biochemical investigation showed a monoclonal peak (3.1 g/L) in the γ-globulin region. Immunoglobulin G kappa monoclonal protein was confirmed by immunofixation. The kappa/lambda free light-chain ratio was 5.2 (reference range: 0.26–1.65), compatible with a diagnosis of MGUS. Tranexamic acid was prescribed in the case of a bleeding episode and the patient remains under close follow-up observation by the hematology clinic.

Case 6. An 81-year-old male patient with cardiovascular disease and obstructive sleep apnea syndrome was referred to a hematologist by his general practitioner due to frequent epistaxis (nosebleeds) occurring in the 2 previous years, with the need for nasal tamponade at the emergency department. The complete blood count revealed an increased red blood cell count (6.10^{12}/L), increased hemoglobin level (176 g/L), and increased hematocrit concentration (54.9%), which raised the suspicion for PV. However, further studies on *BCR/ABL* p210 and *JAK2* V617F mutations were normal, supporting the diagnosis of secondary erythrocytosis due to cardiovascular disease, which is one condition that can cause AVWS. The patient was counseled, and instructions were given for handling future bleeding episodes. Tranexamic acid was also prescribed to treat further bleeding episodes.

Case 7. A 22-year-old female patient was referred to the hematologist for bleeding evaluation. She reported the development of apparently spontaneous subcutaneous hematomas, unrelated to trauma or physical activity, during the last 3 years. Additional examination showed increased thyroid-stimulating hormone (TSH) and thyroid peroxidase (> 1000 U/mL) levels, consistent with a diagnosis of autoimmune thyroiditis, and the patient was referred to the endocrinologist. Her hypothyroidism was treated and, 1 year later, normal TSH values were recorded together with normalization of coagulation test findings for VWF:Ag (69%), VWF:GPIbM (86%), fibrinogen (2.58 g/L), and CRV (< 1 mg/L).

Results

Coagulation Workup for AVWD Diagnosis

In this case series, coagulation studies showed normal PT and prolonged APTT (Cases 1–5). Mixing study revealed corrections for both immediate and incubated APTT tests, indicating a mild deficiency of FVIII in Cases 1, 2, 3, and 5. FIX, FXI, and FXII levels were normal. Follow-up assessments demonstrated severely decreased (< 35%) VWF activity in four of seven patients (►**Table 1**), fulfilling the criteria for VWD diagnosis. Both decreased VWF:Ag and VWFGPIbM levels in Cases 1 and 5 and normal VWF:Ag levels with low VWF:GPIbM levels in Cases 2 and 3 were observed. In patient 6, the levels of VWF:Ag, VWFGPIbM, and FVIII:C were increased, while a decreased VWF function/antigen ratio (VWFGPIbM/VWF:Ag) was recorded. High-dose ristocetin-induced platelet aggregation was decreased in two patients (cases 1 and 5), while low-dose ristocetin-induced platelet aggregation was normal. In Case 7, the levels of VWF:Ag and VWFGPIbM were both decreased with a normal VWF function/antigen ratio. Complete blood count and platelet aggregation studies were normal.

VWF multimeric analysis (►**Figs. 1 B-F**) revealed decreased HMW multimers, supporting AVWS in all instances (Cases 1–6). In Case 6, during the visual investigation of gel, we did not detect any abnormalities in the VWF pattern, yet densitometric data provided additional information about the VWF multimeric structure. Multimeric analysis (►**Fig. 1 H**) showed a normal distribution pattern, suggesting type 1 AVWS. We noted that patients with lower HMW multimers by densitometric evaluation presented with more severe bleeding complications.

Discussion

We herein describe the clinical and laboratory data of seven patients with AVWS. All cases were discussed in a multidisciplinary meeting involving both clinical and laboratory experts. In all cases, the laboratory findings and lack of previous lifelong bleeding episodes and family history suggested AVWS.

Earlier studies have documented that MGUS,[13-15] NHL,[16] ET,[17,18] and autoimmune hypothyroidism[19] are associated with AVWS. The pathogenesis of AVWS is variable but may have an overlapping mechanism among patients with different underlying disorders.[4,20,21]

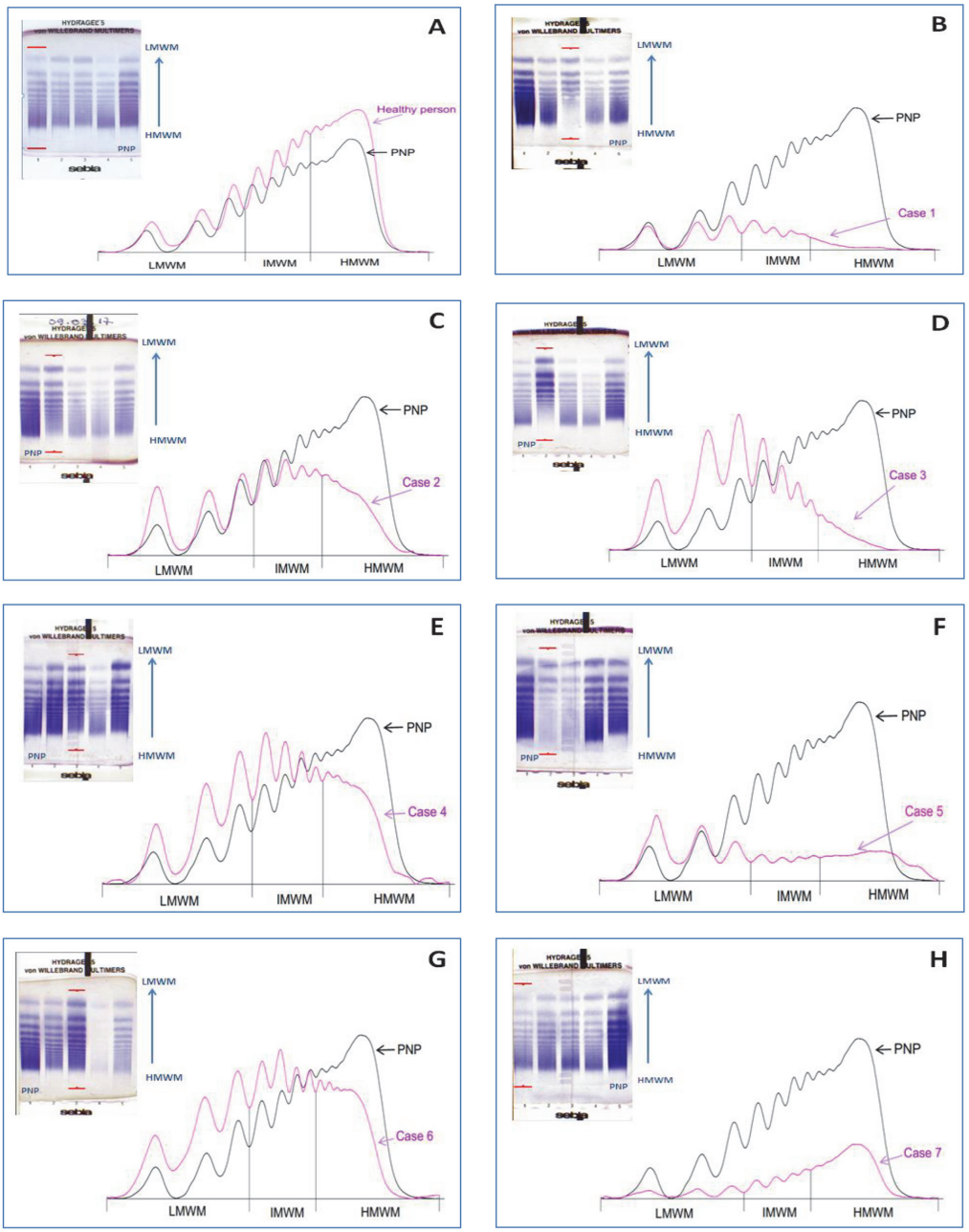

Fig. 1 Electrophoresis gels and densitograms: A—healthy person, B—Case 1, C—Case 2, D—Case 3, E—Case 4, F—Case 5, G—Case 6, H—Case 7. LMWM, low-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers; HMWM, high-molecular-weight multimers; PNP, pool normal plasma.

In our series, six patients showed a type 2–like phenotype with decreased VWF activity to the Ag ratio and a loss/decrease of HMW multimers. One patient had a type 1 VWD phenotype. Recently developed diagnostic algorithms, based on standard laboratory assays, may assist clinicians in the diagnostic workup and help differentiate between AVWS

and VWD types 1 and 2. As reported by Federici et al, using data from the AVWS 2004 International Registry,[22] AVWS is often correlated with a reduced ratio of VWF:RCo/VWF:Ag. The same findings were observed in our study in all cases except in Case 7, where the patient's clinical presentation was caused by the decreased synthesis of VWF.

Notably, our study demonstrated that VWF multimer analysis aids in the diagnosis of AVWS as an important, valuable tool. We further observed in our study that decreases in the level of VWFGPIbM and ratio of VWFGPIbM /VWF:Ag were associated with the selective loss or decrease of HMW multimers. Our study is in agreement with the report by Tiede et al,[23] which suggested that a reduced VWF:RCo/Ag ratio in AVWS indicates inhibitory antibodies or a selective loss or decrease in HMW multimers. Separately, research conducted in Germany[24,25] reported that, in 207 patients with cardiovascular disorders associated with AVWS and a loss of the HMW multimers, only 44% showed a ratio below 0.7 and noted that those patients would have likely been misdiagnosed without multimer analysis. In addition, VWF multimer analysis has been reported by Chen and Nichols as the most sensitive and specific method available for detecting such AVWS or acquired VWF abnormality without definite bleeding symptoms.[26] In line with a previous study,[27] our results revealed that AVWS is also associated with a decreased response to the higher ristocetin concentration (Cases 1 and 5).

The incidence of AVWS is possibly underestimated in the clinic. For example, as seen in the retrospective report by Mital et al on ET patients, AVWS may develop as frequently as in every fifth patient with ET.[28] Furthermore, AVWS should be considered in all patients with new-onset bleeding whenever the laboratory findings suggest VWD, particularly in the presence of an AVWS-associated disorder. AVWS testing is also recommended prior to surgery or an intervention characterized by a high risk of bleeding in any individual with an AVWS-associated disorder. Treatment of the patient's underlying condition can lead to remission of AVWS. Strategies to prevent and/or treat bleeding episodes should also be put into place, including the use of VWF-containing FVIII concentrates, desmopressin, and tranexamic acid. Treatment success will depend largely upon the underlying pathogenesis of the disorder. Therefore, investigation of the VWF multimers presents profound clinical significance in suspected AVWS.

The gold standard for the detection of structural abnormalities of VWF is the multimeric assay.[29] We assessed the VWF multimeric pattern in gels and quantified multimeric fractions using Sebia analysis software program. This method is easy to use and could prove very useful in future laboratory workup required for the diagnosis of AVWS. Interestingly, the densitometric evaluation of VWF multimers showed that patients with lower HMW multimer values presented with more severe bleeding complications. However, most AVWS patients do not bleed until they experience additional triggers like invasive procedures or trauma.[4] Therefore, correct identification of patients with AVWS is a prerequisite for determining the applicable guidance on clinical management.[30]

Conclusions

Our data also demonstrate that the diagnosis of AVWS is complex and requires extensive laboratory evaluation.[31] Our data support that VWF multimer analysis should be included in the AVWS diagnostic algorithm. Interdisciplinary collaboration and complex laboratory evaluations are of paramount importance for the early recognition of AVWS and the selection of appropriate clinical management protocols.

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Conflicts of Interests
None declared.

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ESTABLISHING REFERENCE INTERVALS FOR VON WILLEBRAND FACTOR MULTIMERS

USPOSTAVLJANJE REFERENTNIH INTERVALA ZA MULTIMERE FAKTORA VON WILLEBRAND

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Summary

Background: von Willebrand factor (VWF) multimers (VWF:MM) methodologies are technically difficult, laborious, time consuming, non-standardized and results vary between laboratories. A new semi automated VWF:MM assay is available for routine use (Sebia). Due to lack of reference values for VWF:MM fractions, results interpretation can be challenging in some cases. The aim of this study was to determine reference intervals for low molecular weight (LMWM), intermediate molecular weight (IMWM) and high molecular weight (HMWM) multimers.
Methods: By the international cooperation initiated between 4 countries (Estonia, Latvia, France, and USA) 131 samples of relatively healthy individuals were analyzed for VWF:MM (in total 51 males and 80 non-pregnant females aged 17–69 years). Reference intervals were calculated according to CLSI C28-A3 standard.
Results: The proposed reference intervals for VWF:MM were calculated for LMWM 10.4–22.5%, IMWM 22.6–37.6%, HMWM 45.6–66.6%. Age related differences were

Kratak sadržaj

Uvod: Metodologija multimera (VWF:MM) von Willebrand faktora (VWF) tehnički je teška, naporna, digotrajna, nestandardizovana i rezultati se razlikuju u različitim laboratorijama. Novi poluautomatski VWF:MM test (Sebia) dostupan je za rutinsku upotrebu. Zbog nedostatka referentnih vrednosti za VWF:MM frakcije, tumačenje rezultata može u nekim slučajevima biti izazovno. Cilj ove studije bio je da se odrede referentni intervali za multimere niske molekularne mase (LMWM), srednje molekularne mase (IMWM) i visoke molekularne težine (HMWM).
Metode: Međunarodnom saradnjom započetom između 4 zemlje (Estonija, Letonija, Francuska i SAD) 131 uzorak relativno zdravih pojedinaca analiziran je na VWF:MM (ukupno 51 muškarac i 80 žena koje nisu bile trudne u dobi od 17–69 godina). Referentni intervali su izračunati prema CLSI C28-A3 standardu.
Rezultati: Predloženi referentni intervali za VWF:MM izračunati su za LMWM 10,4–22,5%, IMWM 22,6–37,6% i HMWM 45,6–66,6%. Starosne razlike su primećene u

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List of abbreviations: VWF, von Willebrand factor; VWF:MM, von Willebrand factor multimers; LMWM, low molecular weight multimers; IMWM, intermediate molecular weight; HMWM, high molecular weight multimers; VWD, von Willebrand disease

seen in IMWM and HMWM ($p<0.001$ and 0.038). There was no gender related difference observed. Geographically LMWM results of France were different from the other regions ($p<0.05$).

Conclusions: Quantification of VWF:MM fractions, in addition to qualitative assessment of VWF:MM patterns, has the potential to aid in differential diagnosis of von Willebrand disease (VWD) subtypes. The reference values calculated in this study can be used in future research to establish clinical decision limits.

Keywords: von Willebrand factor, von Willebrand factor multimers, quantitative analysis, reference intervals

IMWM i HMWM ($p < 0,001$ i 0,038). Nije uočena razlika vezana za pol. Geografski rezultati LMWM iz Francuske bili su različiti od ostalih regiona ($p < 0,05$).

Zaključak: Kvantifikovanje frakcija VWF:MM, pored kvalitativne procene VWF:MM uzoraka, može da pomogne i u diferencijalnoj dijagnozi podtipova von Willebrandove (VWD) bolesti. Referentne vrednosti izračunate u ovoj studiji mogu se koristiti u budućim istraživanjima za utvrđivanje granica kliničkih odluka.

Ključne reči: von Willeberandov faktor, multimeri von Willebrandovog faktora, kvantitativna analiza, referentni intervali

Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder with an approximate prevalence of about 1–2 % in the general population (1–3), although the true incidence is unknown (4). VWF plays an important role in regulation of normal hemostasis and facilitates progression of bleeding or thrombotic disorders with platelet and endothelial dysfunction (5–6). VWD arises due to structural and/or quantitative abnormalities of von Willebrand factor (VWF), a large multimeric glycoprotein with adhesive functions through binding to FVIII, to platelet surface glycoproteins, and to constituents of subendothelial connective tissue (5–7).

VWD is classified into 3 main types: type 1, a partial quantitative deficiency; type 2, a qualitative defect that is further subdivided into 4 categories, 2A, 2B, 2N, and 2M; and type 3, a complete absence of VWF (1). Correct classification of the type/subtype of the VWD is important in patients' management and the therapeutic approach (1).

As VWF has diverse functions, laboratory testing for VWD and other VWF-related disorders (i.e., thrombotic thrombocytopenic purpura (8) or a variety of cardiac lesions that result in clearance of larger multimers, such as aortic regurgitation, mitral insufficiency, and hypertrophic cardiomyopathy (9)) require complex laboratory assessment (3, 10). The first-line tests typically include evaluation of VWF antigen (VWF:Ag), different VWF activity (VWF:Ac) assays (e.g. ristocetin cofactor assay (VWF:RCo), VWF activity measured as VWF binding to the glycoprotein Ib (GPIb) receptor on the platelet surface (VWF:GPIbM), collagen binding (VWF:CB) etc.) and factor VIII activity (FVIII:C) (4).

VWF multimeric assay is a second-line analysis used in the diagnosis and classification of different VWD subtypes (11). VWF circulates in plasma as low, intermediate, and high molecular weight (LMWM, IMWM, and HMWM, respectively) multimers (12, 13). The absence of HMWM is the cardinal feature that distinguishes type 1 from type 2A and 2B VWD, whereas the different subtypes of type 2 VWD can be differentiated by more subtle alterations of the inner structure of smaller multimers (4, 10, 11).

Historically, VWF multimers are analyzed by in-house developed electrophoresis techniques and densitometric analysis of Western blots (7, 14). These methodologies are technically difficult, laborious, time consuming and non-standardized (2, 12). The development of a relatively rapid semi-automated commercial VWF multimer kit assay (Hydragel 5/Hydragel 11 von Willebrand multimers, Sebia, France) may represent a first step toward standardization. This method was already shown to provide adequate information for characterization and classification of congenital VWD subtypes (12, 14, 15). Moreover, results correlate with the clinical status, diagnosis of inherited or acquired VWD, if used and interpreted by experienced professionals (12, 14).

In addition to qualitative interpretation of multimer patterns, the Sebia PHORESIS software allows quantification of VWF:MM band patterns, and calculation of the percentage values of each molecular weight multimer fraction. Quantitative multimer analysis might be needed for the detection of subtle abnormalities and changes following therapeutic interventions (7, 16). Due to lack of reference values for VWF:MM fractions, result interpretation can be challenging in some cases.

Thus, in the present study we used densitometry to determine reference intervals for LMWM, IMWM and HMWM fractions.

Materials and Methods

Study subjects

To collect a larger sample size an international cooperation was initiated between 4 countries (Estonia, Latvia, France, and USA). The list of participating institutions were as follows: L1 (two institutions from Baltic countries: L1A – Laboratory of North Estonia Medical Centre, Tallinn Estonia; L1B – Riga East University Hospital, Riga, Latvia), L2 (Department of Biology, Foch Hospital, Suresnes, France), and L3 (University of Utah / ARUP Laboratories, Salt

Lake City, Utah, United States). Both Estonian and Latvian samples were analyzed in the Laboratory of North Estonia Medical Centre, thus accounted as one group L1.

In total 134 healthy volunteers were recruited for this study, but after outlier exclusion 131 samples were analyzed: 51 males and 80 non-pregnant females aged 17–69 years.

Acceptance criteria: no history of hemorrhagic episodes; no usage of any interfering medication for at least 10 days before blood collection; normal VWF results (VWF:Ag; VWF:Ac – VWF:GPIbM (L1), VWF:GPIbR (L2) and VWF:RCo (L3); VWF:Ac/VWF:Ag ratio); written consent provided. Blood donor plasmas were not used because the questionnaire for blood donors do not include information regarding family bleeding history, individual mild bleeding episodes and are not screened for VWD routinely. The study was performed according to the Declaration of Helsinki and was approved by appropriate local or national ethical committees or local Institutional Review Board at each institution.

Sample collection and specimen processing

Samples for the reference interval studies were collected from apparently healthy individuals according to the participating institutions' locally approved venous blood sampling procedures and in concordance with ethical laws of each participating country. Briefly, peripheral venous blood specimens were collected into light blue-top vacuum tubes [3.2% sodium citrate tubes (BD Vacutainer, L1A, L3 or Sarstedt, L2) or 3.8% NC Buffered Citrate (Vacutest KIMA srl, L1B)], centrifuged (within 2 hours after sampling) at a speed and time required to consistently produce platelet-poor plasma (residual platelet count less than 10 x10^9L):

L1A – 1500 g for 15 minutes at room temperature

L2B – 1500 g for 15 minutes at room temperature, aliquoted, stored frozen at -70 °C and transported on dry ice to L1A

L2 – 2000 g for 15 minutes at 15 °C (twice)

L3 – 1700 g for 15 minutes at room temperature

Samples were aliquoted and stored frozen (at least –20 °C) until testing (within 30 days). Aliquots were thawed in a water bath (+37 °C) for 5 minutes and mixed well before testing.

VWF multimers method and densitometry

The VWF multimers method, developed by Sebia (France), is described in detail elsewhere (3, 4). It was used by the participating laboratories without deviation from the original Sebia assay protocol. In brief, citrated plasma samples were analyzed on the Hydrasys 2 instrument (Sebia, France) with ready to use SDS agarose gels (Hydragel 5 von Willebrand multimers, Sebia). Densitometry of VWF multimer patterns was carried out with a transmission scanner (Sebia Gelscan Instrument) which allows scanning and data storage of the results. Data acquisition is performed by a bidimensional calibrated CCD sensor. The instrument, when connected to a PC with the Sebia PHORESIS software, allowed the operator to display the gel images, curves, curves overlapping, and quantification of multimer band patterns according to the manufacturer recommendation (LMWM 1-3 bands; IMWM 4–7 bands; and HMWM 8th band and above).

The percentage values of each molecular weight multimer fraction was provided by the software. The calculation was made by applying the ratio of the area of each fraction and the total area under the curve. The multimer patterns of the plasma samples studied were, if necessary, compared with the reference pool pattern analyzed on the same gel. The total area under the curve of each sample was directly proportional to the amount of antigen (VWF:Ag).

Statistical analysis

All statistical analysis was performed with MedCalc® software (MedCalc Software, Belgium) version 18.11.6. and IBM SPSS statistics version 23. Descriptive statistics was used to analyze demographic data and laboratory characteristics. The data was analyzed according to age, gender and geographic location. The results were expressed as median (interquartile range [IQR]). The difference between variables was tested using the Mann-Whitney test. P values of <0.05 were considered statistically significant.

Reference intervals were established using a robust method following CLSI C28-A3 standard to calculate the 2.5th and 97.5th percentiles and associated 90% confidence intervals (CI) for each VWF multimeric fraction. Data distributions were tested for normality by Shapiro-Wilk test. Outlier detection was performed by Grubs double sided and Tukey methods.

Results

Study subjects

Data and samples were collected from 131 healthy volunteers (51 males and 80 non-pregnant females), from Baltic Region (L1), France (L2) and United States (L3). The demographic characteristics and laboratory findings are summarized in *Table I*.

Table I Characteristics of study groups and corresponding results of VWF:MM fractions.

	L1 (n=31)	L2 (n=64)	L3 (n=36)
Age range (years)	18–69	17-62	19–61
Age, median (IQR)	34 (23–46)	40.5 (30.3–51.8)	30 (24.3–36.0)
males/females	7/24	27/37	17/19
LMWM, % median (IQR)	15 (12.7–17.2)	16.1 (14.5–19.1)	14 (12.4–16.0)
LMWM lowest / highest value	9.8–23.0	10.7–23.3	9.7–19.9
IMWM, % median (IQR)	29.2 (26.7–31.2)	29 (27.2–30.6)	30.7 (26.3–34.2)
IMWM lowest / highest value	22.8–36.4	21.4–35.8	21.3–38.6
HMWM, % median (IQR)	55.4 (51.1–60.2)	54.5 (52.2–58.1)	55.9 (51.3–59.6)
HMWM lowest / highest value	43.2–66.2	45.1–65.2	44.4–68.2

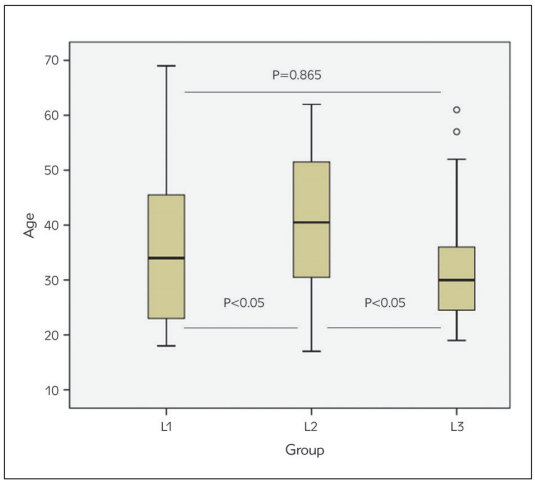

Figure 1 Age differences between subjects of different geographical locations. L1, samples from Baltic region; L2, samples from France; L3, samples from United States.

Participants' age was between 17 and 69 years. Subjects from L3 were younger than from L1 and L2: medians (IQR) were 30 (24.3–36.0), 34 (23–46) and 40.5 (30.3–51.8), respectively. As presented in *Figure 1*, there was no significant difference in age between L1 and L3 (P=0.865), but the differences between L2 vs L1 and L2 vs L3 were statistically significant (p<0.05).

To assess possible differences in VWF multimers fractions data from the 3 participating regions was compared.

Age related difference in VWF multimers fractions

VWF multimers patterns were analyzed for age-related differences and are shown in *Figure 2*. Visually LMWM tend to increase with increasing age, although changes are not statistically significant. IMWM variations were found to be statistically significant (P<0.001), but values fluctuate with two intervals with increasing values, and one shift of decreasing values. HMWM tend to decrease with increasing age, and this finding is statistically significant (P=0.038).

Gender related difference in VWF multimers fractions

As shown in *Figure 3A*, there was no significant difference between males and females in VWF multimers structure: LMWM (P=0.067), IMWM (P=0.507), HMWM (P=0.060).

Geographical locations related difference in VWF multimers fractions

Table I and *Figure 3B* summarize the results of the VWF structure related parameters.

The LMWM were higher in group L2 (16.1 [14.5–19.1]) than in group L1 (15 [12.7–7.2]) and group L3 (14 [12.4–16.0]). The differences between L2 vs L1 and L2 vs L3 were statistically significant (p<0.05) but clinically irrelevant, difference between L1 vs L3 was insignificant (P=0.260). There was no significant difference in IMWM and HMWM between geographical locations.

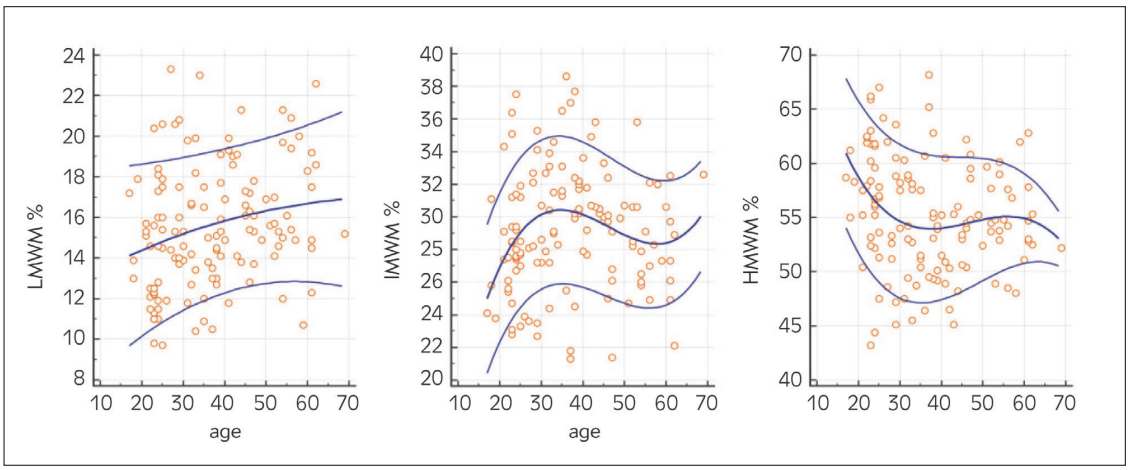

Figure 2 Age-related differences of LMWM, IMWM and HMWM in study population. LMWM, low-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers HMWM, high-molecular-weight multimers. Blue lines represent 0.1, 0.5 and 0.9 centiles.

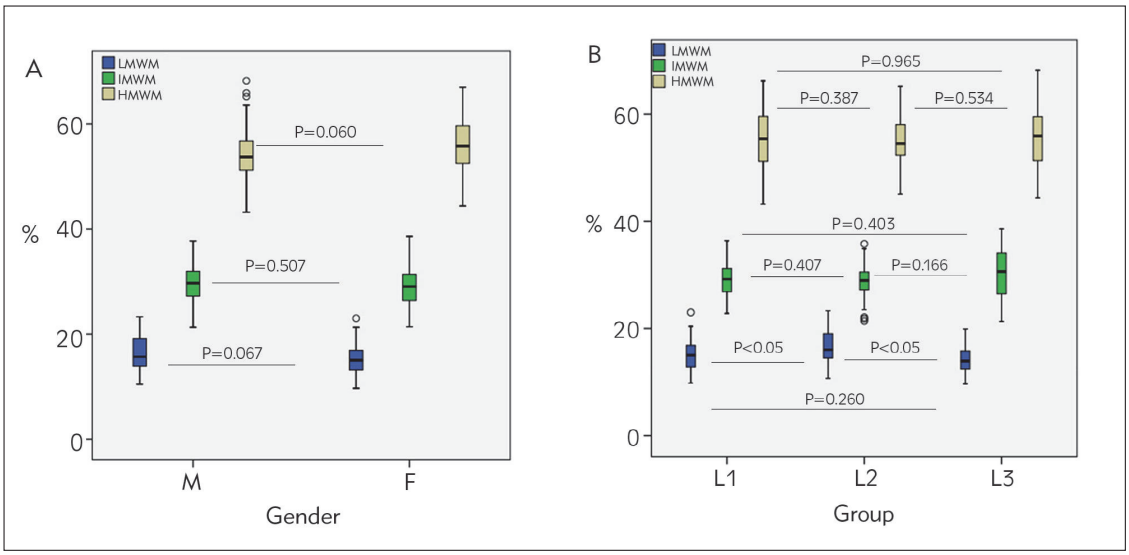

Figure 3 Differences of LMWM, IMWM and HMWM percentage values by gender (A) and between geographical locations (B). L1, samples from Baltic region; L2, samples from France; L3, samples from United States; LMWM, low-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers HMWM, high-molecular-weight multimers.

Calculation of reference intervals

Values of the three testing locations for the LMWM, IMWM and HMWM were distributed normally, thus reference values were calculated based on a normal distribution.

The proposed reference intervals for VWF:MM are presented in *Table II*.

Table II Proposed reference intervals for VWF:MM.

	Low Molecular Weight	Intermediate Molecular Weight	High Molecular Weight
Lower limit, % [90% CI]	10.4 [9.9–11.0]	22.6 [21.8–23.3]	45.6 [44.5–46.7]
Upper limit, % [90% CI]	22.5 [21.5–23.5]	37.6 [36.4–38.7]	66.6 [65.1–68.0]

Discussion

VWF multimeric analysis is essential for diagnosis and subtyping of VWD and acquired von Willebrand syndrome (AVWS) (5, 9, 17–19). There is still a need for interlaboratory standardization of this method. Indeed, interlaboratory comparability and reproducibility of this analysis are insufficient due to the predominant use of locally developed VWF multimer methods by laboratories worldwide (22, 25). The new semi-automated VWF multimer technique can help in standardization (26): it helps to reduce the interlaboratory variability and the variability between different measurement runs. Densitometry could contribute to its standardization by offering a reproducible quantification and additional visualization of VWF multimer patterns and permitting a precise quantitative comparison of sample patterns with those of a reference plasma curve (20).

Several independent investigators have previously reported on the analytical performance evaluation of the new Sebia technique with either 5-gel and 11-gel formats (3, 12, 14, 15, 18, 20, 21, 23). Details of analytical performance of the Sebia method are beyond the scope of our current study. In brief, this new assay provides a clear pattern of VWF multimer distribution on the gels and densitometry scans. It demonstrates acceptable performance results and has the major advantage of being performed within one working day.

In published data for evaluation of the accuracy of the new Sebia assay researchers have used different approaches. They have compared plasma samples from patients presenting with different types of VWD with samples from healthy volunteers (21), commercial Standard Human Plasma (23), donors and commercial frozen normal donor plasmas (14). Reference intervals were not originally defined by the manufacturer. Due to lack of reference values for VWF:MM fractions, results interpretation can be challenging in some cases. HMWM have the greatest role in VWF functional activity (13), therefore reference intervals for HMWM are most important in clinical decision making.

In 2018, Bowyer et al. (14) investigated multimeric patterns in 51 samples collected from healthy volunteers and using commercial frozen normal donor plasma (Cryocheck; Precision Biologic, Halifax, NS, Canada). In this study ranges for HMWM varied 35–58.5%, but authors noted that Gaussian distribution was not observed for HMWM. Importantly, the storage condition for the commercial Cryocheck Normal Donor Set is at -40 to -80 °C. Storage and transport issues that allowed plasmas to reach temperatures outside of this range potentially could have affected the establishment of HMWM lower intervals using this donor set.

A group of researchers from Belgium (21) has calculated normal reference intervals for VWF multimers fractions using samples from 40 healthy volunteers. They have reported intervals for HMWM as 40.8–63.2%.

The intervals determined in these previous studies were similar to our results, but they were calculated using a relatively low powered sample size. According to the CLSI guidelines C28–A3 (24), the sample size can be considered to be representative when it is larger than 120, therefore in the current study we established the reference intervals of LMWM, IMWM and HMWM fractions in 131 relatively healthy adults, in order to obtain a more acccurate result.

An interesting finding was the relationship of certain multimer fractions with the age of study individuals. The tendency of LMWM to increase and HMWM to decrease with increased age is seen in our data. Meanwhile, IMWM values are variable during adult life. Nevertheless, definitive conclusions cannot be made due to the small sample size of the study. Discovered tendencies, especially the tendency of HMWM to decrease with increasing age, could potentially be analyzed in detail in future larger studies.

It should be noted that multimer fraction separation and their percentage values calculation is based on the scanned gel and are not directly measured quantitatively, thus an interpretation of »gray zone« should be considered in future studies evaluating clinical decision making possibilities.

To conclude, the quantification of VWF:MM fractions is an additional valuable tool to supplement the qualitative visual assessment of VWF:MM patterns. It potentially has the value to aid in differential diagnosis of VWD and AVWS subtypes. The reference values calculated in this study can be used in future research to establish clinical decision limits.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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