Performance evaluation and multicentre study of a von Willebrand factor activity assay based on GPIb binding in the absence of ristocetin

Juergen Patzke[a], Ulrich Budde[b], Andreas Huber[c], Adriana Méndez[c], Heidrun Muth[a], Tobias Obser[d], Ellinor Peerschke[e], Matthias Wilkens[a] and Reinhard Schneppenheim[d]

The functional activity of von Willebrand factor (VWF) is most frequently measured by using the ristocetin cofactor assay (VWF:RCo). However, the method's drawbacks include unsatisfactory precision, sensitivity and availability of automated system applications. We have developed an alternative assay (INNOVANCE VWF Ac*) that is based on the binding of VWF to recombinant glycoprotein Ib (GPIb). Two gain-of-function mutations were introduced into a GPIb fragment, allowing an assay format without ristocetin. Fully automated assay applications are available for the BCS/BCS XP systems and the Sysmex CS-2000i*, Sysmex CA-7000, Sysmex CA-1500 and Sysmex CA-560 systems. The INNOVANCE VWF Ac* assay measuring range extends from 4 to 600% VWF for all systems except the Sysmex CA-560 system. Within-device precision values were found to be between 2 and 7%. The limit of detection was below 2.2% VWF. In a study on the BCS XP system, a total number of 580 sample results yielded a correlation to the VWF:RCo assay of *r* equal to 0.99 (slope = 0.96). Very similar results were observed when von Willebrand disease samples type 1, 2A, 2B, 2M, 2N and 3 were investigated with the new assay and the VWF:RCo assay. The excellent performance data and comparability to VWF:RCo, together with the ease of use, led us to the conclusion that the ristocetin cofactor assay can be replaced by the new GPIb-binding assay to reliably diagnosing patients with von Willebrand disease.

***Not available for sale in the U.S. Product availability varies by country. *Blood Coagul Fibrinolysis* 25:860–870 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Blood Coagulation and Fibrinolysis 2014, **25**:860–870

Keywords: activity, GPIb, von Willebrand disease, von Willebrand factor, von Willebrand factor:RCo, VWF:GPIbM

[a]Siemens Healthcare Diagnostics Products GmbH, Assay Development, Marburg, [b]Hemostaseology, Medilys Laborgesellschaft mbH, Hamburg, Germany, [c]Kantonsspital Aarau, Zentrum für Labormedizin, Aarau, Switzerland, [d]Department of Paediatric Haematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany and [e]Center for Clinical Laboratories, The Mount Sinai Hospital and Medical School, New York, USA

Correspondence to Dr Juergen Patzke, Siemens Healthcare Diagnostics Products GmbH, Emil-von-Behring-Str. 76, D-35041 Marburg, Germany
Tel: +49 6421 394162; fax: +49 6421 393018;
e-mail: juergen.patzke@siemens.com

Received 30 April 2014 **Accepted** 26 May 2014

Introduction

Von Willebrand factor (VWF) is a very large multimeric protein with a range of multimers from 500 kDa to more than 20 000 kDa and a length of up to 2 μm [1]. It binds in the presence of shear stress to collagen surfaces and to platelets, thereby supporting the formation of a hemostatic plug. If VWF function is disturbed, patients suffer from an increased risk of bleeding. The most frequent disorder is the hereditary Von Willebrand disease (VWD), observed in approximately 1% of the general population. At least 1% of these patients have clinically significant bleeding problems. Acquired VWF deficiencies are also known, but they are less frequent.

A number of laboratory tests are required for the diagnosis of VWD and its different types 1, 2A, 2B, 2M, 2N and 3 [2]. The most commonly used assays are VWF ristocetin cofactor assay (VWF:RCo), VWF antigen assay (VWF:Ag), VWF collagen binding assay (VWF:CB), multimer analysis of VWF, ristocetin-induced platelet aggregation assay (RIPA) and the VWF factor VIII binding assay (VWF:FVIIIB). A so-called 'activity' assay that is based on particle agglutination is also frequently used. However, this measures the binding of an antibody to a functional epitope of VWF [3,4]. The VWF:Ag assay measures the quantitative amount of the VWF protein, but functional defects and abnormalities in molecular size distribution are not detected. The VWF:RCo assay is still the assay of choice for the detection and quantification of functional defects as in VWD type 2A, 2B and 2M. The assay was developed in the 1970s [5,6]. It uses stabilized platelets and the peptide ristocetin from Amycolatopsis lurida. VWF binds to the GPIb receptor of the stabilized platelets if ristocetin is present. An agglutination of platelets is induced and measured as a decrease in turbidity.

The VWF:RCo assay has several limitations. Its precision is unsatisfactory with frequently observed coefficients of variation of up to 20% [2] or higher. The lower limit of the measuring range is typically 10–20%, which is not low enough for phenotyping of severe forms of VWD. Furthermore, a VWF amino acid polymorphism (p.D1472H) was recently shown to affect the results of the VWF:RCo assay

0957-5235 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/MBC.0000000000000169

probably due to decreased binding of ristocetin to the VWF A1 domain, and was identified as the probable cause for spuriously lower VWF activity/antigen ratios dependent on the presence of the H-allele [7]. Another disadvantage is the limited availability of VWF:RCo assay applications on automated systems.

There are several approaches for alternative activity assay designs that have been published or presented as posters and or even commercialised [7–15]. Our approach for an improved activity assay (VWF:GPIbM) was to use a recombinant GPIb alpha (GPIbα) fragment with gain-of-function mutations. The mutated GPIb binds to VWF without the support of ristocetin. A microparticle agglutination type of assay was developed to make the assay widely available on automated coagulation systems.

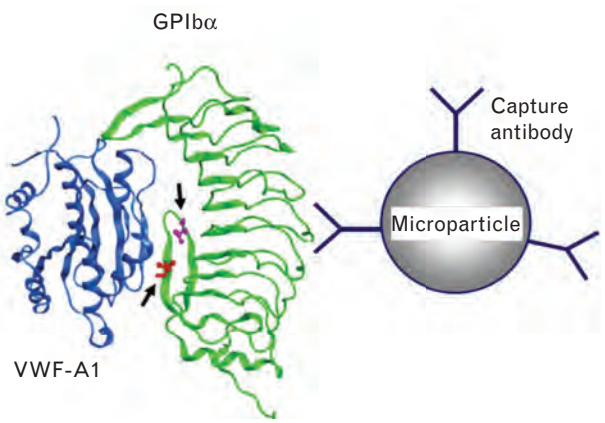
Materials and methods

GPIbα fragment

A recombinant GPIbα fragment was constructed. It is expressed in human embryonal kidney (HEK) cells and secreted by the cells in the culture medium. Purification is performed with affinity chromatography.

The gain-of-function mutations were introduced into the GPIbα fragment (Fig. 1). Both mutations are known from the platelet-type or Pseudo VWD that is characterized by spontaneous binding of VWF to platelets with the mutant GPIb [16]. Both mutations are located in the β-hairpin loop, which plays a functional role in the binding of GPIb to the A1 domain of VWF. Although the wild type receptor requires ristocetin to bind VWF *in vitro*, the mutated receptor shows maximum binding even in the complete absence of ristocetin (Fig. 2).

Fig. 1

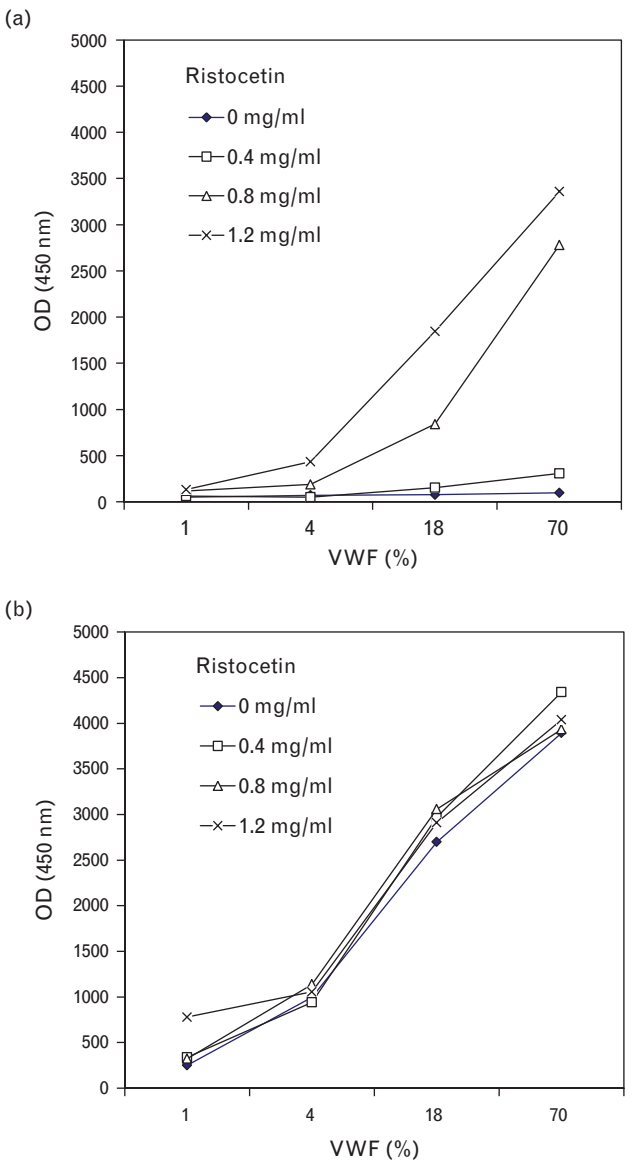


Assay principle of VWF:GPIbM. A monoclonal anti-GPIb antibody is coupled to the surface of polystyrene microparticles. The antibody captures the recombinant GPIbα fragment that has two gain-of-function mutations included. When mixed with a sample containing active VWF, the microparticle-GPIbα complex binds spontaneously to the A1 domain of VWF. No ristocetin or shear stress is needed to enhance the binding reaction. Due to the high number of A1 binding sites of the VWF molecule as well as the numerous antibody binding sites on the particle, microparticle agglutination can take place and is measured turbidimetrically. Reproduced by courtesy of C. Baldauf.

Materials

The following kits and reagents were obtained from Siemens (Marburg, Germany): INNOVANCE VWF Ac test kit (VWF:GPIbM), vWF Ag test kit (VWF:Ag), BC von Willebrand Reagent (VWF:RCo), Standard Human Plasma, Control Plasma N, Control Plasma P, Owrens Veronal Buffer (OVB).

Fig. 2



Ristocetin dependence of the wild type and mutated GPIbα fragment. An ELISA format of the VWF:GPIb assay is used to investigate the dependence of the binding reaction on the ristocetin concentration. (a) Wildtype recombinant GPIb fragment (b) Mutant recombinant GPIb fragment.

The coagulation systems BCS/BCS XP, Sysmex CA-1500, Sysmex CA-560, Sysmex CA-7000 and Sysmex CS-2000i/2100i were provided by Siemens (Marburg, Germany). Assay applications are available for Sysmex CA-500 series models with 575 nm detection capability only.

VWF:GPIbM assay

The VWF:GPIbM assay (INNNOVANCE VWF Ac) kit is composed of three different liquid reagents. Reagent I contains polystyrene microparticles. The surface is covered with a monoclonal mouse antibody against the GPIbα fragment. The ingredients of Reagent II are a heterophilic blocking reagent, detergent and polyvinylpyrrolidone. Reagent III contains the recombinant GPIbα fragment.

On the BCS XP system, 13 μl of Reagent III, containing the GPIbα fragment, is mixed on board the system with 70 μl of Reagent II, 30 μl Owrens Veronal Buffer and 15 μl of the patient's plasma sample. VWF of the sample binds to the gain-of-function GPIbα fragment without any support of shear stress, ristocetin or any other agent. After 120 s of incubation, Reagent I (40 μl), containing the microparticles, is added as the starting reagent. The binding of the microparticles to the VWF/GPIbα complex is measured as an increase in turbidity, which is caused by particle agglutination. The measuring time is 55 s. Calibration of the assay is performed by the system by diluting Standard Human Plasma with OVB. An extended measuring range is achieved by increasing the sample volume or by prediluting the sample.

Samples

Citrated human plasma was used for all studies. Refer to the respective study description below for more details.

Dilution linearity

The procedure and evaluation was performed for all system applications according to the CLSI guideline EP06-A [17]. One plasma sample with 700–800% VWF (upper limit of the measuring range: 600% of norm) was diluted with OVB to obtain at least 10 levels of VWF activity. The measured values were plotted against the theoretical values and the deviation of the plotted curve from the ideal straight line was calculated for each measured VWF level. This procedure was repeated by using a sample with 150–190% VWF.

Limit of blank/Limit of detection

Five samples containing no VWF (buffer, VWF-deficient plasma) as well as five samples containing very low levels of VWF were measured on two systems of each system type on 3 days in double determination. The CLSI guideline EP17-A [18] was followed to calculate the limit of detection and limit of blank.

Measuring range/assay protocols

One assay protocol was established for screening purposes. It covers 25–125% VWF on the Sysmex CA-560 system and 15–150% VWF on all other systems. If VWF activity exceeds these limits, the range is extended by automated sample predilution on the Sysmex CA-560 system and all other systems up to 480 and 600% of norm, respectively. If the VWF activity underruns the lower limit, a dedicated and calibrated second, low-range assay protocol is performed automatically. The low-range assay protocol of the Sysmex CA-560 system is not calibrated; it uses the calibration curve of the screening assay protocol. The lower end of the low measuring range is 6% of norm on the Sysmex CA-560 system and 4% norm on all other systems.

The calibrator for the VWF:GPIbM assay is Standard Human Plasma, which is calibrated against the VWF:RCo value of the International Standard for Blood Coagulation Factor VIII and VWF in Plasma. The results are reported as % of norm (100% of norm = 1 IU/ml).

Precision

The precision of the VWF:GPIbM assay was measured on 20 days using four sample aliquots per day by following the recommendations in the CLSI guideline EP5-A2 [19]. The samples used were commercial controls (see section Materials) or plasma pools, partially diluted with VWF-deficient plasma (in-house preparation) or spiked with Haemate obtained from CSL Behring (Marburg, Germany).

Interference

The possible interference of haemoglobin and bilirubin was investigated by spiking a control plasma with the pure substance. Bilirubin was obtained from Sigma-Aldrich (Hamburg, Germany). Haemoglobin was isolated from fresh red blood cells.

Triglyceride interference was investigated either by spiking plasma with triglycerides (Lipovenoes obtained from Fresenius, Bad Homburg, Germany) or by using native, lipemic samples. For these samples, interference was identified as the difference of results of uncentrifuged and centrifuged samples (18 000*g*, 15 min).

Interference of rheumatoid factors and human antimouse antibodies (HAMAs) was investigated by measuring samples with increased rheumatoid factor and HAMA values untreated and after a treatment with a heterophilic blocking tube (HBT; Scantibodies, Santee, California, USA). Samples with increased rheumatoid factor and HAMA levels were obtained from Trina (Nänikon, Switzerland), Clinisys (Atlanta, Georgia, USA), Pro-MedDx (Norton, USA), Scantibodies and Milan (Magden, Switzerland).

Control recovery/recalibration frequency

Control Plasma N and Control Plasma P were measured on 20 working days. Four vials were tested every day.

The calibration was performed on the first day and no recalibration was necessary during the measuring period.

Reference range

Citrated human plasma was obtained from healthy donors at the blood donation centre of Siemens (Marburg, Germany) and at the study site in New York (USA). Reference ranges were determined on all systems by calculating the 2.5th–97.5th percentile of the results obtained from more than 119 donors with blood O or non O, respectively.

Method comparison

Citrated plasma samples were collected at the study sites in Hamburg, Aarau and New York. The diagnosis and classification was performed by the study sites.

The following patient groups and sample numbers were included in the method comparison study ($n = 580$): normal controls ($n = 240$) and patients with elevated VWF values as well as patients not classified ($n = 147$), VWD type 1 (n = 82), 2A ($n = 25$), 2B ($n = 21$), 2M ($n = 12$), 2N ($n = 6$), acquired von Willebrand syndrome ($n = 8$) and patients treated with DDAVP ($n = 39$). The samples were measured by using the VWF:GPIbM assay and BC von Willebrand Reagent on the BCS XP system. Most of the samples were also measured by using the VWF Ag reagent on the BCX XP system. In Hamburg, seven type 3 VWD samples were investigated.

Statistical analysis

Method comparison was evaluated using a Passing–Bablok regression analysis. Reference ranges were calculated nonparametrically according to IFCC [20]. Furthermore, a nested analysis of variance model with random effects was used to evaluate the precision performance data and to calculate the coefficients of variation in agreement with CLSI guideline EP5-A2 [19]. Statistical evaluations were performed with SAS-Software Version 9.1 (SAS Institute, Cary, North Carolina, USA).

Results

Ristocetin dependence

The VWF:GPIbM assay uses a recombinant mutated GPIbα fragment that binds to the A1 domain of VWF as well as to the anti-GPIb antibody on the surface of microparticles (Fig. 1). The subsequent agglutination of the microparticles correlates with the VWF activity of the plasma sample and is measured as an increase in turbidity.

The dependence of the assay of ristocetin was investigated in an ELISA setup (Fig. 2). When a wild-type GPIb fragment was used, ristocetin was needed to obtain a measurable binding of VWF of the sample to the GPIb fragment present on the surface of the ELISA wells. Replacement of the wild-type GPIb fragment by the mutated GPIb fragment renders the assays completely independent of ristocetin. Maximum binding is observed even in the absence of ristocetin.

Measuring range

The linearity of the method was evaluated by plotting the measured values of a series of sample dilutions against the theoretical values. No sample result of the plotted curve differed from the ideal straight line by more than 11% rel. (>30% VWF) or more than 3% abs. (≤30% VWF) within the intended measuring range.

The limit of blank and limit of detection, calculated according to the CLSI guideline EP17-A, were less than or equal to 1.1% VWF and less than or equal to 2.2% VWF, respectively, for all the system applications.

These results allowed the establishment of a measuring range from 4 to 600% of norm on the BCS/BCS XP, Sysmex CA-1500, Sysmex CA-7000 and Sysmex CS-2000i/2100i systems and from 6 to 480% of norm on the Sysmex CA-560 system.

Precision

Eighty sample aliquots were measured on 20 working days in two runs per day. The VWF levels of the samples ranged from 9 to 450% of norm. The mean within-device CV of all measurements and systems was 3.6%. No major difference was observed between the systems or between different VWF levels of the samples. Only one within-device CV result was more than 6.6%. Even at a level of 9% VWF, very low CVs between 1.9 and 6.6% were observed (Table 1).

Table 1 Within-device CV on different systems

		System					
	VWF (%)[a]	CA-1500	CA-7000	CS-2x00i	BCS XP	CA-560	Mean
Plasma pool	9.0	6.6	2.4	1.9	1.8	4.5	3.4
Control plasma P diluted	10.7	3.2	3.4	3.2	2.7	3.2	3.1
Plasma pool	17.7	5.8	10.7	3.6	3.2	3.8	5.4
Control plasma *P*	32.3	3.5	4.8	4.6	2.4	3.4	3.7
Plasma pool	33.0	4.3	2.7	5.2	1.3	2.0	3.1
Plasma pool	84.6	2.8	2.3	4.7	1.7	3.3	3.0
Control plasma *N*	86.7	1.7	2.3	4.1	2.9	1.9	2.6
Plasma pool	127	2.2	6.2	4.5	4.4	3.2	4.1
Plasma pool	448	2.6	3.9	3.7	4.9	4.0	3.8
Mean	94.3	3.6	4.3	3.9	2.8	3.3	3.6

[a] VWF (%) determined by using the CA-1500 system.

Control recovery/recalibration frequency

The recovery of Control Plasma N, Control Plasma P and Control Plasma P 1:3 diluted with OVB was investigated over a period of 4 weeks. In total, 80 vials of controls were tested. The calibration curve was measured at the beginning and no remeasurement was necessary during the experiment. All control results were within the assigned range (Fig. 3). It is expected that the calibration curve is usable for a period of at least 4 weeks.

Correlations between different reagent lots and between different systems

Figure 4 shows the excellent correlation of results obtained by using different reagent lots on the same system. The correlation of the Sysmex CA-1500 system with the BCS XP system is shown in Fig. 5 (slope = 1.02, $r = 0.99$). The correlation results from all other systems (Sysmex CS-2000i*, CA-7000 and CA-560 Systems) to the BCS XP system are very similar (data not shown). For all systems, the correlation coefficient r was between 0.98 and 1.00 and the slope was between 1.02 and 1.08.

Reference range

The reference range of a German population (Marburg) was examined on the BCS XP system for blood group non O runs from 61 to 179% and for blood group O from 46 to 146%. As expected, higher values for blood group non O than for blood group O were observed. The reference range established in New York – also on a BCS XP system – was nearly identical to the Marburg reference range (Table 2).

Interference

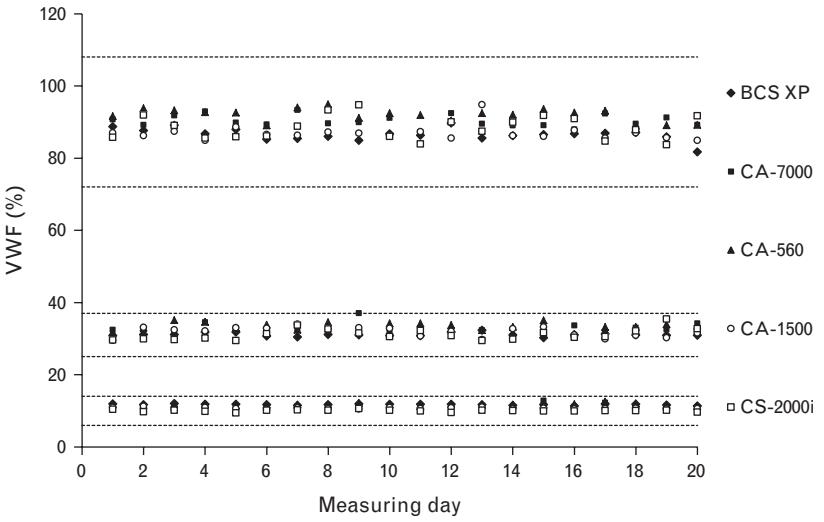
No interference of bilirubin was observed up to 60 mg/dl and of haemoglobin up to 1000 mg/dl on all systems. These concentrations were the highest levels tested. Triglyceride interference was variable on the different systems and assay protocols. On the Sysmex CA-560 system, turbid samples could not be measured with the assay protocol for the low range. Such samples should be centrifuged to remove the lipids. All other systems and assay protocols are at least free of interference up to 312 mg/dl triglycerides.

Normal samples and samples with rheumatoid factors (41–1450 IU/ml) were measured untreated and treated with a HBT. The treatment did not change the result in any of the rheumatoid factor samples (Fig. 6a). The same experiment was performed by using normal samples and samples with increased HAMA values (1–3614 ng/ml). In one out of 26 HAMA samples, the treatment considerably lowered the measured VWF level from 19 to 5% indicating the presence of interference in this sample (Fig. 6b).

Method comparison

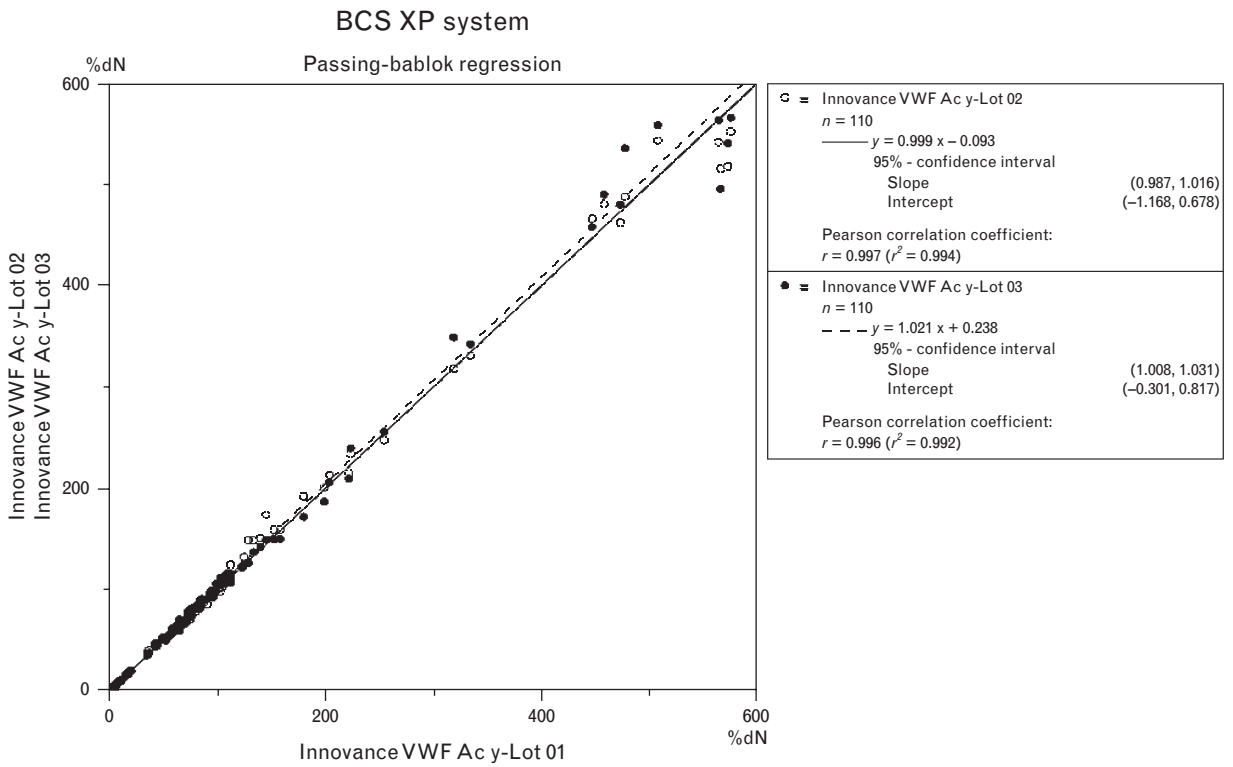
Results from normal and pathological samples from all three study sites were pooled (Fig. 7a). Both VWF:GPIbM and VWF:RCo (BC von Willebrand) were measured on the BCS XP system. A correlation coefficient of 0.99 and a slope of 0.96 were observed. In the range below 200% VWF, a slope of 0.95 and a correlation coefficient of r equal to 0.98 was calculated (Fig. 7b). The results from samples classified as VWD type 1, 2A, 2B, 2M or 2N are shown in Fig. 8 and Table 3. For all types,

Fig. 3



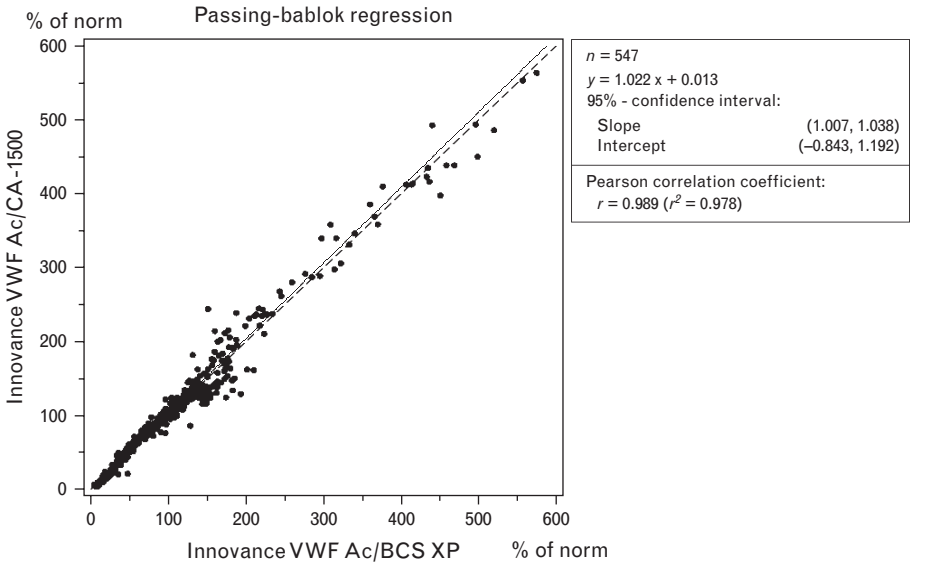
Control recovery over a 20-day measuring period. One normal control and two pathological controls were measured on 20 days within a time period of 1 month on 5 different automated systems with the VWF:GPIbM assay application. Assigned confidence ranges as defined by the manufacturer are marked by the dashed lines. Calibration of the assay was performed before starting the measurement. Recalibration was not required.

Fig. 4



Lot-to-lot variability. Sample results of the VWF:GPIbM assay on the BCS XP system by using reagent lots 2 and 3 were correlated with the results of reagent lot 1.

Fig. 5



Comparison of Sysmex CA-1500 System and BCS XP Systems. A large number of samples ($n = 547$) from three study sites were measured either on the Sysmex CA-1500 system or the BCS XP system by using the VWF:GPIbM assay.

Table 2 Reference range of von Willebrand factor by using INNOVANCE VWF Ac on the BCS XP instrument

	Blood group O		Blood group non O		All	
	Marburg	New York	Marburg	New York	Marburg	New York
n	129	119	134	122	263	241
n female	63	71	67	67	130	138
n male	66	48	67	55	133	103
Age (years)	19–64	19–72	19–64	20–68	19–64	19–72
Median (% of norm)	80.0	79.7	121.8	118.3	100.2	99.3
Mean (% of norm)	85.0	84.8	117.3	116.4	101.4	100.8
97.5 percentile (% of norm)	145.6	148.1	179.1	176.1	173.2	162.5
2.5 percentile (% of norm)	46.3	44.0	61.4	61.3	47.8	45.2

the VWF:GPIbM assay correlated very well to the ristocetin cofactor assay. Also, the activity/antigen ratio of the new assay correlates well with the VWF:RCo assay (Fig. 9). Two type 1 samples with unexpected low ratios of 0.4 were observed by using the VWF:GPIbM result and one type 2A sample with an unexpected high ratio of 1.2 was observed by using the VWF:RCo result. Type 2M 'smeary' was shown as a group separate from the classical type 2M. This subtype of 2M is known for activity/antigen ratios well above 0.7.

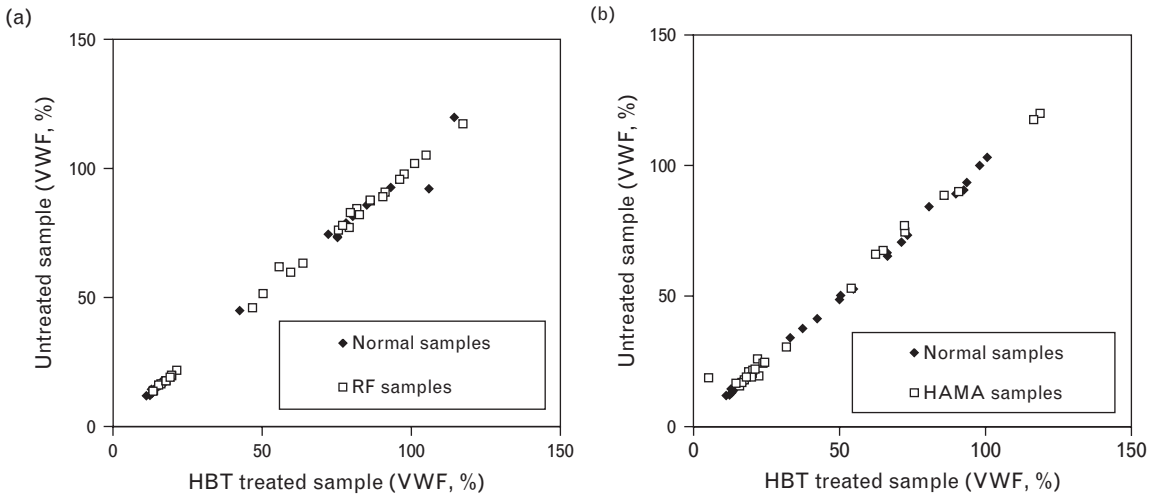
All seven type 3 VWD samples measured at the study site in Hamburg were below the lower end of the VWF:GPIbM measuring range of the BCS XP system (4% of norm).

Discussion

Ristocetin has been known for a long time to be a problematic substance because of its unspecific binding to many proteins [21]. The introduction of two gain-of-function mutations in the GPIb fragment enabled us to develop a VWF activity assay free of ristocetin. Spontaneous binding of VWF to GPIb is known from the platelet type VWD wherein one of both mutations is present. In the new assay without ristocetin, VWF binding is sufficiently fast to initiate microparticle agglutination and to allow a very short measuring time of approximately 1 min. On the basis of such a strong binding reaction, a very sensitive and precise assay could be developed. The use of simple, turbidimetric measurements allowed the application of the assay on a variety of automated coagulation systems.

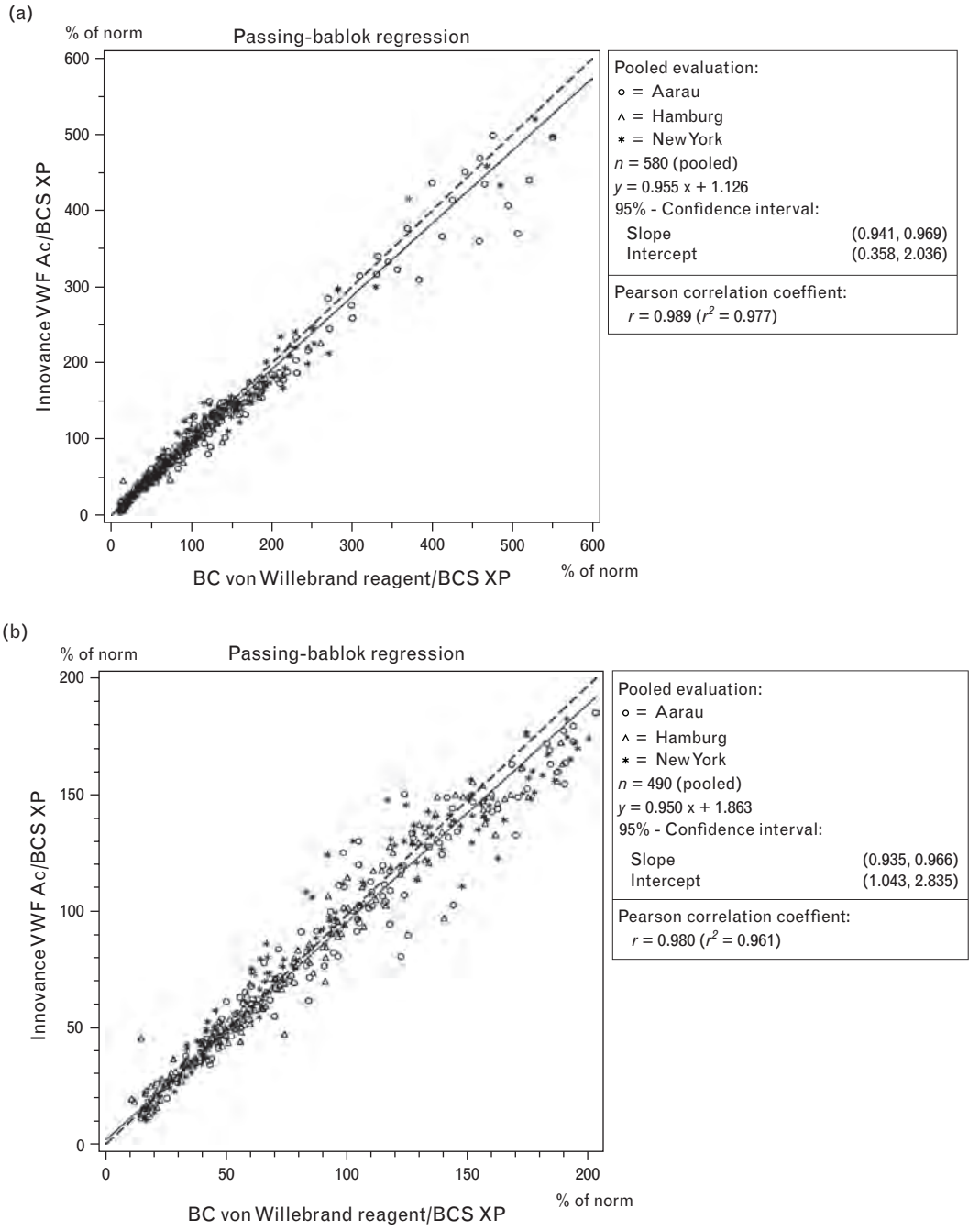
The measuring range on all systems was examined by investigating dilution linearity and limit of detection. Samples for the precision and method comparison studies were selected with VWF levels spanning the entire measuring range. Because of the excellent performance, the new assay is perfectly suited for decision-making in the range between 4 and 70% of norm. Very strong deficiencies can be detected and a reliable activity/antigen ratio can be calculated even in the very low range of VWF levels. Within-device CVs were mostly between 2 and 7%. In comparison, for another GPIb-based VWF

Fig. 6



Interference of rheumatoid factors and human antimouse antibodies. Normal samples and samples with the interferent were measured untreated and treated with a heterophilic blocking tube to remove possible interference. (a) 30 samples containing 41–1450 IU/ml (mean = 636 IU/ml) RF and 20 normal samples. (b) 26 samples containing 1–3614 ng/ml (mean = 397 ng/ml) HAMA and 26 normal samples.

Fig. 7



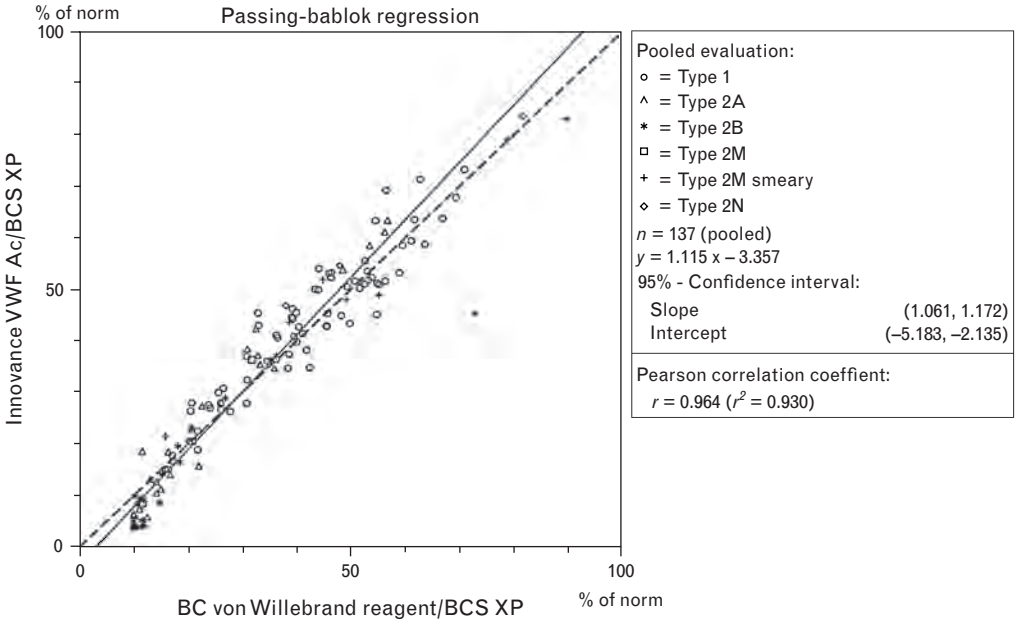
Method comparison between VWF:GPIbM (INNOVANCE VWF Ac Assay) and VWF:RCo (BC Von Willebrand Reagent). The samples originated from normal blood donors and from patients with decreased or increased VWF levels. Sample results from the study sites in Hamburg, New York and Aarau were included. Measuring range of VWF:RCo: 10–600%. (a) Complete range from 10 to 600% ($n = 580$). (b) Range from 10 to 200% ($n = 490$).

assay, reproducibility CVs between 7 and 18% were reported [15].

However, the lower limit of the measuring range of 4% of norm is still not sufficiently low for a definite diagnosis of VWD type 3 defined as having 'undetectable' VWF. Because of the extremely low incidence of samples with such low VWF levels, this should not cause a problem for most clinical laboratories. Such samples should be reinvestigated using other methods such as multimer analysis or a VWF:Ag ELISA.

The automated predilution of the samples allows the measurement of samples with very high VWF levels.

Fig. 8



Method comparison of von Willebrand disease samples between VWF:GPIbM (INNOVANCE VWF Ac Assay) and VWF:RCo (BC von Willebrand Reagent). Sample results from the study sites in Hamburg, New York and Aarau were included ($n = 137$). Four sample results with values above 100% are not shown in the diagram to allow for a better graphical differentiation in the lower range. Results of VWF:RCo/VWF:GPIbM of these samples were 95/113, 101/99, 109/113, 117/121% VWF. VWF:GPIbM results below 4% and VWF:RCo results below 10% were arbitrarily set to 4 and 10%, respectively.

Increased VWF levels are probably a risk factor for thrombosis [22] and the new assay can also be used for this purpose. The automated predilution of the sample is also useful if an interference is suspected for a specific sample.

The new assay is very robust with respect to interfering factors, reagent stability, day-to-day variation of systems or differences between different system types. The requirements of routine clinical laboratories are clearly met.

Heterophilic antibodies are very rare but they may pose a problem for assays using antibodies, especially in micoparticle-based assays. The addition of blocking antibodies to a reagent can remove most but not all of these interferences. The VWF:GPIbM assay uses mouse antibodies for capturing the GPIb fragment and not for binding the analyte. Furthermore, a heterophilic blocking reagent is added to Reagent II of the assay. These facts contribute to the high robustness of the assay with respect to RF and HAMA interference. However, one sample with an increased VWF value in comparison to the sample treated with a HBT was observed in the presence of HAMAs indicating that HAMA interference is still present in this sample. Therefore, in case of implausible results, a heterophilic antibody interference should be taken into account. We recommend performing a sample predilution for such samples.

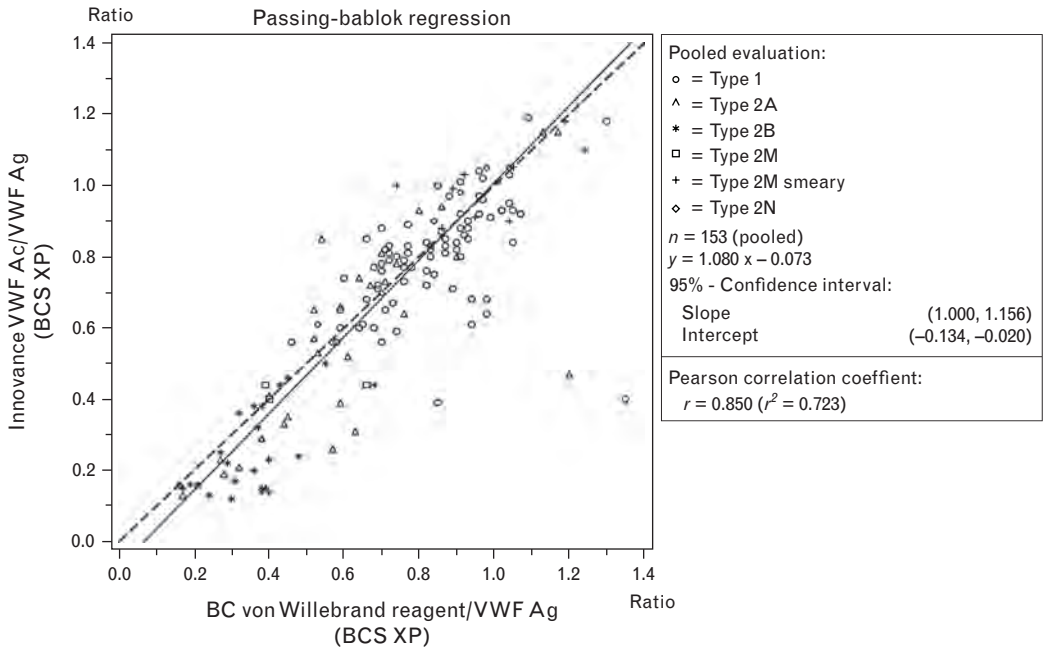
All study results indicate that the new assay is measuring the same functional VWF activity as the ristocetin cofactor assay. The correlation of results is good for normal samples as well as for VWD types 1, 2A, 2B, 2M and 2N and 3 (Fig. 8). The very similar response to functional

Table 3 Comparison of VWF:RCo and INNOVANCE VWF Ac test results

	n	VWF:Ag (% of norm)	VWFRCo (% of norm)	INNOVANCE VWF Ac (% of norm)	INNOVANCE VWF :RCo/VWF Ag VWFAc/VWF :A ratio	ratio
Type 1	79	51 (12–84)	39 (10–71)	38 (4–72)	0.84 (0.46–1.35)	0.80 (0.39–1.19)
Type 2A	32	44 (9–119)	25 (10–95)	25 (4–111)	0.60 (0.16–1.20)	0.56 (0.13–1.15)
Type 2B	24	52 (28–107)	18 (10–73)	14 (4–47)	0.36 (0.17–0.68)	0.26 (0.12–0.50)
Type 2M	3	50 (18–81)	21 (12–32)	21 (8–35)	0.48 (0.39–0.66)	0.43 (0.40–0.44)
Type 2M smeary	9	72 (22–201)	72 (16–172)	72 (22–176)	0.99 (0.74–1.24)	1.00 (0.88–1.18)
Type 2N	6	87 (51–139)	74 (38–117)	79 (44–125)	0.89 (0.53–1.04)	0.93 (0.61–1.05)

VWF:GPIbM results below 4% and VWF RCo results below 10% were arbitrarily set to 4 and 10%, respectively.

Fig. 9



Method comparison of activity/antigen ratios of von Willebrand disease samples. The VWF:GPIbM and VWF:RCo results of von Willebrand disease patient samples (Fig. 8) were divided by the corresponding VWF:Ag results. One type 1 patient sample had an activity/antigen ratio of approx. 1.7 with both assays and is not included in the diagram to graphically enlarge the lower range of ratios.

defects is probably due to the principle of the assay which is the same as in the VWF:RCo assay: the binding of VWF to GPIb. All patient groups showed the same results in both assays apart from the typical scattering of values as expected from the imprecision of the VWF:RCo assay. It can be speculated that the included gain-of-function mutations have a similar molecular effect on the binding process of VWF to GPIb as shear stress has.

Lawrie *et al.* [21] investigated the INNOVANCE VWF Ac assay in comparison to a VWF:RCo assay running on the Sysmex CS-2000i system and obtained very comparable results.

Another assay that also uses a gain-of-function GPIb fragment showed different results with type 2B vWD samples [15]. A mean activity/antigen-ratio of 2.63 was observed in 11 type 2B samples while the mean VWF:RCo/antigen-ratio was 0.66.

Three patients in Fig. 9 with an activity/antigen ratio of VWF:GPIbM of approx. 0.4 showed clearly higher ratios with the VWF:RCo assay axis (0.85, 1.20, 1.35). All three samples had VWF:RCo values close to 10% of norm (<10, 10.2, 13.5%), which is the lower end of the measuring range. It is therefore probable that the high imprecision of VWF:RCo at the lower end of the measuring range is the reason for too high ratios in comparison to the VWF:GPIbM assay.

Conclusion

The new ristocetin-free VWF activity assay INNOVANCE VWF Ac is very reliable, precise, sensitive and fully automated. It is based on the binding of VWF to a recombinant GPIbα fragment. Normal samples as well as samples of VWD type 1, 2A, 2B, 2M and 2N patients correlate very well to the VWF:RCo assay. According to the evaluation, the assay seems superior to the VWF:RCo assay and has the potential to become a valuable tool in the clinical laboratory.

What is known about this topic?

(1) Recently, GPIbα-binding assays were developed as an alternative to the classical ristocetin cofactor assay for the measurement of VWF activity. These assays do not need stabilized platelets, but they still need ristocetin as a cofactor.

What does this paper add?

(1) We have developed an alternative ristocetin-free assay that is based on the binding of VWF to recombinant gain-of-function glycoprotein Ib (GPIb).
(2) The excellent performance data and comparability to VWF:RCo, together with the ease of use, led us to the conclusion that the ristocetin cofactor assay can be replaced by the new GPIb-binding assay.

Acknowledgements

The authors would like to thank the following collaborators for their excellent contributions to the performance verification and clinical validation of the VWF activity assay: Beate Becker-Scheidemann, Kirsten Fischer†, Dirk Greis, Heike Herrmann, Ingo Imhof, Ruth Neunziger, Irene Jennemann-Watzka, Thorsten Keller, Markus Muth, Tina Rausch, Christel Ruckert, Stephanie Schmidt, Holger Sohl.

Conflicts of interest

J.P., H.M. and M.W. are employees of Siemens Healthcare Diagnostics Products GmbH. AH was a recipient of a travel grant from Siemens. R.S. and T.O. developed the recombinant GPIba fragment used by Siemens. U.B., A.M. and E.P. declare no conflicts of interest.

References

1 Sadler JE, Budde U, Eikenboom JC, Favaloro EJ, Hill GH, Holmberg L, *et al.* Update on the pathophysiology and classification of vonWillebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost* 2006; **4**:2103–2114.

2 Nichols W, Hultin MB, James AH, Manco-Johnson MJ, Montgomery RR, Ortel TL, *et al.* Von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Hemophilia* 2008; **14**:171–232.

3 Chen D, Tange JI, Meyers BJ, Pruthi RK, Nichols WL, Heit A. Validation of an automated latex particle–enhanced immunoturbidimetric von Willebrand factor activity assay. *J Thromb Haemost* 2011; **9**:1993–2002.

4 Ahmad S, Ptashkin B, DiGiovanni C, Cines DB, Konkle BA, Cuker A. False normal von Willebrand factor activity by monoclonal antibody-based ELISA in a patient with type 2A(IID) von Willebrand disease. *Thromb Haemost* 2011; **106**:1224–1225.

5 Howard MA, Firkin BG. Ristocetin a new tool in the investigation of platelet aggregation. *Thromb Diath Haemorrh* 1971; **26**:362–369.

6 Allain JP, Cooper HA, Wagner RH, Brinkhous KM. Platelets fixed with paraformaldehyde: a new reagent for assay of von Willebrand factor and platelet aggregating factor. *J Lab Clin Med* 1975; **85**:318–328.

7 Flood VH, Gill JC, Morateck PA, Christopherson PA, Friedman KD, Haberichter SL. Common VWF exon 28 polymorphisms in African Americans affecting the VWF activity assay by ristocetin cofactor. *Blood* 2010; **116**:280–286.

8 Vanhoorelbeke K, Cauwenberghs N, Vauterin S, Schlammadinger A, Mazurier C, Deckmyn H, *et al.* A reliable and reproducible ELISA method to measure ristocetin cofactor activity of von Willebrand factor. *Thromb Haemost* 2000; **83**:107–113.

9 Vanhoorelbeke K, Cauwenberghs N, Vandecasteele G, Vauterin S, Deckmyn H. A reliable von Willebrand factor: ristocetin cofactor enzyme-linked immunosorbent assay to differentiate between type 1 and type 2 von Willebrand disease. *Semin Thromb Haemost* 2002; **28**:161–166.

10 Federici AB, Canciani MT, Forza I, Mannucci PM, Marchese P, Ware J, Ruggeri ZM. A sensitive ristocetin co-factor activity assay with recombinant glycoprotein Ibalpha for the diagnosis of patients with low von Willebrand factor levels. *Haematologica* 2004; **89**:77–85.

11 Vanhoorelbeke K, Pareyn I, Schlammadinger A, Vauterin S, Hoylaerts MF, Arnout J, Deckmyn H. Plasma glycocalicin as a source of GPIbalpha in the von Willebrand factor ristocetin cofactor ELISA. *Thromb Haemost* 2005; **93**:165–171.

12 Piñol M, Sánchez T, Sales M, Arnout J, Van Russelt M. New automated ristocetin cofactor activity assay to distinguish type 1 and type 2 von Willebrand Disease (VWD) [Abstract No. PP-TH-635, XXII ISTH Congress]. *J Thromb Haemost* 2009; **7 (Supplement 2)**:1146.

13 Flood VH, Friedman KD, Gill F C, Morateck PA, Wren JS, Scott JP, Montgomery RR. Limitations of the ristocetin cofactor assay in measurement of von Willebrand factor function. *J Thromb Haemost* 2009; **7**:1832–1839.

14 Tous J, Barry RG, Arnout J, Vanrusselt M, Pascual Z, Serra J. New automated chemiluminescent VWF:Ag and VWF:RCo assays: preliminary analytical and clinical performance. *J Thromb Haemost* 2009; **7 (Suppl 2)**:1143; Abstract PP-TH-635 of the XXII ISTH Congress.

15 Flood VH, Gill JC, Morateck PA, Christopherson PA, Friedman KD, Haberichter SL, *et al.* Gain-of-function GPIb ELISA assay for VWF activity in the Zimmerman Program for the Molecular and Clinical Biology of VWD. *Blood* 2011; **117**:e67–e74.

16 Othman M. Platelet-type Von Willebrand disease: three decades in the life of a rare bleeding disorder. *Blood Rev* 2011; **25**:147–153.

17 CLSI/NCCLS. Evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline. CLSI/NCCLS document EP06-A. Wayne, PA: NCCLS; 2003.

18 CLSI/NCCLS. Protocols for determination of limits of detection and limits of quantitation; approved guideline. CLSI/NCCLS document EP17-A. Wayne, PA: NCCLS; 2004.

19 CLSI/NCCLS. Evaluation of precision performance of quantitative measurement methods; approved guideline—second edition. CLSI/NCCLS document EP05-A2. Wayne, PA: NCCLS; 2004.

20 International Federation of Clinical Chemistry, Scientific Committee, Clinical Section, Expert Panel on Theory of Reference Values and International Committee for Standardization in Haematology. Part 5. Statistical treatment of collected reference values. Determination of reference limits. J Clin Chem Clin Biochem 1987; 25:645–656.

21 Scott JP, Montgomery RR, Retzinger GS. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. *J Biol Chem* 1991; **266**:8149–8155.

22 Lazzari MA, Sanchez-Luceros A, Woods AI, Alberto MF, Meschengieser SS. Von Willebrand factor (VWF) as a risk factor for bleeding and thrombosis. *Hematology* 2012; **17 (Suppl 1)**:S150–S152.