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Comparison of von Willebrand factor platelet-binding activity assays: ELISA overreads type 2B with loss of HMW multimers

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Essentials:

- Several new assays have recently become available with different novel measuring principles to ascertain von Willebrand factor (VWF) activity.
- The international COMPASS-VWF study was set up to compare these assays in a blinded fashion for normal controls as well as for well-characterized von Willebrand disease (VWD)

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patients. Good overall correlation was reported previously. In this study, we dissected assay behaviors further to see if there are differences for any particular VWD subtypes.

- All assays performed equally for normal controls, but discrepant results were found for some VWF mutations including higher activity in type 2B patients by all ELISAs; and for the mutation p.V1665E by the VWF:Ab assay.
- This study proves that various measuring principles may reflect VWF activity differently, and that there is an urgent need for a more extensive study to understand such assay-specific differences for all VWD subtypes.

Abstract.

Background: A number of new assays with different measuring principles are available to measure VWF GPIb-binding activity, but little is known about how these assays might behave differently for subtypes of VWD.

Objectives: The **COMPASS-VWF** study was designed to compare all available VWF GPIb-binding activity assays for von Willebrand factor. We specifically searched for particular assay behavior differences.

Patients/Methods: To sort out random differences from systematic assay behavior deviations, all assays were performed in different laboratories on the same samples in a blinded fashion. Samples from 53 normal controls and 42 well-characterized VWD patients were re-analysed in this study to dissect assay-specific discrepancies.

Results: No assay behavior differences were found for 53 normal controls. For VWD patients, we found the following systematic assay behavior patterns: (i) All ELISA assays for VWF:GPIbR as well as VWF:GPIbM are insensitive to detect the low VWF activity of VWD type 2B patients with loss of HMW multimers. (ii) VWF:Ab assay reports higher activity for the p.V1665E mutation than all other assays, and (iii) all ristocetin-based assays (including VWF:RCo using fixed platelets) but the AcuStar assay report discrepantly low VWF activity for the p.P1467S polymorphism. No systematic assay-specific difference was observed for either the particle agglutination VWF:GPIbM assay, or the AcuStar assay using magnetic beads.

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Conclusions: Different assay principles may lead to discrepant results for certain VWD types or mutations. Therefore, a more extensive study for a large number of patients is needed to better characterise the incidence and relevance of such assay specific differences.

KEY WORDS:

von Willebrand Factor, Clinical Laboratory Techniques, Enzyme-linked Immunosorbent Assay, von Willebrand Disease, Blood Coagulation Disorders

Introduction. Shortcomings of the ristocetin cofactor activity assay (VWF:RCo), the former gold standard to measure VWF to platelet GPIb-binding, led to the development of several new assays with improved performance characteristics. Recent publications reported an overall good correlation between the new assays, and VWF:RCo. All of the new assays, including VWF:GPIbR by the HemosIL latex agglutination assay [1-3], and the HemosIL AcuStar assay [1, 4-7]; the VWF:GPIbM Siemens assay [4, 8-13], and the VWF:Ab assay [14-18] performed well, and yielded results close to the (g)old standard VWF:RCo. **Information about the new assays has recently been reviewed [19-23]**. However, detailed information is still limited about how these assays compared to VWF:RCo and to each other. In particular, it remains unknown, whether the new assays, with different measuring principles, behave differently for certain subtypes of VWD. Knowledge about such assay dichotomy would obviously be very important when interpreting assay results for individual patients or certain groups of patients. Therefore, the von Willebrand factor Subcommittee of the International Society for Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SSC) designed a collaborative study, designated COMPASS-VWF, involving expert laboratories from several countries to compare all available tests with each other and with the VWF:RCo assay. **Overall comparisons of this study have recently been reported**[24] and, like previous reports, found good overall correlation between all assays with clinically insignificant minor differences[24]. In this paper we re-analysed data from the COMPASS-VWF study, and dissected assay results for individual patient samples to look for systematic differences between the VWF activity assay types, which are labeled using the nomenclature recently recommended by the VWF subcommittee of the ISTH SSC [25].

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Methods: An independent international multicenter study was organized by the ISTH SSC VWF Subcommittee (Comparison of Assays to Measure VWF Activity, **COMPASS-VWF**)[24]. Eight laboratories participated (Germany: 3 labs, Italy: 2 labs, Hungary, UK and USA 1 lab each). Each of the participating centers was assigned a laboratory identification (Lab 1 through Lab 8), and provided with 95 frozen and blinded, double-spun platelet-poor plasma samples from 95 individuals (dispatched on dry ice). Each Laboratory performed assays available for them for routine use (summarized in **Table 1**). The blinded samples consisted of 53 samples from normal healthy individuals, and 42 samples from 42 individual VWD patients. The latter group was comprised of: 11 type 1, 21 type 2, 6 type 3 patients as well as 4 recombinant VWF proteins (diluted in type 3 VWD patient's plasma: WT; P1467S; D1472H; and a "heterozygote" D1472H, i.e. a 1:1 mixture of WT and mutant VWF). Only samples from thoroughly characterized patients were included, and availability of the molecular defect resulting in the particular VWD type was required for all patients. All measurements were performed in duplicates. The limited amount of the samples did not allow for repeat testing.

The frozen samples sent to Lab 7 and Lab 8 were inadvertently thawed during transportation, and were excluded from the previous study[24]. However, since Lab 8 performed the measurements anyway, we examined the possible effect of thawing on VWF:Ag and activity assays run by Lab 8: VWF:GPIbR and VWF:GPIbM – both by house-made ELISA (data not shown). Although the incidental thawing did not cause a systematic shift in VWF activity (data not shown), we did not include data from thawed samples in any of the analyses, except to illustrate the striking difference for VWD type 2B patients with ELISA methods (see **Table 2**). This observation led us to test the phenomenon in 15 more VWD type 2B patients, not initially included, in a single experiment in Lab5 for VWF activities by three commercial assays as well as two home-made ELISA assays for VWF:GPIbR and VWF:GPIbM (see **Table 4**).

Participating labs measured VWF:RCo and VWF:Ag as well as one or more of the following commercially available new VWF activity assays: VWF:GPIbR (IL HemosIL® Von Willebrand Factor Ristocetin Cofactor Activity [Instrumentation Laboratory, Bedford, USA]- Labs 1,2,3, and 5; IL HemosIL® AcuStar Von Willebrand Factor Ristocetin Cofactor Activity - Labs 1,2,3, and 5);

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VWF:GPIbM (INNOVANCE® VWF Ac [Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany] - Labs 1 through 4); and VWF:Ab (IL, HemosIL® von Willebrand Factor Activity – Labs 1,2 and 5). In addition, two laboratories performed in-house ELISA measurements (VWF:GPIbR – Lab 8,[26] and VWF:GPIbM – Labs 6 and 8 [27]). Lab 5 used in-house ELISAs to measure the VWF:GPIbR[28] and VWF:GPIbM[29] activity in VWD type 2B patients (**Table 4**).

Calibrators and instrumentation for each assay was as follows: *VWF:RCo*: BC Von Willebrand Reagent, Siemens (Labs 1 through 5), and on the Siemens BCS XP or Sysmex® CS-5100 (Sysmex, Kobe, Japan) platform (Labs 1, 2 and 4). Lab 2 and 3 used laboratory developed test applications on Siemens BCS XP and Sysmex CS-2000i (Sysmex, Kobe, Japan) platforms, respectively, with in-house validated lower limits of detection of 5% and 4%, respectively. Otherwise, values below 10% VWF are outside the measuring range of the VWF:RCo assay for any Siemens platforms as defined by the provider. *VWF:GPIbM: INNOVANCE VWF Ac* assay, Siemens (Labs 1-4) on the Siemens BCS XP or Sysmex® CS-2000i (Sysmex, Kobe, Japan) platform using Siemens Standard Human Plasma for calibrating both assays. Lab 5 used the ACL 9000, Instrumentation Laboratory platform for determining *VWF:RCo* [30] using a pooled normal plasma, which had previously been calibrated against the World Health Organization Fifth Standard for FVIII/VWF assays. *VWF:Ag*: HemosIL von Willebrand factor antigen on the ACL TOP500, Instrumentation Laboratory (Labs 3, 5), or the ACL AcuStar platform, Instrumentation Laboratory (Labs 3, 5) or the Siemens vWF Ag on Siemens BCS® XP (Lab 1, 3, 4) ; *VWF:GPIbR*, by the *HemosIL latex agglutination* assay was measured on the ACL TOP500 platform, Instrumentation Laboratory, (Labs 1,2,3 and 5); and *VWF:Ab* by the *HemosIL VWF activity* assay was measured on the ACL TOP500 platform, Instrumentation Laboratory (Labs 1, 2 and 5), using the IL Calibration plasma for calibration for all IL assays listed above; *VWF:GPIbR* by the *HemosIL AcuStar* assay (Labs 1,2,3 and 5) was determined on the ACL AcuStar platform using the calibrators included in each kit.

Data management: Results were submitted to the organizing centre, sample blinding was decoded and entered into the database.

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(i) Outliers were removed for all analyses, as follows: for each plasma sample, measurements from all activity assays across all labs were pooled, and the outliers were identified by Grubbs' test with $p < 0.01$ for each plasma sample. In this way a total of 14 measurements (0.7% of data points) were removed[24]. *(ii)* Screening for systematic differences between assays was performed using the following strategy: For each assay result for each patient sample, weighted percent distance between assays was screened for using the formula $100*(Mean^{assay}–Mean^{others})/Mean^{others}$, where $Mean^{assay}$ is the average of results performed in different laboratories using the VWF activity assay in question, and $Mean^{others}$ is the average of all VWF activities performed with all assays (the thawed samples from Lab8 were not included in this analysis) other than the assay in question (**Figure 1**). The *weighted percentage distance* formula was designed to show the weighted (percentage) difference between the average of any particular sample's VWF activity by a particular assay from the average of VWF activity of the same sample as measured by all other assays. As can be seen from the formula, percentage distance is zero % for values that are equal to the mean of the other assays, and -100% for values that are 0 IU/dl, regardless of the actual value of the other assays. Incidentally thawed samples were not included in this analysis to avoid any uncertainty about the differences. Cutoffs were placed arbitrarily to 120% for values that were higher than the mean of the other assays, and to -70% for values that were lower. These cutoffs identified 11 VWF activities of which 3 turned out to be false positive (either non-systematically different or different in a clinically meaningless manner). Bringing the cutoffs closer to zero only increased the number of false-positive samples without identifying any more true positive samples (data not shown). *(iii) regression* analysis was performed to compare VWF antigen and activity results of samples that were inadvertently thawed to all other samples. This comparison was done on healthy donor samples only to decrease the number of variables on the comparison. No systematic effect of thawing was detected (**data not shown**).

Analysis: The analysis was conducted inMicrosoft Excell (weighted percent distance) and in R package 'mcr' [31].

Results:

I. Screening for VWF activity assays with systematically different behavior for any particular patient sample.

Using the weighted percent distance test described under Methods, we identified eleven samples with one or more assays showing markedly different VWF activity compared to the rest of the assays (**Figure 1 and Table 2**).

Of these, three were found to be ***falsely selected*** for the following reasons:

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(i) Although GPIbR (HemosIL latex agglutination) was higher for T(1)-10, than measured with any other assay, this was due to a single high measurement in Laboratory 5 (**Table 3)**, and not to a systematic difference of assay behavior. The outlying high measurement was missed by our initial statistical screening for outliers because of the low number of measurements for each sample.

(ii) (ii) T(2)-4 had clinically meaningless slight differences between VWF activity measured by the different assays. It was selected by our weighted percent distance screening because of the combination of a zero value by GPIbM-ELISA (L6) assay and a slightly higher value by VWF:GPIbR. The T2-4 row in **Table 2** clearly shows that the slight differences do not reflect an important assay behavior difference.

(iii) (iii) Finally, T(2)-17 was only selected because of the technical feature of the percent weighted difference screen to give a value of 100% for any zero measurement. The T2-17 row in **Table 2** clearly shows now difference at all between the different VWF activity results. Therefore, the three "falsely" selected samples T(1)-10, T(2)-4 and T(2)-17 were excluded from further analysis, and we focused on the remaining 8 samples (**Figure 1** and **Table 2 & 3**). The following assay behavior patterns were identified, listed below.

True discrepancies included the following observations:

A. ***Discrepantly high VWF activity detected by all ELISA assays for type 2B VWD***. Two patients were included in the COMPASS-VWF study with type 2B VWD: T(2)-2 with the p.R1341Q heterozygous mutation and T(2)-15 with the p.R1308C heterozygous mutation. Their VWF activity was in the range of 6-9 and 7-13 IU/dl, respectively measured by the standard VWF:RCo assay as well as all commercially available new assays, including the VWF:GPIbM assay from Siemens, which uses a recombinant GPIb construct with two gain-of-function (GOF) mutations causing spontaneous binding to VWF. In sharp contrast, all three ELISA assays, two measuring VWF:GPIbM and one VWF:GPIbR activity, detected much higher levels (**Table 2**). Thus, the difference seemed to lie in the ELISA assay principle, rather than the presence or absence of the GOF mutation in the GPIb construct. Although the VWF:GPIbR ELISA (L8) and the VWF:GPIbM ELISA (L8) assays were performed on samples that had

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incidentally thawed during transportation, the phenomenon could not be attributed to thawing, for thawing did not lead to a general increase of VWF activity (**data not shown)**. Also, the VWF:GPIbM ELISA (L6) assay that used samples which were not thawed, showed discrepantly high results for the type 2B samples. To further refine and confirm these results, we tested an additional 15 VWD type 2B patients with bead-based and ELISA-based VWF activity assays (**Table 4**). These patients were not included in the original COMPASS-VWF study, but were specifically included to gain insight into the observed discrepancy. In agreement with the two 2B patients described above, nine of the 15 patients had strikingly higher values measured by either VWF:GPIbR or VWF:GPIbM ELISA than any of the bead-based activity assays. These nine patients had different 2B mutations, but all had a loss of HMW multimers. In contrast, the other six patients, who had preserved HMW multimers, had little difference between bead- or ELISA-based VWF activity. Thus, we conclude that ELISA methods give discrepantly higher values for type 2B VWF activity, and this phenomenon is only present for the typical 2B patients with lack of HMW multimers, while it is not seen with the 2B New York/Malmoe subtype, where HMW multimers are preserved.

B. ***Discrepantly high VWF:Ab results for p.V1665E***. Three unrelated type 2A VWD patients were included with this single point mutation, T(2)-7, T(2)-8 and T(2)-9; and all three had consistently higher VWF activity by the VWF:Ab assay, then measured by any other tests (**Table 2 & 3**). Therefore, the VWF:Ab assay seems to be less sensitive for the detection of the VWF activity defect caused by p.V1665E.

C. ***Falsely low VWF activity for samples with the p.P1467S by all but one (the AcuStar) assays using ristocetin***. One of the criticisms to the VWF:RCo assay has been the falsely low VWF:RCo activity for individuals with the p.P1467S polymorphism, a genetic variant affecting the VWF ristocetin binding site, but not affecting VWF hemostatic functions. Indeed, all assays NOT using ristocetin correctly measured the VWF activity of the VW-Rec-01 sample, a recombinant VWF protein with a homozygous p.P1467S mutation reconstituted in type 3 patient plasma, whereas VWF:RCo and VWF:GPIbR by both a house-made ELISA and the HemosIL latex agglutination assay detected falsely low activity levels. Surprisingly, however,

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the AcuStar assay, which also uses ristocetin-activation of plasma VWF with a chemiluminescence detection on magnetic beads, did not fall for this error. In contrast, there was no apparent discrepancy between different VWF activity levels of the recombinant sample with a homozygous p.D1472H mutation (**Table 2**, last row).

D. ***Discrepant high VWF activity by the GPIbR latex agglutination assay (HemosIL) for two patients: VWD type 2M from homozygous p.C2362F and VWD type Vicenza from p.R1205H***. The discrepant high values were clearly due to assay behavior, since all labs performing VWF:GPIbR by the HemosIL assay detected discrepant values for the two patients, patient T(2)-10 (homozygous p.C2362F) and patient T(1)-11 (heterozygous p.R1205H), (**Table 3**). We note that these discrepant values were detected in single patients, and not in their counterparts with identical mutations and multimer patterns (see **Fig 1 and Fig2**).

II. Multimer analysis of the selected samples. To further characterize the samples, we performed a multimer analysis on all samples. **Figure 2** shows the multimer pattern of all samples with a discrepant activity, along with normal plasma and patient samples with similar VWD subtype. We were especially interested in finding an anomalous multimer pattern for samples T(1)-11 T(2)-10 that showed discrepant VWF activity with the VWF:GPIbR latex agglutination assay (HemosIL) without a plausible explanation. However, multimer patterns were similar to the other Vicenza and 2M smeary patient samples, without any perceivable differences.

Discussion. The introduction of new assays to measure VWF activity revolutionized the laboratory diagnosis of VWD. Indeed, the new assays allow for simpler, more reliable measurements compared to the (g)old standard VWF:RCo. Although the new assays use different measuring principles, in general, there has been good correlation between the new assays and VWF:RCo assay[1-18]. We recently reported the initial results of the COMPASS-VWF study[24], an independent international comparison organized by the VWF Subcommittee of the ISTH SSC, which compared VWF activity

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assays performed in different expert laboratories, and only found minor differences in the overall performance of the assays. In this report, we attempted to analyze systematic assay behavior differences in our database obtained with the COMPASS-VWF study. It is not easy to sort out random assay measurement deviations from actual assay behavior differences. First, we removed what can be considered outlier measurements, in other words, errors. These can be pipetting errors, transcription errors, or even sample mixes. Using the Grubbs test to objectively, statistically define such outliers, 0.7% of all data points were removed.

To identify non-random deviations, we devised the "percentage distance" screening strategy – see Methods section. The VWF activity results identified with this strategy were then manually reviewed to verify that the differences were indeed (i) systematic, i.e. all measurements by a particular assay were different, not driven by an erratically high or low figure, and (ii) the difference was clinically meaningful. The screening threshold was arbitrarily set to 120% and -70%, because lowering these thresholds only increased the false positive samples without yielding any true systematic differences (data not shown). With this threshold, we identified eight samples with true systemic discrepancies, while three samples were false positive. Analysis of the true discrepancies resulted in the following observations:

1. **No systematic assay behavior difference was seen with normal controls**. This observation underlines the notion that the new assays basically measure VWF activity reliably[24], and any assay behavior differences reflect the relationship of different assaying principles to a few specific VWF defects.
2. **VWD 2B is read discrepantly high** by all ELISA assays. Since the description of VWF:GPIbM[27] using mutations in the GPIb construct recapitulating platelet-type VWD, where a GOF mutation brings about spontaneous (i.e. without ristocetin activation) binding between VWF and GPIb, it had been noted that type 2B patients had elevated VWF:GPIbM/VWF:Ag ratios compared to VWF:RCo/VWF:Ag[27]. Along the same lines, another study of 32 type 2B patients found that an elevated VWF:GPIbM/VWF:VWFRCo ratio could be used to identify type 2B patients.[29] While both studies used ELISA systems to measure VWF:GPIbM, they concluded that GPIbM reads higher for type 2B VWF activity,

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perhaps, it was thought, because the GPIb construct with its GOF mutation binds too avidly to the GOF mutation of type 2B VWF. The current study, however, makes it clear that this may not be the reason. What causes unexpectedly high readings is probably not the presence or absence of the GOF mutation in the assay, but the ELISA technique itself. Indeed, all three ELISA assays (two of them GPIbM with, and one GPIbR without the GOF mutation) measured significantly higher values than all the other assays. On the other hand, the automated, latex-bead-based GPIbM did not show the same phenomenon in spite of the presence of GOF mutations.

To further confirm this unexpected result, we tested 15 additional type 2B patients in a single laboratory (**Table 4**). In agreement with our initial finding, all patients with a loss of HMW multimers had strikingly higher ELISA readings for both VWF:GPIbR and VWF:GPIbM, when compared to all other bead-base assays supporting the conclusion that the ELISA methodology is responsible for the discrepant high readings (**Table 4**). Interestingly, this difference was only present for 2B patients with a loss of HMW multimers, and was not seen with the 2B New York/Malmoe subtype, where HMW multimers are preserved. We note that in an early report,[32] the VWF:GPIbR (ELISA) of 13 VWD type 2B patients was not different from VWF:RCo. The activities were compared in aggregate and multimer structure was not reported.[32] The reason for the discrepancy to our findings is unclear. The difference between latex and ELISA-based VWF activity for the VWD type 2B patients with loss of HMW multimers remains unexplained at this time, but seems to have to do with differences in the physico-chemical characteristics of the two systems.

3. **Discrepantly high results *for p.V1665E using the VWF:Ab assay.*** The VWF:Ab assay uses an antibody that binds the A1 domain of VWF in a way that is reported to recapitulate the A1 domain – GPIb binding. This can be interpreted as the REF-VIII:R/2 monoclonal Ab used in the assay providing a binding surface similar to (molecular mimicry of) the platelet GPIb binding site. However, the extent of the similarity is unknown at this time. Thus, it is possible that some structural changes in the A1 domain will affect the binding of VWF to GPIb and the REF-VIII:R/2 mAb differently. Therefore it is instructive to find such VWF defects in real life

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patients. The p.V1665E mutation is located in the A2 domain, and results in loss of high and medium-sized multimers[33]. How this particular mutation enhances the binding to the antibody is unclear, although mutations are known to cause distortions of distant tertiary structures. The p.C1272D mutation with a similar loss of multimers (**Fig. 2**) was correctly detected by the VWF:Ab assay (VWF:Ab: 3.3; mean of all others: 3.1), thus, insensitivity to multimer loss is unlikely to be the cause. VWD type 2M mutations have already been reported to be missed by the VWF:Ab assay[18]. Specifically, three patients with the p.G1324A (G561A in the old nomenclature used in the original report) mutation, which affects the A1 domain GPIb binding-site, seems to also differentially alter VWF-GPIb and VWF-REF-VIII:R/2 mAb binding[18]. We should finally note that – while significantly higher than measured by all other assays – the VWF:Ab still identified the three p.V1665E mutation patients' VWF activity as abnormal (16.3-20.1 IU-dl, **Table 2**), thus, these patients would not be missed if the VWF:Ab was used as part of a screening strategy.

4. ***Falsely low VWF activity for samples with the p.P1467S by all but one (the AcuStar) assays using ristocetin.*** One of the disadvantages of the VWF:RCo is that it falsely reports low VWF activity for individuals with the p.P1467S polymorphism [26, 34], a common VWF variant in certain populations, that results in no functional defect and is not associated with a bleeding phenotype. The reason for the false readings lies with the fact that the p.P1467S polymorphism affects the binding of ristocetin itself, and thus, interferes with the in vitro test results only, not with the physiologic VWF activation by high shear forces. Therefore, falsely low readings by assays that use ristocetin was expected, and confirmed previous reports in a blinded multi-laboratory setting. However, it came as a surprise that one of the ristocetin-triggered assays, the AcuStar VWF:GPIbR test was not affected by this phenomenon. The reason for this is unclear. It may be related to the ristocetin concentration used in the assay, but the actual concentration used in the assay is not public information, and we could not obtain specifications from IL. Furthermore, we did not see the same discrepancy with the D1472H sequence variation (as reported before[35]). We do not have a clear explanation

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for the lack of discrepancy in our study, but feel that it could be due to slight differences in post-translational modifications between the native and recombinant VWF.

5. ***Discrepant high VWF activity by the GPIbR latex agglutination assay (HemosIL) for two patients: VWD type 2M from homozygous p.C2362F and VWD type Vicenza from p.R1205H***. While clearly due to assay behavior differences, since all labs performing VWF:GPIbR by the HemosIL assay detected discrepant values for the two patients, patient T(2)-10 (homozygous p.C2362F) and patient T(1)-11 (heterozygous p.R1205H), these discrepant values were detected in single patients, and not in their counterparts with identical mutations (T(2)-11 and T(2)-17 for p.C2362F; T(1)-4, T(1)- 6 to 10 for p.R1205H; **Fig. 1**) and multimer patterns (**Fig. 2**). Thus, in these two cases, it is unlikely that the mutations themselves would explain the discrepant assay behavior. Limited sample volume precluded further testing of the problem, therefore we can only speculate that a possible explanation is some interfering substance (e.g. heterophil antibodies) for which the HemosIL VWF:GPIbR is more sensitive than the other assays. Again, this remains a speculative explanation at this point.

Taken together, our results show that in general the new VWF activity assays correlate well with each other and the VWF:RCo assay, while there are significant behavior differences for certain particular mutations, and such differences should be taken into account in interpreting results. One of the strengths of our study is the fact that only molecularly characterized patients were enrolled. In fact, several recurrent mutations were studied for both type 1 and type 2 VWD patients. While this design facilitates conclusions made for a particular defect (e.g. p.V1665E) or subtype (e.g. VWD type 2B), only a narrow spectrum of VWD mutations could be included and, therefore, generalizability to the multitude of other VWD mutations is limited. Clearly, more information is needed, before the new assays can reliably be used interchangeably for all patients.

Author Contribution:

A. Szederjesi prepared the manuscript and performed laboratory tests, and collected, decoded and analyzed data; L. Baronciani, P. Colpani and A. S. Lawrie performed laboratory tests and contributed to the manuscript; U. Budde, R. Schneppenheim and R. Montgomery participated in designing the

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study, performed laboratory tests and contributed to the manuscript; G. Castaman participated in designing the study, collected patient samples and contributed to the manuscript; Y. Liu performed most of the statistical analysis and contributed to the manuscript; F. Peyvandi participated in designing the study and contributed to the manuscript; J. Patzke performed laboratory tests, participated in designing the study and co-chaired the Study Steering Committee; I. Bodó designed the study, chaired the study Steering Committee, analyzed data and oversaw the writing of the manuscript.

Conflict of interest:

R. Schneppenheim has a patent 'Bestimmung der von Willebrand Faktor Aktivitaet in Abwesenheit von Ristocetin' with royalties paid by Siemens Diagnostics. G. Castaman reports having received honoraria for advisory board participation and for speaking from Baxalta (now a part of Shire), Bayer HealthCare, Biogen, CSL Behring, Novo Nordisk, Pfizer, Kedrion and Werfen; and has received research support directly to his Institution from CSL Behring and Pfizer. These relationships have no bearing on the current manuscript. J. Patzke has a patent EP2167978B1 issued and is an employee of Siemens Healthcare Diagnostic Products GmbH. F. Peyvandi reports personal fees from Freeline, Kedrion Biopharma, LFB, Octapharma, Ablynx, Bayer, Grifols, Novo Nordisk, Sobi and Shire, and has been a member of advisory boards for Ablynx and F. Hoffmann-La Roche, outside the submitted work. R. Montgomery reports medical advisory fees from Shire, CSL, Octapharma, Biogen and Grifols, outside the submitted work. In addition, R. Montgomery's inventions have been patented and owned by BloodCenter of Wisconsin (9678089, 9046535, 8865415, 8318444 and 8163496).

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Figure legends.

Figure 1. **Weighted percentage distance** between assays. For each assay result for each patient sample, weighted percent distance between assays was calculated using the formula $100*(Mean^{assay}–Mean^{others})/Mean^{others}$, where $Mean^{assay}$ is the average of results performed in different laboratories using the VWF activity assay in question, and $Mean^{others}$ is the average of all VWF activities performed with all assays other than the assay in question (the incidentally thawed L8 sample results were not included in the calculation). As can be seen from the formula, percentage distance is zero % for values that are equal to the mean of the other assays, and -100% for values that are 0 IU/dl, regardless of the actual value of the other assays. Cutoffs were placed arbitrarily to 120% for values that were higher than the mean of the other assays, and to -70% for values that were lower. Patient samples identified using this screen are labeled with arrows, and the corresponding mutations are shown. Red arrows with an X point to falsely identified discrepancies (see text).

Figure 2. Multimer analysis of patient samples. **Panel A**, medium resolution, **panel B**, low resolution multimer gels of all patients with the Vicenza phenotype included in the COMPASS-VWF study. The horizontal red arrow points to the patient showing ultra-high multimers. **Panel C**, medium resolution multimer gel analysis of patients with VWD type 2A and 2M smeary phenotypes. The corresponding mutations are shown below the gels. **Panel D**, medium resolution multimer gel analysis of the two VWD type 2B patients (two left lanes) and recombinant samples taken up in the plasma of a patient with VWD type3 (no VWF present). All gels contain normal plasma lanes for comparison.

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Table 1. Summary of the VWF activity assays used by each Laboratory.

VWF activity	Description of assay principle	Assays	Detection	Laboratories
VWF:RCo	Ristocetin cofactor activity: The assay uses platelets and ristocetin	**Siemens: BC von Willebrand reagent on various platforms (see Methods)**	**Agglutination of lyophilized platelets**	**L1, L2, L3, L4, L5**
VWF:GPIbR	The assays are based on the ristocetin-induced binding of VWF to a recombinant WT GPIb fragment	**IL: HemosIL®** VWF Ristocetin Cofactor Activity	**Latex bead agglutination**	**L1, L2, L3, L5**
		IL: HemosIL® AcuStar VWF Ristocetin Cofactor Activity	**Chemiluminescence detection of VWF bound to magnetic beads**	**L1, L2, L3, L5**
		In-house ELISA	**Sandwich ELISA**	**L5#, L8***
VWF:GPIbM	The assays are based on the spontaneous binding of VWF to a gain-of-function mutant GPIb fragment	**Siemens: INNOVANCE®** VWF Ac	**Latex bead agglutination**	**L1, L2, L3, L4**
		In-house ELISA	**Sandwich ELISA**	**L5#, L6, L8***
VWF:Ab	All assays that are based on the binding of a monoclonal antibody (*mAb*) to a VWF A1 domain epitope	**IL: HemosIL®** VWF Activity	**Latex bead agglutination**	**L1, L2, L5**

*Performed on samples that incidentally thawed during transportation.

#L5 performed house-made ELISA testing on the additional 15 type 2B patients, not on the initial set of samples.

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Table 2. Different VWF activity assay results of eleven patient samples selected for systematic discrepancy using the $100*(Mean^{assay}–Mean^{others})/Mean^{others}$ formula (see text). Average of measurements from all labs performing a particular assay is shown. All three ELISA assays were only performed by one lab each, and therefore, the ELISA columns represent single measurements. Values below or above the arbitrary cutoffs of 120% and -70% are labeled in red bold. Some high values resulted in other values not far from the mean of others to be technically slightly below 70% or above 120% in the weighted percentage difference analysis - these are pointed out in blue – see also **Fig 1**. To facilitate comparison, the order of columns is identical to the order of assays in **Fig. 1**. Note that the ELISAs of the last two columns are not included in **Fig 1**. The last row (R-4) is included for comparison only. In contrast to a previous report,[35] no significant assay discrepancy was detected for the recombinant D1472H VWF in our study.

Patient ID+	VWD type & mutation	VWF:RCo (IU/dl)	VWF:GPIbM INNOVANCE (IU/dl)	VWF:GPIbR AcuStar (IU/dl)	VWF:GPIbR HemosIL (IU/dl)	VWF:Ab (IU/dl)	VWF:GPIbM ELISA (L6) (IU/dl)	VWF:GPIbR ELISA (L8) (IU/dl)*	VWF:GPIbM ELISA (L8) (IU/dl)*	Mean	Non-discrepant mean#
xT1-10	1 R1205H	4.0	6.3	4.9	**18.3**	11.4	2.4	3.9	3.5	6.8	5.2
T1-11	1 R1205H	8.0	19.0	12.4	**48.2**	15.7	11.1	7.9	10.2	16.6	12.0
T2-2	2B R1341Q	8.1	8.8	8.3	8.8	6.0	**34.6**	**31.8**	**56.2**	20.3	8.0
xT2-4	2M Y1312D	4.0	1.9	1.8	**7.7**	2.0	**0.0**	4.5	< 1.6	2.7	2.5
T2-7	2A V1665E	6.2	7.6	9.1	8.0	**18.6**	4.4	13.1	6.8	9.2	7.9
T2-8	2A V1665E	4.0	5.6	9.9	12.1	**20.1**	4.7	2.2	10.5	8.1	7.0
T2-9	2A V1665E	4.3	7.3	9.2	6.0	**16.3**	5.0	14.1	5.9	8.5	7.4
T2-10	2M C2362F	4.0	7.6	5.6	**77.0**	5.6	4.5	1.6	7.0	14.1	5.1
T2-15	2B R1308C	6.8	8.4	11.5	9.6	12.7	**45.6**	**36.1**	**69.0**	25.0	9.8
xT2-17	2M C2362F	4.0	1.9	1.9	3.6	3.1	**0.0**	< 1.6	< 1.6	2.0	2.3
R-1	P1467S	**11.9**	46.7	45.3	**11.6**	50.1	48.3	**5.1**	80.4	37.4	54.2

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R-4	D1472H	32.9	32.6	34.8	18.7	40.0	36.1	56.3	38.9	36.3	N/A

[+]These patient IDs were created for easier understanding after breaking the code (once all measurements were completed and reported). Initial patient IDs were random-generated.

*Samples sent to Lab 8 were inadvertently thawed during transportation. Lab 8 performed home-made ELISAs for VWF:GPIbR and VWF:GPIbM. Since throrough testing found that thawing did not lead to a general increase of VWF activity (**Data not shown**) we included the two ELISA test results in Table 2, for it was felt that the striking discrepantly high VWF activity of the two 2B patients is unlikely to be due to the effect of thawing. These two assays were not considered in the weighted percentage analysis (**Fig. 1**). Red **x**: These three patients were erroneously selected by our screening strategy (see text and **Table 3**).

[#]This column contains means calculated with the values that did not show discrepancy. To highlight the difference, for patients that had more than one discrepant assays, all discrepancies were left out (unlike for the calculation of the percentage weighted distance).

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Table 3. Individual measurements from each lab are shown for the average values selected for systematic discrepancy using the 100*(Mean^{assay}–Mean^{others})/Mean^{others} formula (see text and **Table 2**). Columns in **Table 3** represent expansions of the cells labelled in red in **Table 2**. Each cell in **Table 2** represents the mean of a patient sample measured with the given method in several laboratories, except the last three columns which show single measurements in the laboratory indicated in the column heads. **Table 3** shows the individual laboratory values that served to calculate the mean (also shown in the last row). For most patients, there was little variation between laboratories, except for patient T1-10, first column, Lab5, labelled in red.

SAMPLE: / ASSAY:	x T1-10 GPIbR HemosIL (IU/dl)	T1-11 GPIbR HemosIL (IU/dl)	x T2-4 GPIbR HemosIL (IU/dl)	T2-7 Ab (IU/dl)	T2-8 Ab (IU/dl)	T2-9 Ab (IU/dl)	T2-10 GPIbR HemosIL (IU/dl)	R1 RCo (IU/dl)	R1 GPIbR HemosIL (IU/dl)
Lab1	10.1	50.0	12.2	17.6	18.9	15.8	84.1	11.7	13.0
Lab2	6.5	58.8	5.7	20.2	19.5	18.0	90.6	10.4	16.2
Lab3	12.4	48.8	7.0	ND	ND	ND	73.3	2.0	4.2
Lab4	ND	ND	ND	ND	ND	ND	ND	18.2	ND
Lab5	**44.0**	35.0	6.0	18	22.0	15.5	60.0	17.0	13.0
Mean	**18.3**	**48.2**	**7.7**	**18.6**	**20.1**	**16.3**	**77.0**	**11.9**	**11.6**

ND, not performed in the laboratory.

Red **x**: These two patients were erroneously selected by our screening strategy.

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Table 4. VWF activity in 15 VWD type 2B patients. Patients 1-9 have classic 2B with loss of HMW multimers, while patients 10-15 have preserved HMW multimers (e.g. 2B New York/Malmoe). Bold faced are the ELISA activities of which the average is at least 100% higher than that of all bead-based assays. All results are the mean of two or three separate measurements.

Pt#	Mutation	VWF:GPIbR IL (IU/dl)	VWF:GPIbR AcuStar (IU/dl)	VWF:GPIbM INNOVANCE (IU/dl)	VWF:GPIbR ELISA (L5) (IU/dl)	VWF:GPIbM ELISA (L5) (IU/dl)
1	p.V1316M/WT	5.10	10.35	8.15	**33.50**	**39.00**
2	p.V1316M/WT	8.00	6.25	5.40	**18.50**	**28.00**
3	p.R1341Q/WT	13.93	13.25	11.40	**26.00**	**49.50**
4	p.R1341Q/WT	23.65	*29.80*	18.65	**49.00**	**59.50**
5	p.R1306W/WT	5.95	9.80	9.50	**28.50**	**40.00**
6	p.R1306W/WT	6.97	4.80	5.85	**11.00**	**18.00**
7	*p.R1308C/WT*	18.90	*27.45*	10.05	**95.50**	**101.00**
8	*p.R1308C/WT*	14.75	11.40	11.30	**39.50**	**39.00**
9	p.H1268D/WT	21.30	23.90	17.00	**49.00**	**60.00**
10	p.R1308L/WT	21.20	16.10	23.20	20.50	37.00
11*	S1263S-P1266L/WT	63.20	52.60	66.65	65.00	61.00
12	p.S1263S-P1266L/WT	42.00	53.10	63.80	65.00	56.00
13	p.S1263S-P1266L/WT	42.90	49.80	61.50	75.00	59.00
14	p.V1245V-P1266L/WT	24.80	23.05	26.40	33.00	26.50
15	p.V1245V-P1266L/WT	67.50	60.45	70.15	44.50	69.00

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* Pt 11 also harbored three additional substitutions: p.V1229G & p.N1231T & p.V1245V.

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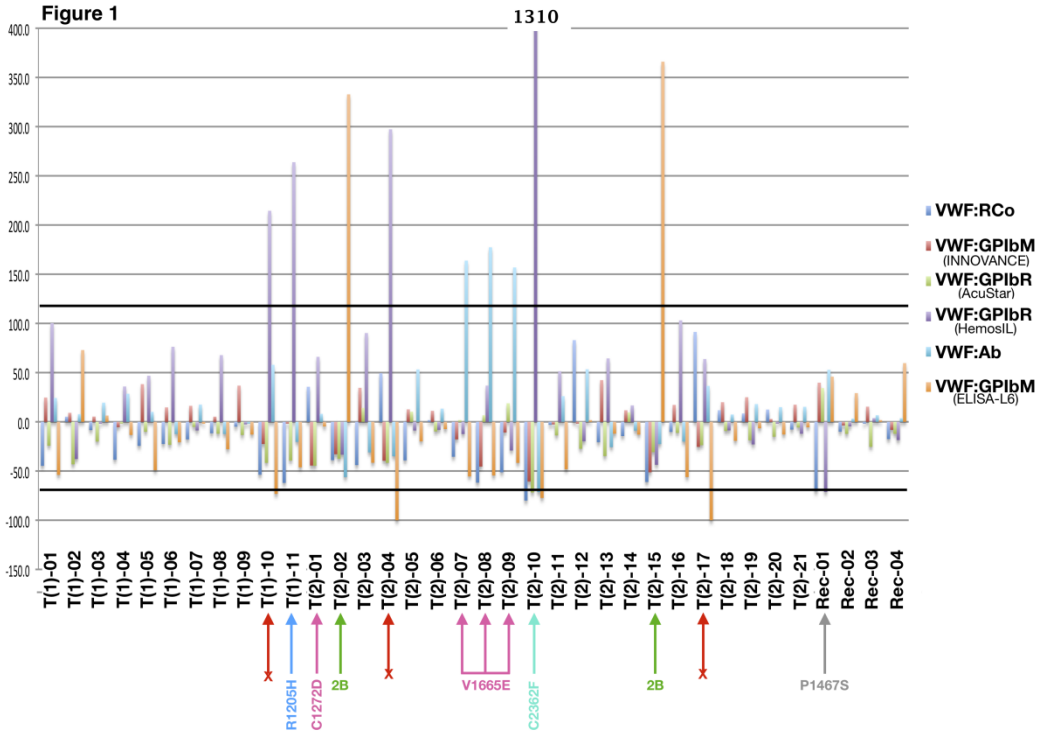

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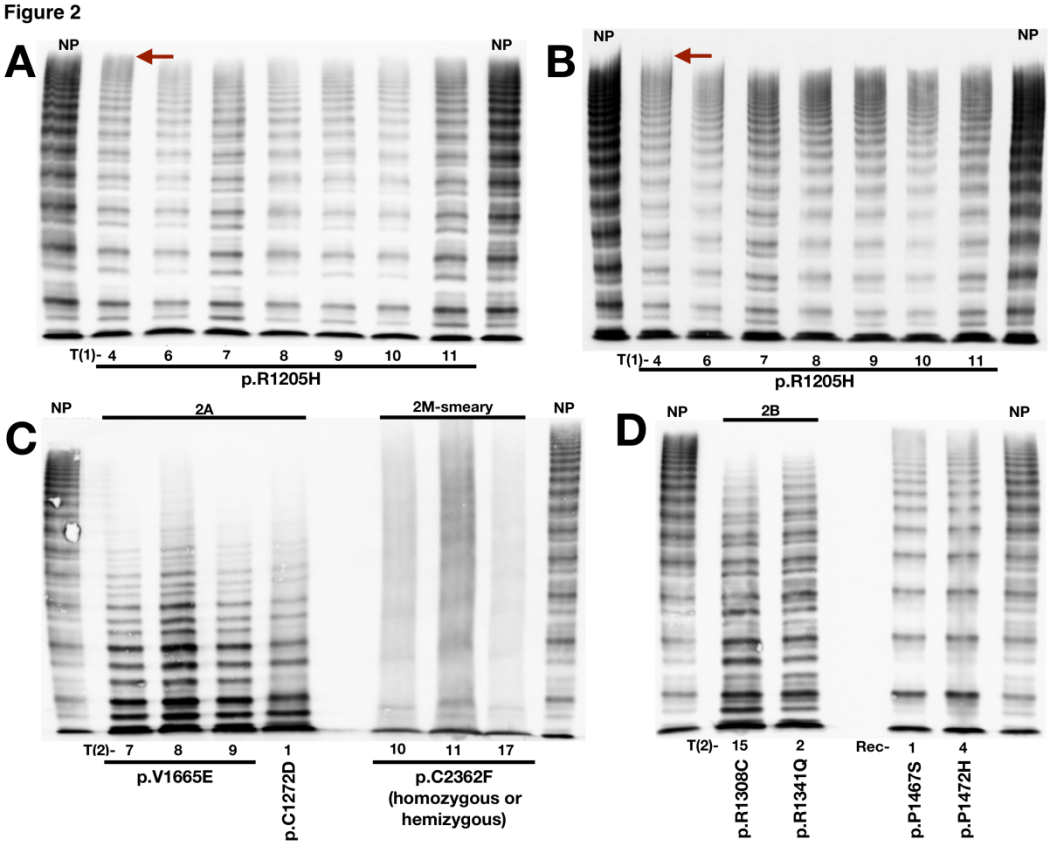
Figure 2



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