FRETS-VWF73 rather than CBA assay reflects ADAMTS13 proteolytic activity in acquired thrombotic thrombocytopenic purpura patients

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Summary
Collagen-binding activity (CBA) and FRETS-VWF73 assays are widely adopted methods for the measurement of the plasmatic activity of ADAMTS13, the von Willebrand factor (VWF) cleaving-protease. Accurately assessing the severe deficiency of ADAMTS13 is important in the management of thrombotic thrombocytopenic purpura (TTP). However, non-concordant results between the two assays have been reported in a small but relevant percentage of TTP cases. We investigated whether CBA or FRETS-VWF73 assay reflects ADAMTS13 proteolytic activity in acquired TTP patients with non-concordant measurements. Twenty plasma samples with non-concordant ADAMTS13 activity results, <10% using FRETS-VWF73 and ≥20% using CBA, and 11 samples with concordant results, <10% using either FRETS-VWF73 and CBA assays, were analysed. FRETS-VWF73 was performed in the presence of 1.5 M urea. ADAMTS13 activities were also measured under flow conditions and the VWF multimer pattern was defined in order to verify the presence of ultra-large VWF due to ADAMTS13 deficiency. In FRETS-VWF73 assay with 1.5 M urea, ADAMTS13 activity significantly increased in roughly 50% of the samples with non-concordant results, whereas it remained undetectable in all samples with concordant measurements. Under flow conditions, all tested samples showed reduced ADAMTS13 activity. Finally, samples with non-concordant results showed a ratio of high molecular weight VWF multimers higher than normal. Our results support the use of FRETS-VWF73 over CBA assay for the assessment of ADAMTS13 severe deficiency and indicate urea as one cause of the observed differences.

Keywords
ADAMTS13 activity assay, TTP, autoantibodies, von Willebrand factor, flow assay

Introduction
Thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy caused by the presence of highly thrombogenic ultra-large (UL) von Willebrand factor (VWF) multimers in the microcirculation (1, 2). The acquired form of TTP is associated with the severe deficiency of ADAMTS13, the VWF-cleaving protease, caused by anti-ADAMTS13 autoantibodies (3, 4). Severe ADAMTS13 deficiency (activity below 10% of normal) either at the time of the initial episode or during remission has been reported to be predictive of a risk for relapse (5–7). Notably, Kremer-Hovinga et al. found a 10 fold higher risk of relapse among patients with ADAMTS13 activity <10% at presentation (4% vs 41% estimated risk at 7.5 years) (7). Therefore, determining the presence of a severe deficiency of ADAMTS13 is critical in the management of TTP patients.

Since the discovery of ADAMTS13 role in the pathogenesis of TTP, many assays for the measurement of ADAMTS13 activity have been developed (5). Collagen-binding (CBA) and FRETS-VWF73 assays are two widely adopted methods (8, 9). In a recent study, we compared the performance of these assays for the measurement of ADAMTS13 activity in patients with thrombotic microangiopathies (10). Despite a good agreement between the two assays, the results were not concordant in about 10% of the tested samples (10). In these samples, severe ADAMTS13 deficiency was detected by the FRETS-VWF73 assay but not by the CBA assay. Moreover, we observed a higher frequency of anti-ADAMTS13 antibodies in the group with the non-concordant samples (10). These findings suggested that the use of a denaturing agent (i.e., urea) in CBA might dissociate complexes between ADAMTS13 and anti-ADAMTS13 antibodies, thus generating falsely high ADAMTS13 activity results (10). The aim of this study was to test this hypothesis and to investigate whether CBA or
FRETS-VWF73 assay results reflect VWF cleavage in samples from acquired TTP patients with non-concordant results.

Materials and methods

Samples

A total number of 567 samples of patients diagnosed with acquired TTP, referred to our centre and enrolled in the Milan TTP Registry (www.tttpdatabase.org) until June 2012, were tested for ADAMTS13 activity using CBA and FRETS-VWF73 assays. Citrate-anticoagulated plasma samples were collected as previously described (11), during an acute episode of TTP before plasma therapy or during disease remission. The diagnosis of TTP was made on the basis of previously reported criteria (11, 12). Pooled normal plasma (PNP) prepared from 90 healthy donors was used as the common reference standard. The study was carried out with patient informed consent on the experimental nature of this study and after approval by the Institutional Review Board of the Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico. The cohort analysed herein was enlarged with respect to our previous study (10), and further analyses were performed.

Study design and definition of study groups

A summary of the study design is given in Figure 1. Samples were classified in two groups according to ADAMTS13 activity assays outcomes: (1) samples with non-concordant results showed ADAMTS13 activity <10% using CBA and FRETS-VWF73 assay and ≥10% using CBA assay; (2) samples with concordant results showed ADAMTS13 activity < or ≥10% using both assays. The two groups of samples were compared for the presence of anti-ADAMTS13 antibodies.

Additional analyses were carried out in a subgroup of samples of non-concordant (group A) and concordant (group B) samples: (a) the measurement of ADAMTS13 activity using FRETS-VWF73 assay in the presence of 1.5 M urea; (b) the measurement of ADAMTS13 activity under flow conditions (flow-based assay); (c) the evaluation of the VWF multimer pattern. With regards to group A, samples presenting ADAMTS13 activity ≥20% by CBA were arbitrarily selected to take into account the variability of the assays. None of the selected samples collected during an acute TTP episode presented high level of bilirubin (about 100 µM) that could interfere with the FRETS-VWF73 assay (13).

Laboratory measurements

ADAMTS13 activity under static conditions

In-house CBA was performed as previously described (8, 11), with some modifications mainly concerning the final concentration of urea (2.3 M). Test plasmas were diluted in 5 mM Tris-HCl (pH 8.3), containing 2.5 M urea and 1 mM Pefabloc SC (Roche, Mannheim, Germany). Fifty µl of plasma dilutions were incubated with 10 µl of 50 mM BaCl₂ and incubated for 1 hour (h) at 37°C to achieve partial degradation of endogenous VWF. Subsequently, 50 µl of sample mixtures were added to 50 µl of purified VWF concentrate (Laboratoire Francais du Fractionnement et des Biotechnologies, Lille, France) previously diluted in 5 mM Tris-HCl (pH 8.3), containing 2.5 M urea and incubated for 20 h at room temperature. FRETS-VWF73 was performed as previously reported (9, 10). In both assays, ADAMTS13 activities were calculated against a standard curve of serial dilutions of PNP and expressed as percentage of PNP. The limit of detection (LOD) of CBA and FRETS-VWF73 assays were <6% and <3%, respectively.

In order to explain the reason of non-concordant measurements obtained by CBA and FRETS-VWF73 assays, the latter was performed in samples from groups A and B under denaturing conditions. Different concentrations of urea ranging from 1.5 M to 2.5 M were added to sample buffer and tested using FRETS-VWF73 assay. A concentration of 1.5 M urea was used in the final experiments.

ADAMTS13 activity under flow conditions

In order to determine whether either CBA or FRETS-VWF73 assay results would reflect the ADAMTS13 proteolytic activity in patients with non-concordant results, an additional assay, the more physiological flow-based assay, was performed. Given the lower sensitivity of flow-based methods (14) and the large volume of plasma needed, this flow-based assay was performed in 10 plasma samples with the highest difference in activity measurements (ADAMTS13 activity <10% by FRETS-VWF73 assay and ≥30% by CBA assay) and in four samples with undetectable ADAMTS13 activity revealed in both assays. One sample from a congenital TTP patient with undetectable ADAMTS13 activity and antigen was tested as negative control. The flow-based assay was carried out as previously reported (15). Briefly, platelet-decorated VWF strings were perfused for 180 seconds (s) with Zn²⁺ and Ca²⁺ containing Hepes-buffered saline (HBS) buffer, recalcified PNP or TTP patient plasma diluted 1:1 in HBS buffer. VWF strings were counted every 10 s and the percentage of remaining VWF strings in function of time was calculated (15).

Anti-ADAMTS13 antibodies

Total anti-ADAMTS13 immunoglobulin (Ig) G and anti-ADAMTS13 inhibitors were detected by western blot and ELISA (11, 16) and by a CBA-based mixing assay (11), respectively.

VWF multimer analysis

To investigate which ADAMTS13 activity assay reflects the degree of VWF proteolytic cleavage, VWF multimers were evaluated in samples from groups A and B collected during disease remission, as they are usually consumed in the acute phase (17). The qualitative and quantitative evaluation of VWF multimers carried out herein has been described in detail elsewhere (17).
Statistical analysis

Data were expressed as mean ± standard deviation (SD) for continuous variables and proportions for categorical variables. The difference of the medians and its 95% confidence interval (CI), the Mann-Whitney U-test and the Fisher’s Exact test were used to compare continuous and categorical variables in different study groups, respectively. The McNemar’s test was used to compare matched results in the same samples of FRETS-VWF73 experiments in the presence and absence of urea. To this purpose, results were categorised according to the above mentioned criteria for the definition of study groups: ADAMTS13 activities measured in the presence of urea were considered non-concordant with those measured by CBA when ADAMTS13 activity was <10%. Conversely, ADAMTS13 activities measured in the presence of urea were considered concordant with those measured by CBA when ADAMTS13 activity was ≥10%. Values below the LOD were assigned a value equal to 50% of the LOD. A p-value of 0.05 was considered statistically significant.

Results

Anti-ADAMTS13 antibodies in different study groups

Of the 567 samples analysed, 11% (n=65) showed ADAMTS13 activity <10% by FRETS-VWF73 assay and ≥10% by CBA and 89% (n=502) showed ADAMTS13 activity < or ≥10% by both assays (Figure 1). The frequency of anti-ADAMTS13 antibodies by western blot analysis or ELISA was higher in non-concordant samples compared with concordant samples (52/65 vs 309/502; Fisher’s Exact test, p=0.0037). Anti-ADAMTS13 IgG titres and the presence of anti-ADAMTS13 inhibitors were assessed in 52 and 43 non-concordant samples with anti-ADAMTS13 antibodies and in 143 and 135 concordant samples with anti-ADAMTS13 antibodies and ADAMTS13 severe deficiency, respectively (Figure 1). The group with the non-concordant samples presented a lower anti-
ADAMTS13 IgG titre (median difference, 95% CI: –25%, –35% to -15%; Mann-Whitney U-test, p<0.0001) (▶ Figure 2) and a lower anti-ADAMTS13 inhibitor frequency (4/43 vs 86/135; Fisher’s Exact test, p<0.0001) compared with the group with the concord-ant samples. Further analyses were performed on a subgroup of non-concord-ant (group A) and concordant (group B) samples (▶ Figure 1). All results of these analyses are summarised in ▶ Table 1 and de-scribed below.

FRETS-VWF73 assay under denaturing conditions
Experiments were performed to evaluate the influence of different concentrations of urea on the measurement of ADAMTS13 activity of PNP using FRETS-VWF73 assay. In the presence of 2.5 M urea, which was similar to the concentration used in CBA to unfold VWF (2.3 M), ADAMTS13 activity of PNP was abolished. A concentration of 1.5 M urea, which gave an approximately 30% reduction of ADAMTS13 activity, was chosen for further experi-ments (data not shown). Eight out of the 19 samples from group A displayed ADAMTS13 activity values above 10% when urea was added (▶ Table 1, bold numbers and ▶ Figure 3). In contrast, the addition of urea to samples of group B did not influence the outcome of ADAMTS13 activity levels: all 11 samples had ADAMTS13- and VWF-related measurements in selected subgroups
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Table 1: ADAMTS13- and VWF-related laboratory data of selected TTP samples with non-concordant results between CBA and FRETS-VWF73 as-says. Flow assay results were expressed as the percentage of remaining VWF strings after 180 s of sample perfusion. Bold numbers indicate samples presenting ADAMTS13 activity values ≥10% by FRETS-VWF73 assay in the presence of 1.5 M urea (“FRETS-VWF73 urea”). Units and normal ranges are indicated between parentheses.

<table>
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<tr>
<th>Sample</th>
<th>Disease phase</th>
<th>CBA (% , 50–158)</th>
<th>FRETS-VWF73 (% , 45–138)</th>
<th>Flow assay (remaining VWF strings, %)</th>
<th>HMW VWF ratio (0.85–1.21)</th>
<th>ULVWF</th>
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*†Samples collected from the same patient. A acute; R, remission; CBA, collagen binding assay; HMW, high molecular weight; VWF, von Willebrand factor; ULVWF, ultra-large von Willebrand factor; +, positive; -, negative; ND, not done.

Figure 3: Paired comparisons of ADAMTS13 activity values measured by FRETS-VWF73 assay in absence (-) and presence (+) of urea. Data of 19 samples with non-concordant and 11 samples with concordant results between CBA and FRETS-VWF73 assays are depicted.
ADAMTS13 activities <10% in the presence and absence of urea (McNemar’s test, p=0.0133) (Figure 3). In samples from group A, the antibody levels between samples showing ADAMTS13 activity ≥10% and those with ADAMTS13 <10% were not significantly different (median difference, 95% CI: –3.5%, –8.9% to 1.9%; Mann-Whitney U-test, p=0.8026).

**ADAMTS13 activity under flow conditions**

Next, a more physiological test was used to determine ADAMTS13 activity. In this assay, ADAMTS13 mediated proteolysis of UL-VWF multimers anchored to endothelial cells under flow conditions, is measured. The disappearance of these VWF strings was determined in the presence or absence of PNP by calculating the percentage of remaining strings in function of time. In the presence of buffer alone, 91 ± 4% (n=10) of the released VWF strings remained attached to the endothelial surface after 180 s of perfusion (Figure 4). Conversely, perfusion with PNP resulted in the gradual disappearance of VWF strings, with 49 ± 12% (n=11) of the strings remaining after 180 s of perfusion. When a congenital TTP plasma sample was perfused, 92% of VWF strings persisted after 180 s, indicating that the VWF strings cleavage was ADAMTS13-specific (Figure 4).

ADAMTS13 activity of 10 samples from group A and four samples from group B was next determined. The percentage of UL-VWF strings that remained after 180 s of perfusion was 84 ± 5% (mean ± SD) for group A (non-concordant samples) and 87 ± 4% (mean ± SD) for group B (concordant samples) (Figure 4), reflecting low ADAMTS13 activities, close to the situation with congenital TTP plasma resulting in about 90% of remaining strings after 180 s.

**VWF multimer analysis**

All samples with non-concordant results showed a high molecular weight (HMW) VWF multimers ratio higher than normal range (mean ± SD, 1.40 ± 0.10; normal range: 0.85-1.21), due to the presence of UL-VWF multimers (except for sample no. 13) (Table 1 and Figure 5).

**Discussion**

In this study we investigated the cause of non-concordant results between ADAMTS13 activity assays in patients with acquired TTP. To this purpose, we (a) compared the prevalence of anti-ADAMTS13 antibodies and inhibitors in a large cohort of samples with non-concordant and concordant measurements; (b) performed the FRETS-VWF73 assay in the presence of 1.5 M urea, (c) measured ADAMTS13 activity under flow conditions, and (d) evaluated the pattern of VWF multimers in a selection of samples with anti-ADAMTS13 antibodies. We found that (a) non-concordant samples presented a higher frequency of anti-ADAMTS13 antibodies, although with lower titre and inhibitory activity; (b) about half of the tested samples showed significantly increased...
ADAMTS13 activity when urea was added to FRETS-VWF73 assay; (c) ADAMTS13 activity measured under flow conditions was consistent with a severe deficiency of the enzyme; (d) the HMW VWF multimer ratio was higher than in normal samples.

ADAMTS13 activity level below 10% of normal is associated with a higher risk of recurrence in TTP patients (5–7). Our study reports considerable differences between CBA and FRETS-VWF73 assay results. This may lead to spurious attribution of non-severe deficiency of the enzyme in a small but relevant percentage of samples, in which a high frequency of anti-ADAMTS13 antibodies was found. This group of samples showed both a lower anti-ADAMTS13 IgG titre and a lower frequency of ADAMTS13 inhibitors, suggesting that anti-ADAMTS13 antibodies may interfere differently in CBA and FRETS-VWF73 assays. In a study by Mackie et al. comparing CBA and FRETS-VWF73 assays in 76 samples (18), 17% of samples showed ADAMTS13 activity ≤10% by only one of the assays. In contrast to our study, samples with moderate ADAMTS13 deficiency (11–55% activity) frequently showed lower levels by CBA than FRETS-VWF73 assay and no association was found with anti-ADAMTS13 IgG level (18). However, in the study by Mackie et al., the experimental conditions employed in CBA were different in terms of buffer molarity, urea and VWF concentrations. Discrepancies in ADAMTS13 activity results obtained by different methods have been reported also by Froehlich-Zahnd et al., who described the case of a patient with consecutive bouts of acquired TTP secondary to HIV (19). The authors showed that flow-based assay results were more in accordance with FRETS-VWF73 than with quantitative immunoblotting assay results, being also supported by the endogenous VWF multimer analysis. Moreover, they reported evidences of the influence of anti-ADAMTS13 antibodies on non-concordant measurements (19).

Another finding of this study concerned the role of urea in determining the observed differences. The addition of urea did not influence ADAMTS13 activity levels in samples with concordant results between CBA and FRETS-VWF73 assays. On the contrary, about half of the samples with non-concordant measurements showed increased ADAMTS13 activity when FRETS-VWF73 was performed in the presence of 1.5 M urea. It is worth considering that ADAMTS13 activity levels measured in these samples using CBA were higher than those obtained using FRETS-VWF73 assay in the presence of urea. This result may depend on the different concentration of urea used (2.3 M in CBA assay versus 1.5 M in FRETS-VWF73 assay). Overall, these findings support the conclusion that urea is clearly one cause of non-concordant results, at least in a group of samples. However, half of the observed discrepancies remain to be explained. The lower anti-ADAMTS13 IgG titres and anti-ADAMTS13 inhibitor frequency found in samples with non-concordant results suggest that anti-ADAMTS13 antibodies with lower affinity may be present in this group of samples. Consequently, it is reasonable to conclude that urea present in CBA could dissociate such weaker immune complexes, generating higher ADAMTS13 activity results.

CBA and FRETS-VWF73 assays measure ADAMTS13 activity in static conditions, which do not reflect the in vivo physiologic flow conditions, required for conformational changes in VWF that allow its proteolysis by ADAMTS13. Moreover, CBA assay exploits denaturing conditions whereas FRETS-VWF73 assay uses a synthetic peptide as VWF substrate. Flow-based assay results in acquired TTP samples with non-concordant outcomes between the two static assays were consistent with a severely reduced ADAMTS13 activity. The data of the flow-based assay were hence more related to the data of the FRETS-VWF73 assay than to the data of the CBA assay. Hence, the FRETS-VWF73 assay might better reflect the actual ADAMTS13 activity in these patient samples than the CBA assay. This conclusion is also supported by the presence of UL-VWF multimers in this group of samples. Notably, flow-based assay results suggest that the different type of substrate, full-length– (CBA and flow-based assay) or peptide-based (FRETS-VWF73) VWF, is not a cause of the observed differences, at least in the analysed samples.

This study has limitations. First, a relatively small number of samples with different measurements have been analysed. On the other hand, TTP is a rare disease and this type of particular samples represents only a low percentage of cases (about 10%). Secondly, as previously reported, the flow-based assay may only be reliable in discriminating ADAMTS13 levels higher or lower than 20%, without measuring a precise value of ADAMTS13 activity (14). Nevertheless, it has the great advantage of employing a physiological substrate (full-length VWF, anchored to endothelial cells and decorated with platelets), under physiological conditions (flow).
In conclusion, this study showed that FRETS-VWF73 rather than CBA assay reflects ADAMTS13 proteolytic activity in acquired TTP samples with non-concordant ADAMTS13 activities measured by the two assays. The presence of urea in CBA is likely a cause of these differences. Therefore, to our opinion, assays that do not require denaturing agents may be advised when assessing the severe deficiency of ADAMTS13. Most importantly, clinicians and biologists involved in ADAMTS13 testing should be aware of the different experimental conditions employed by each assay and of the advantages and disadvantages that this entails. Likewise, it is important to use the same assay for the diagnosis of TTP patients at acute state, during the follow up and disease remission in order to prevent the misinterpretation of data due to methods variability.

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Conflicts of interest
None declared.

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