Discrepancies between ADAMTS13 activity assays in patients with thrombotic microangiopathies

Ian Mackie1; Katy Langley1; Andrew Chitolie1; Ri Liesner2; Marie Scully2; Samuel Machin1; Flora Peyvandi1

1Haemostasis Research Unit, Haematology Department, University College London, UK; 2Haematology Department, University College London Hospitals, London, UK

Summary

ADAMTS13 activity assays are sometimes useful in confirming the clinical diagnosis or to distinguish different thrombotic microangiopathies (TMA). We investigated the commonly used clinical assays for ADAMTS13 activity. 159 samples from normal subjects or acquired TMA patients were studied in collagen binding (CBA), Fret and chromogenic peptide substrate assays. Frozen aliquots of pooled normal plasma gave similar values by CBA, Fret-VWF73 peptide, Fret-VWF86 and chromogenic VWF73 ELISA (chr-VWF73). Two lyophilised commercial calibrants gave lower ADAMTS13 activity by CBA than peptide substrate assays. The addition of solid HEPES to normal plasma caused a significant fall in CBA, but not Fret-VWF73 activity and might partly explain the differences, since lyophilised plasmas are often HEPES buffered. Normal plasmas showed good agreement between CBA and Fret assays, although chr-VWF73 gave slightly higher values. In acquired TMA, there was reasonable agreement between assays for samples with <11% ADAMTS13 activity (83% of samples showed agreement between CBA, Fret-VWF73 and chr-VWF73), but samples with moderate deficiency frequently showed lower CBA levels (only 41–52% agreement). However, there were also some discrepancies among the peptide substrate assays, with Fret-VWF86 sometimes giving slightly higher values than the VWF73 substrate assays. An international reference plasma might improve standardisation, but is not the only problem. It is unclear which assay has greatest clinical utility, this may depend on the nature of the sample. If the activity does not match the clinical picture, an alternative method should be performed. Where therapeutic monitoring is required, the same activity assay should be used throughout.

Keywords

ADAMTS13, TMA, TTP, ADAMTS13 activity assay

Introduction

Thrombotic microangiopathies (TMA) are a group of rare, potentially life-threatening disorders characterised by microangiopathic haemolytic anaemia (MAHA), thrombocytopenia and variable levels of ischaemic organ damage. The group includes thrombotic thrombocytopenic purpura (TTP) and haemolytic uraemic syndrome (HUS). TTP is characterised by platelet clumping in the microcirculation, causing organ ischaemia and damage. In most cases, the pathophysiology appears to be related to congenital or acquired (autimmune) deficiency of the metalloprotease, ADAMTS13, that normally regulates von Willebrand factor (VWF) molecular size and hence function (1). Decreased ADAMTS13 activity allows the continued presence of ultra-large molecular size VWF in plasma, facilitating increased platelet adhesion and activation. HUS is characterised by acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia (2). It has been divided into infection induced HUS and atypical HUS (aHUS), where there is no obvious infection-related aetiology. ADAMTS13 levels sometimes show a mild reduction, but profound deficiency is rare in HUS, although abnormalities of the complement system are relatively common. Rapid differential diagnosis of these diseases is essential so that appropriate treatment can be delivered rapidly (2).

ADAMTS13 activity assays may be helpful in confirming the clinical diagnosis, particularly in difficult cases, as well as in the prediction of relapse. However, ADAMTS13 deficiency is not specific for TTP and some cases of aHUS can have reduced ADAMTS13 activity. A variety of different ADAMTS13 activity methods are available (3), but they are poorly standardised and often only performed in specialist laboratories. ADAMTS13 activity assays vary in terms of: the substrate (full-length VWF, or peptide substrates based on the cleavage site within the VWF A2 domain), the reagent composition, presence of denaturing reagents, plasma dilution, reaction times and calibrants. Multi-centre studies comparing ADAMTS13 assays showed that assays based on full length VWF substrate gave good performance characteristics and assays based on VWF peptide substrates showed excellent performance; while the use of common, frozen plasma as calibrator reduced the variability (4-6). However, some of these studies only included a relatively small number of laboratories, or assessed mixtures of a single, familial TTP patient and pooled normal plas-
mas, which could have introduced analytical bias; while local methods were used by specialist laboratories rather than commercial kits.

With a straightforward, reliable, rapid method available in hospital laboratories, ADAMTS13 assays might have greater clinical utility. As a first step towards improving the standardisation of ADAMTS13 assays, we decided to compare the commonly used assays and all available commercial kits, in a large number of samples from patients being investigated for TMA.

Materials and methods

Blood samples

In total, 159 citrated plasma samples, stored at -80°C were studied (details in Table 1). Twenty-one TMA patients from UCLH and 55 from other hospitals were referred to our laboratory between July 2009 & May 2010 for investigation of ADAMTS13, as part of the patient’s clinical management. The results from 128 samples (comprising the first sample received from each patient plus the normal controls) were analysed in detail.

Plasma Haemoglobin (Hb) was measured using a HemoCue Plasma/Low Hb photometer (HemoCue Ltd, Dronfield, UK) after conversion to azidemethaemoglobin by the addition of sodium nitrite and sodium azide, at 570 nm; with absorbance at 880 nm used to compensate for the degree of turbidity.

The effect of HEPES buffering of plasma was studied by adding various amounts of solid HEPES to normal plasma, mixing and then removing any solid materials by centrifugation at 2000 g for 15 minutes (min).

ADAMTS13 activity assays

Pooled normal plasma (PNP) was used as calibrant for the in-house assays and the manufacturer’s calibrant for each commercial method. Frozen aliquots of in-house normal and abnormal quality control (QC) plasmas (which were the materials routinely used for QC of the in-house assays and had been selected on the basis of availability of suitable amounts of material and providing plasmas with normal and decreased, but detectable ADAMTS13 levels) were used in each assay. The latter was plasma from a patient with acquired TTP (not included in the clinical samples above), between plasma exchange treatments with an IgG antibody to ADAMTS13. All available commercial ADAMTS13 activity assays were performed on a single lot number of kits, according to the manufacturer’s instructions. All fluorescence assays were performed using a FluoSTAR microtitre plate reader (BMG Labtech Ltd, Aylesbury, UK).

In-house collagen binding assay (CBA) (7, 8)

Plasma samples were diluted 1/6 in Tris/BSA Buffer and incubated overnight at 37°C with plasma derived VWF concentrate (Laboratoire Francais du Fractionnement et des Biotechnologies, Lille, France) in the presence of barium ions, urea and Pefabloc SC (Pentapharm, Basel, Switzerland) (overall dilution in reaction mix was 1/12). A calibration curve was prepared by incubating various dilutions of PNP with the above reactants. Replicate tubes containing EDTA were used as negative controls for patient and calibrant samples. Residual VWF was measured using microplates coated with recombinant human type III collagen (Cambridge Bioscience, Cambridge, UK). Normal Range: 55-160% (n=130 normal subjects).

In-house fluorescence resonance energy transfer (FRET) assay (Fret-VWF73) (9)

Plasma samples were diluted 1/25 in Bis-Tris buffer containing Pefabloc SC and added to 4 µM Fret-VWF73 peptide substrate (Peptanova GmbH, Sandhausen, Germany) (overall dilution 1/50), in the wells of a Fluorunc C96 white maxisorp plate (VWR International Ltd, Lutterworth, UK). Fluorescence (355 nm excitation, 460 nm emission) was monitored every 5 min and the rate of fluorescence change was calculated for data on the linear portion of the rate curve (5-60 min). A linear fit calibration curve was prepared using PNP prediluted in ADAMTS13 inactivated plasma. Normal Range: 60-123% (n=54 normal subjects).

Actifluor assay (Fret-VWF86) (American Diagnostica Inc., Stamford, CT, USA)

This assay used an 86aa FRET substrate (VWF86-ALEXA FRET), based on the VWF A2 domain (10, 11). Test samples were diluted 1/2 in ADAMTS13 inactivated plasma and then 1/5 in buffer (composition not disclosed by manufacturer); VWF86-ALEXA FRET was added to each sample (overall dilution 1/20) and the change in fluorescence was monitored in a kinetic method, at 37°C for up to 20 min (using 485 nm excitation; 530 nm emission). The rate of change of fluorescence was calculated from the linear portion of the rate curve and a standard curve was plotted using the calibrant provided (diluted in ADAMTS13 inactivated plasma) with a point to point fit. Manufacturer’s normal range: 666 ± 135 ng/ml. In order to facilitate comparison with the other methods, results were converted to percent activity by calculations based on the manufacturer’s normal values (yielding a normal range: 60-140%).

Technozym activity ELISA (Chr-VWF73) (Technoclone, Vienna, Austria)

This assay used a chromogenic GST-VWF73 substrate, which was bound to ELISA microplates using a monoclonal anti-GST antibody; and an HRP conjugated monoclonal anti-N10 antibody which is specific for the ADAMTS13 cleavage site in the substrate (12). Samples were diluted 1/30 in assay buffer. Lyophilised calibrators and controls were provided (calibrated against a pool from 300 normal donors, assumed to have 100% activity). Manufacturer’s normal range 40-130%.
Table 1: Blood samples studied.

<table>
<thead>
<tr>
<th>159 Samples</th>
<th>38 Healthy normal subjects</th>
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<tbody>
<tr>
<td>7 Congenital TTP patients</td>
<td></td>
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<tr>
<td>7 Family Members</td>
<td></td>
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<tr>
<td>76 TMA patients</td>
<td></td>
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<tr>
<td>40 TMA – at presentation (probable TTP)</td>
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<tr>
<td>4 TTP – Treated (according to local practice; i.e. plasma exchange and immunosuppression).</td>
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<tr>
<td>19 TTP – Remission</td>
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<tr>
<td>13 aHUS</td>
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<tr>
<td>31 Further samples from 17 of the TMA patients above</td>
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</table>

ATS-13 assay (GTI Diagnostics, Waukesha, WI, USA)

This assay used a 73aa Fret substrate (Fret-VWF73); based on the VWF A2 domain (11). Test samples were diluted approximately 1/8 in specimen diluent (contents undisclosed) and mixed with substrate in the wells of a microplate (overall dilution 1/16). Calibrants and quality control materials were provided frozen at -20°C. Fluorescence was measured for 25 min (355 nm excitation, 460 nm emission) by a kinetic method. The manufacturer did not provide a normal range.

Other methods

IgG anti-ADAMTS13 was measured by an in-house ELISA assay (13, 14). Microtiter plates were coated with 5 µg/ml recombinant ADAMTS13 (rADAMTS13; Baxter Bioscience, Vienna, Austria). Samples were diluted 1:100 with phosphate-buffered saline (PBS)/bovine serum albumin (BSA). Bound IgG was detected using rabbit anti-human IgG conjugated with horseradish peroxidase (HRP). Results were expressed relative to a standard curve using an index plasma with high anti-ADAMTS13 IgG antibody (normal cut-off <6.1%, n=49 normal subjects).

ADAMTS13 antigen was measured using Imubind® ADAMTS13 ELISA kits (American Diagnostica). This employs a monoclonal anti-ADAMTS13 capture antibody and a biotinylated polyclonal antibody against full length ADAMTS13.

We calculated a mean value of 602 ng/ml (2 SD range: 382-823 ng/ml) in 38 normal subjects. To facilitate comparison of activity and antigen values, the results were converted to a percentage using the mean normal value (thus yielding a normal range of: 64-136%).

Data analysis and statistics

Data was assessed for normality using the Shapiro-Wilk test. ANOVA, paired t-test and Pearson's correlation (r) were used for Gaussian data; Friedman's test, Wilcoxon rank sum paired test, and Spearman's correlation (Rs) for non-parametric data. A probability (p) value of <0.05 was taken as indicating statistical significance.

The degree of agreement between assays was investigated by dividing data into three bands (0-10, 11-55, >55%) on the basis that activity values <10% are generally considered to be compatible with TTP diagnosis and the lower cut-off value for the in-house CBA was 55%. Agreement was assessed using the kappa (k) statistic, where k <0.2 shows poor; k =0.2-0.4 fair; k =0.4-0.6 moderate; k =0.6-0.8 good; and k =0.8-1.0 very good agreement.

Results

The sensitivity limits were taken as 5% for the CBA, Fret-VWF73 and Fret-VWF86 assays; and 2.5% for Chr-VWF73, based on the lowest concentration that was consistently detectable compared to buffer blank (results previously obtained in our laboratory, data not shown).

The ADAMTS13 assay using the ATS-13 method was withdrawn by the manufacturer after the study had started, due to a temporary packaging problem with the kit. This caused the contamination of the exterior of the reagent vials with fine, electrolytic, particulate matter resulting in an unacceptably high variability in fluorescence measurements between replicates. The packaging has since been changed to resolve this issue (personal communication from manufacturer).

Calibration

Aliquots of a frozen PNP were used to calibrate the in-house CBA and Fret-VWF73 assays, with an arbitrary potency of 100% (this plasma had 96% ADAMTS13 antigen with activity:antigen ratio 1.04). Each commercial method used the manufacturer’s own lyophilised calibrant. When PNP was assayed as a test sample in the Fret-VWF86 and Chr-VWF73 assays (Table 2) it gave slightly higher than expected values. When the commercial calibrants were assayed as test samples in the CBA assay much lower values were obtained against the PNP calibrant than those expected.

Quality control

Frozen aliquots of normal and abnormal QC plasmas gave similar mean values between assays (Table 2); precision was good for the normal (4-7.5%), but variable for the abnormal plasma (6.1-18%), although this was influenced by the relatively small number of observations. The Fret-VWF86 and Chr-VWF73 kits showed good reproducibility using the lyophilised control samples provided by the respective manufacturer (coefficient of variation [CV] 3.0%; mean ADAMTS13 level 113.9%; and CV 3.7%; mean level 66.0%, respectively).

Effect of HEPES buffering

Due to the variability observed between commercial lyophilised and local frozen calibrants, we assessed the impact of HEPES buffering...
buffer, which is often added to commercial normal plasmas (typical concentration range 34-42 mM) to stabilise the pH during lyophilisation. When solid HEPES was added to normal plasma, a pH range of 7.2-7.5 was observed after lyophilisation, whereas in the absence of HEPES the pH was approximately 8.0. ADAMTS13 by CBA assay decreased from 100% in the absence of HEPES, to 71, 56, 35%, for 21, 42 and 84 mM HEPES, respectively, but in the Fret-VWF73 assay activity was 100-110%. However, the pH of each plasma, as well as commercial calibrants, became the same after dilution in CBA assay buffer.

**Samples from TMA, congenital TTP, and healthy normal subjects**

There was a reasonable correlation (Figure 1A) between the CBA and each of the three peptide substrate assays (range of Rs values: 0.84-0.94). However, CBA gave significantly lower values than each of the other activity assays (p<0.0001). Normalisation of the Fret-VWF86 and Chr-VWF73 data according to the ADAMTS13 value measured for PNP in each assay failed to abolish the differences between assays (data not shown).

**Analysis of data according to the type of sample**

**Healthy normal subjects**

Samples from normal subjects showed a reasonable degree of correlation (r=0.62-0.88) between all activity assay types, but higher mean values and range were observed for the Chr-VWF-73 assay (t-test p<0.0001 for comparison with each assay) (Table 3.

**Congenital TTP cases**

A total of 14 samples from seven patients and seven of their family members, all with a documented ADAMTS13 gene mutation and no detectable IgG anti-ADAMTS13 were analysed (Figure 1C).

The median (and range) were 42.4% (1-73), 14.0 (5-45), 28.5 (5-82), 28.0 (5-65), and 12.8 (43-58) for antigen, CBA, Fret-VWF73, Fret-VWF86 and Chr-VWF73, respectively. Six of seven probands had low, but variable activity in the different assays, but one case with a type II like defect had low CBA activity and little or no reduction by the peptide substrate based assays. Some family members also showed higher activity in peptide substrate assays.

**Acquired TMA samples**

The median and range for all activity assays in the 107 samples from different TMA patients are shown in Table 2. Since samples from some patients were collected on different occasions to facilitate longitudinal analysis, the following data is derived from the first sample only (n=76). When the data was divided into three bands (0-10, 11-55, >55%), as described above, there was good to moderate agreement between the assays (kappa =0.53-0.79). Best agreement was observed for samples with <11% or >55% ADAMTS13 activity, but samples with a mild deficiency of ADAMTS13 frequently showed lower levels by the CBA than peptide substrate assays (Figure 1D).

Most samples with severe reduction of ADAMTS13 activity showed good agreement between the CBA, Fret-VWF73 and Chr-VWF73 assays, with 83% of these samples showing <11% activity by each assay type. However, the Fret-VWF86 assay only showed agreement in 45% of samples. In comparison, only 41 or 52% of samples with ADAMTS13 between 11-55% showed agreement between the CBA and either the Fret-VWF73 or Chr-VWF73 assays.

ADAMTS13 antigen values were available in 38 of the 76 TMA samples, including those with and without a clear discrepancy between assay methods (median 65.3%, interquartile range 59.9, range 2-136). There was no obvious difference in the proportion of discrepant activity values between samples with low or normal ADAMTS13 antigen values.

IgG anti-ADAMTS13 was measured in 73/76 acquired TMA samples and a wide range (2-123%) of values were obtained. Thirty-two samples gave values above the normal cut-off and these had a median of 22%. An inverse correlation was observed with CBA and Fret-VWF73 assays, with 83% of these samples showing <11% activity.

The discrepancies between assays were further investigated by separating the TMA samples into those collected at presentation,
after treatment and in remission. However, similar patterns of results were still observed in each of these groups of samples (data not shown).

In the 17 patients studied longitudinally (data not shown), discrepancies between activity assays were not observed consistently in particular individuals. Some patients showed good agreement throughout, while others only showed discrepancies at certain time points. There was no obvious association with the level of ADAMTS13 activity, antigen, or IgG anti-ADAMTS13.

Plasma haemoglobin

Plasma haemoglobin (Hb) was measured in 10 samples with visible haemolysis (>0.8 g/l) and 22 further samples selected at random. In those with ADAMTS13 levels in the detectable range of both CBA and Fret-VWF73 assays, 8/9 samples with 0.8–2.5 g/l plasma Hb showed higher levels by the peptide substrate based assay. However, discrepancies were also seen in samples without detectable plasma Hb. Only four samples had marked, visible ict-
rus and there were no obvious effects on ADAMTS13 activity levels.

Discussion

TTP remains a clinical diagnosis, but ADAMTS13 assays may be helpful if the diagnosis is uncertain (e.g. when the clinical presentation is not straightforward an ADAMTS13 value <10% would support a diagnosis of TTP, whereas a normal ADAMTS13 level might suggest aHUS). None of the available ADAMTS13 assays suitable for widespread clinical laboratory use adequately reflect its in vivo activity. Ideally the assay would mimic the flow characteristics of circulating blood, utilise VWF as substrate, not require denaturants and yield rapid results. The collagen binding method uses natural substrate, but lacks shear conditions and is time consuming. Assays utilising VWF peptide substrates take several hours, but the substrates may lack some exosites for ADAMTS13 interaction. Most laboratories use a single method and only see a few TTP patients annually, making it hard to detect discrepancies between assays. Several studies (4-6) have investigated the precision and comparability of assays, but these usually involved few laboratories and small sample numbers. We aimed to evaluate all widely available ADAMTS13 activity assays using a wide range of clinical and normal samples. Activity was similar in each assay type for frozen aliquots of PNP calibrant and the local normal and abnormal quality control samples. However, when the two commercial calibrants were tested using the in-house assays, much lower than expected activity was found by CBA. Commercial calibrator and quality control plasmas are often stabilised with HEPES. We found that the addition of HEPES to normal plasma caused an initial fall in pH, but protected plasma pH after lyophilisation. HEPES addition was associated with a decrease in activity by CBA, but not Fret-VWF73 assay. However, the pH of HEPES buffered and non-buffered plasmas as well as commercial calibrator was similar after dilution in CBA assay buffer, suggesting a direct effect of HEPES. It has previously been demonstrated that the addition of 25 mM HEPES to cell culture medium followed by exposure to light can lead to hydrogen peroxide generation and cytotoxicity (15), so redox changes in the distal domains of ADAMTS13 could be a contributing factor and may have important implications for the preparation of reference preparations.

Normal subjects gave similar mean values in each assay. Agreement between assays was moderate or good for TMA samples, although best correlation was between the peptide substrate assays and activity was frequently lower by CBA. Inter-assay discrepancies were not abolished by correcting results according to PNP potency and were not constant for all sample types. In TMA samples, agreement between assays was best for samples with <11% activity, while samples with moderate ADAMTS13 deficiency (11-55% activity) frequently showed lower CBA levels, although there were also some discrepancies between the other assays. There was no obvious relationship between inter-assay discrepancies and ADAMTS13 antigen level, nor were they explained by the TMA sample being at presentation, after treatment or in remission.

Apart from the calibrant differences, a number of variables in the assay methodologies could have contributed to the discrepancies. Two assays used a FRET detection principle, but one used a slightly longer peptide substrate (VWF73 & VWF86); both assays used ADAMTS13 inactivated plasma as an initial diluent, but the buffer formulation is unclear. The Chr-VWF73 assay used a similar peptide in a spectrophotometric technique and diluted sample was washed from the plate before the colour reaction. The peptide substrate assays did not require denaturants, which were essential in the CBA, which used plasma derived VWF substrate. The dilution factors for test samples and the reaction times (between enzyme and substrate) also differed between the assays and are likely to have contributed to the differences. Other potential factors are: proteins that modify the ADAMTS13:VWF interaction (e.g. factor VIII, and haemoglobin) (16-18); abnormal glycosylation of ADAMTS13 (19); bilirubin interference in Fret substrate assays (20); the effects of leukocyte and plasma proteases on ADAMTS13 and VWF (21-23); and in acquired TTP, a variety of antibodies may be present with differing epitope specificity and concentration (24).

The interactions between ADAMTS13 and VWF are complex and in vivo may involve the initial attachment of ADAMTS13 via its C-terminal domains (25-27), followed by conformational changes and closer association between the proteins, bringing the metalloprotease domain into close proximity with the VWF A2 domain. These interactions are also facilitated by the unfolding of VWF when subjected to in vivo high shear forces. In our subjects with ADAMTS13 mutations, some showed good agreement between CBA, peptide substrate and ADAMTS13 antigen assays, but

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**Table 3: ADAMTS13 activity in 38 healthy normal subjects.**

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<thead>
<tr>
<th></th>
<th>ADAMTS13</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Range</td>
<td></td>
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<tr>
<td>CBA (%)</td>
<td>85.9</td>
<td>21.7</td>
<td>38–138</td>
<td></td>
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<tr>
<td>Fret-VWF73 (%)</td>
<td>91.3</td>
<td>16.2</td>
<td>56–133</td>
<td></td>
<td></td>
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<tr>
<td>Fret-VWF86 (%)</td>
<td>87.5</td>
<td>19.1</td>
<td>48–135</td>
<td></td>
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<tr>
<td>Chr-VWF73 (%)</td>
<td>98.3</td>
<td>18.0</td>
<td>59–145</td>
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<td></td>
</tr>
<tr>
<td>Antigen (ng/ml)</td>
<td>602</td>
<td>110</td>
<td>428–784</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen (%)</td>
<td>100</td>
<td>18.3</td>
<td>71–130</td>
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**Table 4: All acquired TMA samples (n=107).**

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>IQR</th>
<th>Range</th>
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<tbody>
<tr>
<td>CBA (%)</td>
<td>31.0</td>
<td>57</td>
<td>&lt;5 – 171</td>
</tr>
<tr>
<td>Fret-VWF73 (%)</td>
<td>53.0</td>
<td>73</td>
<td>&lt;5 – 129</td>
</tr>
<tr>
<td>Fret-VWF86 (%)</td>
<td>54.0</td>
<td>69</td>
<td>&lt;5 – 167</td>
</tr>
<tr>
<td>Chr-VWF73 (%)</td>
<td>47.2</td>
<td>75</td>
<td>&lt;2.5 – 176</td>
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</table>
others had lower activity by CBA, possibly due to an abnormal exosite-mediated interaction between ADAMTS13 and VWF. We do not know whether any of the acquired TMA cases had ADAMTS13 mutations. In CBA, VWF unfolding is facilitated by urea denaturant, which is not required in peptide substrate assays, and urea could shift the equilibrium between bound and free antibodies in patient plasma. This could also be influenced by dilution in buffer, varying according to antibody affinity and epitope. Patients with antibodies directed against the TSP-1 repeats and CUB domains could therefore be more likely to show decreased CBA activity. ADAMTS13/antibody complexes could also potentially act as competitive inhibitors of free ADAMTS13.

Elevated bilirubin tends to reduce ADAMTS13 activity by Fret assay due to fluorescence quenching (20), whereas CBA and Chr-VWF73 assays are not affected, since bilirubin is not present during spectrophotometric measurement. In our study there were no marked differences for the four samples with visible icterus. Hb has been shown to inhibit ADAMTS13 cleavage (17, 18) of full length VWF and the VWF A2 domain as substrates (Hb levels of (0.05 μg/l showed significant inhibition). However, the effect of Hb on shorter VWF peptide substrates, such as those used in the Fret and chromogenic assays, is unclear. A small number of samples in our study had visible haemolysis (0.8-2.5 g/l plasma Hb) and showed higher ADAMTS13 activity by the peptide substrate assays.

Blood sample quality and handling may be critical for the accurate assessment of ADAMTS13 activity, since sample ageing may lead to leukocyte activation and protease release. Serine proteases can cleave ADAMTS13 causing a C-terminal truncation (21, 23) and reduction of its affinity for and proteolysis of VWF, but the 115aa VWF-A2 domain peptide was less affected. Leukocyte proteases may also cleave VWF at sites adjacent to the ADAMTS13 cleavage site (22). It is unclear whether this occurs in vivo, because of rapid neutralisation by plasma protease inhibitors, although in critically ill patients these may be depleted. The inclusion of protease inhibitor cocktails in assay buffers limits non-specific substrate degradation; but it was unclear whether these were present in Fret-VWF86 and Chr-VWF73 assays.

The degree of discrepancy may vary between different CBA methods, as well as other assays using full-length VWF substrate, depending on the exact reaction conditions. One study (28) compared FRETT-VWF73 with an IRMA using wild-type VWF in 64 TMA patients and 10 normal subjects, with PNP calibrator. All TTP patients had ADAMTS13 <5% and normal plasmas gave close agreement, but HUS samples with more than 30% ADAMTS13, showed 20-30% variability between the assays. Another study (29) compared the VWF multimer assay with FRET-VWF73 in 79 patient samples investigated for TTP. 24/27 TTP patients had deficient ADAMTS13 by the multimer assay and undetectable Fret-VWF73 activity. Fifty-five other patients showed normal activity by multimer assay, but reduced Fret-VWF73 (25-103%). In a multicentre study (6) 30 samples with ADAMTS13 activity from <3 to >100%, were shipped frozen with PNP calibrator. ADAMTS13 was measured by: CBA, immunoblotting, ristocetin cofactor, or immunoradiometry. Severe deficiency (<5%) was identified by all assays in nine TTP samples; but activity varied from <5% to 12% between assays in three further samples. One laboratory detected severe deficiency by CBA in two TTP samples, while other laboratories measured 10-34% activity. As in our study, the authors found less concordance in samples with moderately reduced ADAMTS13, with nothing to indicate a systematic deviation for any assay. In contrast to our results, a recent study showed higher values by CBA than in-house FRET-VWF73.
and chr-VWF73 assays; but different modifications (31) of the CBA method (lower Tris buffer molarity and small differences in urea and VWF concentration) were employed. CBA is a very variable technique and it is likely that small changes in plasma pH, ionic environment in the assay and other factors may influence the results. Sample collection and handling differences may contribute to these changes as well as a variety of in vivo factors.

Our study encompassed all widely used clinical assays for ADAMTS13 and included a large number of blood samples. Samples with very low or normal ADAMTS13 levels generally showed good agreement between assays, but there was great variability in results for samples with only moderately reduced activity. In clinical samples, many of the potential variables discussed above cannot be easily controlled, and each may contribute to the final assay result, with sample quality being critical. Blood samples should be obtained by clean venipuncture, and processed rapidly to separate platelet-poor plasma. Samples should be visually inspected for lipaemia, icterus and haemolysis, with incorporation of protease inhibitors in the reagents. Commercial calibrant and quality control plasmas may not have universal applicability, but international standardisation and the development of external quality assessment schemes are clearly required to reduce some of the variability. It is currently unclear which assay has the greatest clinical utility. There is a potential for misdiagnosis of certain patients with ADAMTS13 gene mutations when full-length VWF assay substrates are not used. If an assay is required to assist the differential diagnosis of TMA and the results do not agree with the clinical picture, an alternative method should be performed. The same type of ADAMTS13 assay should be used throughout therapeutic monitoring. It may be appropriate to perform a rapid, peptide substrate assay, followed by an assay using full-length VWF when the activity only shows moderate reduction. The type of method should always be indicated when reporting clinical assays of ADAMTS13.

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Conflicts of interest
None declared.

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