Chapter 8
Laboratory Assays for ADAMTS13

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Abbreviations

ADAMTS13	A disintegrin and metalloproteinase with thrombospondin type 1 motif 13
BU	Bethesda units
CICs	Circulating immune complexes
CV	Coefficient of variation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FRET	Fluorescence resonance energy transfer
Gp1b	Glycoprotein 1b
GST	Glutathione S-transferase
HMW	High molecular weight
HRP	Horseradish peroxidase
LDT	Laboratory-developed tests
nm	Nanometers
NPP	Normal pooled plasma
RCo	Ristocetin-cofactor activity
RFU	Relative fluorescence units
RIPA	Ristocetin-induced platelet aggregation
TMA	Thrombotic microangiopathy
TTP	Thrombotic thrombocytopenic purpura
vWF	von Willebrand factor

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8.1 Sample Collection and Quality

A disintegrin and metalloproteinase with thrombospondin type 1 motif 13 (ADAMTS13) testing should be performed using sodium citrate platelet-poor plasma (light blue top tube) collected and processed in accordance with collection guidelines for coagulation testing [1]. Sodium citrate anticoagulates via weak chelation of calcium ions that are necessary for coagulation. Ethylenediaminetetraacetic acid (EDTA, purple top tube) is an unacceptable anticoagulant because EDTA is a potent chelator of metal ions and ADAMTS13 is a metalloproteinase dependent on metal ions, such as Ca^{2+}, for function [2, 3]. ADAMTS13 activity testing in EDTA plasma results in marked underestimation of ADAMTS13 activity. Serum samples (collected in red top tubes or gold top serum separator tubes) may also result in underestimation of ADAMTS13 activity because thrombin generated during serum formation (allowing blood to clot) may degrade ADAMTS13 [4]. Normal values obtained from a serum sample would be reassuring, and some laboratories have validated ADAMTS13 testing on serum samples. Plasma should be tested within 4 h of collection or frozen at −70 °C until the time of testing [5]. ADAMTS13 is stable frozen at −70 °C for up to 6 months [5], and multiple freeze–thaw cycles or uncontrolled freeze–thaws should be avoided. Frozen plasmas should be thawed rapidly in a 37 °C water bath and thoroughly mixed before testing [5]. ADAMTS13 testing should ideally be completed within 4 h of thawing a frozen sample [5]. Although prolonged refrigerated or room temperature storage is not generally recommended, ADAMTS13 activity appears to be stable for several days refrigerated, with only minimal losses in activity after 5 days of refrigerated storage [6–8], and levels may not significantly decrease after up to 2 days of storage at room temperature [9].

When considering a diagnosis of thrombotic thrombocytopenic purpura (TTP), samples for ADAMTS13 testing should be drawn prior to the administration of any blood products and before initiation of plasma exchange therapy since these therapies will alter ADAMTS13 levels.

8.2 ADAMTS13 Testing Methods

The first assays for measurement of ADAMTS13 were developed in the 1990s, but testing was not available in clinical laboratories until more recently. In general, measurement of ADAMTS13 involves quantitating either ADAMTS13 activity (the amount of functional protein) or ADAMTS13 antigen (the total amount of protein). Activity assays are preferred for clinical testing since these are capable of identifying both quantitative and qualitative abnormalities. Tests are also available to identify and quantitate neutralizing and non-neutralizing autoantibodies to ADAMTS13 that cause acquired TTP.

8.2.1 ADAMTS13 Activity

The ADAMTS13 protease regulates the size of von Willebrand factor (vWF) multimers when subjected to fluid shear stress or treated with protein denaturants [10–13]. The ADAMTS13 cleavage site is a specific peptide bond (Tyr1605–Met1606) in the A2 domain of vWF [14]. Larger or high-molecular-weight (HMW) vWF multimers are more functional than smaller multimers, with the ability to bind subendothelial collagen and the platelet surface glycoprotein 1b (GP1b) receptor. ADAMTS13 regulation prevents accumulation of ultra-HMW vWF multimers. Patients with TTP have ultra-large vWF multimers, due to lack of ADAMTS13 regulation, which results in vWF-mediated microvascular platelet thrombosis, leading to thrombotic microangiopathy (TMA) and organ ischemia [15, 16].

The first activity assays measured ADAMTS13 activity directly through effects of activated patient plasma ADAMTS13 on purified vWF. The resultant vWF multimeric patterns were visualized after electrophoresis, Western blotting, and detection by an anti-vWF antibody [17, 18]. Other assays have measured ADAMTS13 activity indirectly by measuring the residual function of vWF after incubation with patient ADAMTS13. These assays evaluated vWF functions that are preferentially dependent on the presence of HMW vWF multimers, such as collagen binding activity [19, 20] and ristocetin-induced platelet aggregation (RIPA) [21]. vWF collagen binding assays utilize an enzyme-linked immunosorbent assay (ELISA) methodology to determine the degree of vWF binding to a collagen-coated plate [22]. The degree of vWF binding to collagen is inversely proportional to patient ADAMTS13 activity since reagent vWF that has been processed by patient ADAMTS13 demonstrates less binding due to loss of HMW multimers. In the RIPA assay, platelet aggregation, mediated by HMW vWF multimers, is used to measure the residual function of vWF after exposure to patient plasma ADAMTS13. Ristocetin is an antibiotic reagent used to "activate" the vWF, causing a conformational change that allows it to interact with the platelet GP1b surface receptor, resulting in platelet agglutination and aggregation [23]. The testing is performed on a platelet aggregometer using fresh donor platelets but can also be modified for a commercial automated ristocetin-cofactor activity (vWF:RCo) method that utilizes formalin-fixed platelets [24]. In all of the above-described assays, ADAMTS13 activity is quantified by comparison to a calibration curve made from serial dilutions of normal plasma.

The earliest methods were technically complex and time consuming, taking many hours to days to perform (often 2–3 days) and limiting performance to very specialized research laboratories rather than clinical laboratories. Newer assays have subsequently been developed that have performance characteristics and turnaround times more amenable to clinical laboratories. These assays use synthetic vWF peptide substrates that contain the ADAMTS13 cleavage site, located in the A2 domain of vWF, eliminating the need for the denaturing conditions that were necessary to unfold vWF in the earlier assays [25]. Suitable synthetic substrates that

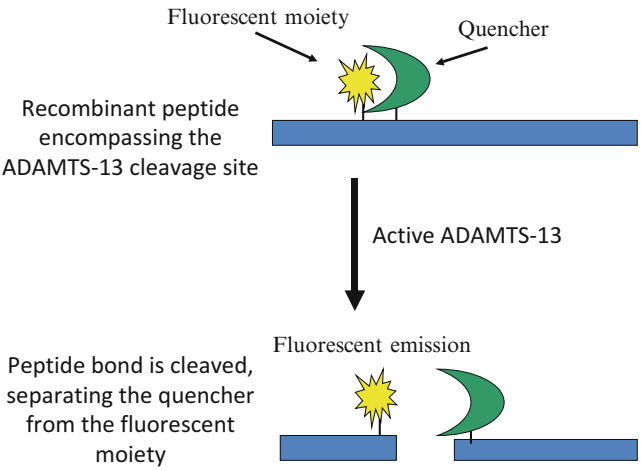

Fig. 8.1 Depiction of a FRET assay utilizing a fluorochrome and quencher that are separated by ADAMTS13 cleavage, resulting in fluorescent emission

have been described include a recombinant A2 domain [25] or shorter peptides containing the minimum portion of the A2 domain necessary for cleavage (D1596-R1668) [14, 26, 27].

Currently, fluorescence resonance energy transfer (FRET) assays, performed in 96-well microtiter plates, are the most widely used activity tests in clinical laboratories. Kokame et al. first described a 73 amino acid FRET substrate, FRETS-VWF73, based on modification of the D1596-R1668 peptide, with 2 amino acid residues flanking the ADAMTS13 cleavage site (Q1599 and N1610) replaced by a fluorescent moiety A2pr (Nma) and quencher A2pr (Dnp), respectively [26]. Nma is excited at 340 nanometers (nm), but when the peptide is intact, the energy is quenched by Dnp. Peptide cleavage by ADAMTS13 results in fluorescent emission, read on a fluorometer at 440 nm. Thus, the amount of fluorescent emission is directly proportional to the ADAMTS13 activity in the patient plasma, and the amount of fluorescent emission is compared to a calibration curve to quantitate activity. This fluorochrome/quencher methodology is used in commercial assays from Technoclone (Vienna, Austria) and Immucor Gamma (Waukesha, WI, USA), while an assay from Sekisui Diagnostics (Stamford, CT, USA) utilizes two fluorochromes located on either side of the ADAMTS13 cleavage site that are uncoupled by ADAMTS13 proteolytic cleavage, resulting in increased fluorescence [27]. Figure 8.1 depicts a FRET assay utilizing a fluorochrome and quencher. Results are often expressed as percentage of normal activity, but may also be expressed as a concentration (such as units/mL or ng/mL) depending on the kit, as there are no official units for activity.

As mentioned above, commercial FRET assays are currently available from several manufacturers. These include Technoclone (Vienna, Austria), Immucor Gamma (Waukesha, WI, USA), and Sekisui Diagnostics (Stamford, CT, USA). The primary differences between these commercial FRET assays include whether the patient's

Table 8.1 Overview of commercial FRET assays

Test	ADAMTS13 capture	Activity substrate	Fluorescent quantification	Analysis time (h)	Maximum tests/kit[a]
Technoclone	Yes	73 aa peptide, FRETS-VWF73	Kinetic	~2.5	40
Immucor	No	73 aa peptide, FRETS-VWF73	Endpoint	~1	40
Sekisui	No	86 aa peptide, ALEXA488-VWF86	Kinetic or endpoint	~1.5	40

[a]Approximation of 40 tests per kit assumes five calibrators and two controls in duplicate, allowing 40 patient samples in duplicate to fill one run on a single 96-well plate. If a plate is used for multiple runs, less total patient samples could be tested

ADAMTS13 is captured on the plate or measured in solution, differences in the activity substrate (vWF peptide with fluorescent tag and quencher or two self-quenching fluorochromes), and method for taking the fluorescent readings, which can entail multiple kinetic readings or comparing a baseline reading to an endpoint reading taken at the end of the reaction incubation. Refer to Table 8.1 for an overview of commercial FRET assays. The Technoclone assay is unique in that the microtiter plate is coated with a monoclonal anti-ADAMTS13 antibody directed against the CUB domain of the protein, which allows capture of patient ADAMTS13 prior to measuring activity. One advantage of the capture approach is that the same microtiter plate can be used to measure ADAMTS13 antigen after the activity has been measured, if desired. However, the assay could be compromised by mutations in the CUB domain that would hamper the ability to capture patient ADAMTS13, causing erroneously low activity results. The other commercial FRET assays measure ADAMTS13 activity in solution, which saves time by eliminating the capture step.

The Technoclone and Sekisui FRET assays are kinetic assays that measure baseline fluorescence followed by several additional readings as the reaction progresses. The Technoclone assay takes one measurement per minute during the reaction for a total of 16 measurements, while the Sekisui assay takes a greater number of readings. The slope of the kinetic curve, expressed as relative fluorescence units (RFU)/second or RFU/minute, is compared against the calibration curve to determine the ADAMTS13 activity. Figure 8.2 shows example data and calibration curve from a kinetic assay. The Immucor Gamma assay utilizes endpoint detection by subtracting the baseline fluorescence (time 0) from the fluorescence at the endpoint of the reaction. This value, which represents the change in fluorescence, is compared to the calibration curve to determine the ADAMTS13 activity. The Sekisui assay also has the option of using endpoint rather than kinetic detection. Evaluation software is generally available from the commercial kit manufacturers to perform the result analysis. In these assays, the reactions are usually performed in duplicate, requiring a certain degree of agreement (coefficients of variation (CVs) <15–20 %) between the 2 wells prior to reporting results. To maintain the accuracy of the assays, strict attention must be paid to incubation and pipetting

a

Time(sec)	A5	A6
0	9.512	9.497
60	9.727	9.825
120	9.975	10.160
180	10.092	10.580
240	10.747	10.296
300	11.127	11.463
360	11.309	12.321
420	11.343	12.244
480	12.085	12.618
540	12.317	13.213
600	12.842	13.769
660	13.633	14.405
720	13.685	14.714
780	14.255	15.078
840	14.616	15.203
900	14.546	15.534

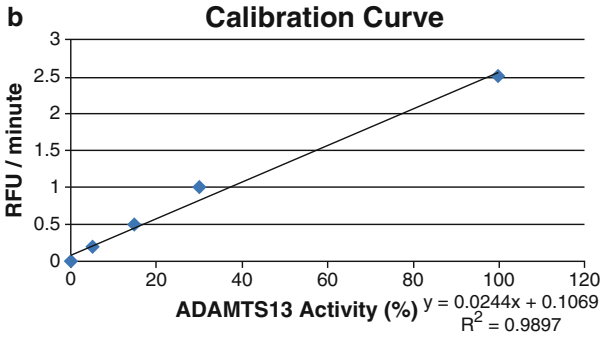

Fig. 8.2 (**a**) Representative data from duplicate wells (A5 and A6) in a kinetic FRET assay. A baseline fluorescence reading is taken (time 0), followed by multiple additional readings as the reaction progresses. The raw data is used to determine the slope of the kinetic curve (RFU/minute), which is compared to the calibration curve to determine activity. (**b**) Example calibration curve showing the relationship between ADAMTS13 activity (% of normal, *x*-axis) and fluorescence (RFU/minute, *y*-axis)

times and temperatures; instructions provided in the package insert of commercial assays should be precisely followed.

ELISA ADAMTS13 activity methods, also performed in a 96-well plate, are similar to the FRET assays in the use of synthetic vWF peptides as activity substrates [28–31]. However, the method for detection of substrate cleavage by ADAMTS13 is indirect rather than direct. A commercial assay is available from Technoclone (Vienna, Austria). In this assay, the activity substrate (vWF73) is labeled with a glutathione S-transferase (GST) tag, termed GST-VWF73, allowing it to be captured to the ELISA plate via an anti-GST antibody and avoiding capture of any patient vWF. When patient plasma is incubated in the wells, patient ADAMTS13 cleaves the vWF peptide exposing a specific amino acid sequence. After incubation, an antibody is added that recognizes the exposed amino acid sequence at the cleavage site. The detection antibody is conjugated to horseradish peroxidase (HRP), and reaction color is developed using an HRP reaction. The optical density of the reaction is read on an ELISA plate reader at 450 nm and is directly proportional to ADAMTS13 activity in the sample, quantitated by comparison to the calibration curve. Possible advantages of ELISA methods are widespread familiarity with ELISA techniques and greater availability of required equipment, as opposed to FRET assays, which require fluorescent plate readers and specific software to read and interpret results. The ELISA methods may also be more easily adapted for ADAMTS13 inhibitor testing. Due to multiple incubation steps, these assays may take longer to perform than the FRET assays (~3 h).

Calibrator material is included in commercial kits or available separately from the kit manufacturers. This may entail a single calibrator that requires dilution to create other levels of calibrator material or a premade series of different calibrator levels. Commercial calibrator material for activity assays is usually made from a large pool of normal donors, defined as having 100 % activity, or calibrated against this type of reference material. The calibration curve generated with each run of testing is validated by testing control material (often included in commercial kits), which usually represents two levels of ADAMTS13 activity (normal and low) with defined ranges for expected results. Patient results are compared against a reference interval to help classify the values as normal or abnormal. Although commercial manufacturers provide an expected range for normal values (approximately 50–150 % of normal), laboratories should establish their own laboratory-specific reference intervals and cutoff values for severe deficiency or validate the manufacturer's suggested interval [32]. For this reason, reference intervals vary slightly from laboratory to laboratory, depending on the test methodology and other variables.

The newer commercial FRET and ELISA activity assays take significantly less time to perform than the historic assays, ranging from 1 to 3 h, depending on specific requirements, such as time needed for ADAMTS13 capture. The reaction time involving incubation of patient ADAMTS13 with the activity substrate is generally quite brief (half an hour or less). Although shorter analysis time can equate to better turnaround time, enhancing clinical utility, it should be noted that many laboratories need to batch samples rather than running individual samples as they are received in order to make the testing cost-effective. TTP is a rare disorder and most clinical laboratories do not test a high volume of samples. Commercial kits are costly and each run of testing must include multiple levels of calibrator (usually at least five) and up to two levels of control material, all in duplicate, meaning that 14 or more wells are used for calibrator and control material each time testing is performed, depending on the specific kit. Kits may include multiple sets of calibrators and controls to allow multiple runs to be performed from a single 96-well plate. However, performing only a single full run per plate maximizes the number of patients that can be tested by minimizing the number of wells that must be used for calibrators and controls. The optimal approach for balancing kit usage with acceptable turnaround times is laboratory specific, based on testing volumes and patient care requirements. For these reasons, many smaller clinical laboratories choose to send this testing to a reference laboratory.

Laboratories may also opt to utilize laboratory-developed tests (LDTs) for ADAMTS13 activity, rather than commercial assays. The FRETS-VWF73 fluorogenic substrate can be purchased directly from several vendors and used in LDT FRET assays. Other LDTs are more novel, using detection methods such as mass spectrometry [33]. In this method, cleavage of the activity substrate by ADAMTS13 results in cleavage products that are detected and quantified by mass spectrometry, a method that measures the mass of molecules that have been converted into ions. The amount of cleavage product generated is directly related to ADAMTS13 activity. Although this method has been cited as having an analytical sensitivity of 0.5 % ADAMTS13 activity [33, 34], it is not widely used and requires expensive

specialized equipment and considerable technical expertise. Other novel assays have also been described, requiring variable amounts of time and degree of technical expertise [35, 36].

Although the newer assays represent a significant advance above the original assays in terms of the required technical time to run the tests, there is interest in identifying even more rapid assays, due to the urgent nature of TTP diagnosis. One such assay has been reported using an automated coagulation analyzer for immunoturbidimetric measurement of residual vWF activity using a latex particle-enhanced monoclonal antibody directed against the platelet-binding domain of vWF after plasma digestion of commercial vWF by patient plasma [37]. This vWF activity method is available as a commercial kit (Instrumentation Laboratory, Bedford, MA, USA). In a comparison of this assay to a reference immunoblot method, there was no statistically significant difference in categorizing subjects with severe ADAMTS13 deficiency (activity <5 % of normal), often considered the diagnostic value for TTP [37]. This type of assay is intriguing due to the relatively rapid turnaround time (~2 h), broad availability of automated coagulation analyzers, and use of a commercial kit for vWF activity, which could facilitate patient testing at local laboratories rather than more distant specialized reference laboratories and allow more cost-effective testing of small numbers of samples.

8.2.2 ADAMTS13 Antigen

Antigen assays detect the amount of ADAMTS13 protein. Commercial ELISA assays for ADAMTS13 antigen are available from both Technoclone (Vienna, Austria) and Sekisui (Stamford, CT, USA). These are traditional sandwich ELISAs that entail antibody capture of patient ADAMTS13 to the plate followed by detection with a labeled secondary antibody that participates in a chromogenic reaction. Analysis times are approximately 3 h. The performance characteristics of antigen assays vary depending on the specificity of the antibodies used for capture and detection of ADAMTS13 (polyclonal versus monoclonal) [38]. Both ADAMTS13 activity and antigen levels are low in subjects with congenital quantitative deficiency of ADAMTS13 (congenital TTP, Upshaw–Schulman syndrome). However, in acquired TTP, activity and antigen levels may not be concordant due to the autoimmune nature of the disease, where the antibodies can demonstrate either activity-neutralizing (inhibit function, approximately 70 %) or non-neutralizing (promote accelerated clearance but do not inhibit function, approximately 30 %) properties [32, 39–41]. Subjects with TTP primarily due to neutralizing antibodies have compromised activity without significant impact on the protein amount (antigen). In a comparison between activity and antigen levels in TTP cases, Starke et al. found a correlation between antigen and activity in 70 % of cases, with the majority of discordant cases having low activity with a normal or high antigen level, presumably due to neutralizing antibodies that form nonfunctional immune complexes [39]. Thus, although many subjects with acquired TTP have decreased ADAMTS13

antigen due to clearance [42], antigen assays should be considered less sensitive for the diagnosis of acquired TTP and should not be used in isolation [39, 43]. Antigen assays may be useful in the diagnosis of congenital TTP or in conjunction with activity and autoantibody assays.

8.2.3 Analytical Sensitivity

There are no definitive criteria for the diagnostic ADAMTS13 level in TTP; thus, TTP remains a clinical diagnosis. However, the lower limit of detection for most FRET assays is approximately 3–6 % [32, 34], which is compatible with the definition of severe deficiency consistent with TTP (<5–10 %) used in the medical literature [32, 44, 45]. The prevalence of severe deficiency in idiopathic TTP patients has been reported to range from less than 20 to 100 %, highlighting challenges with TTP diagnosis [32, 46]. A highly sensitive FRET assay (limit of detection ≤0.3 % activity) utilizing an optimized fluorogenic substrate, FRETS-rVWF71, has recently been described but is not commercially available [47]. Other activity assays may have better analytical sensitivity than most FRET assays. In one study utilizing an activity ELISA, the authors established a detection limit of 0.5 % ADAMTS13 activity [31], and the detection limit of a mass spectrometry method has also been reported as 0.5 % [34]. Highly sensitive activity assays also allow for high sensitivity in ADAMTS13 functional inhibitor studies. ADAMTS13 antigenic tests may also have sensitivity as low as 0.5 % of normal.

In addition to the severe deficiency usually seen in TTP, it is also important to note that mild to moderate ADAMTS13 deficiency can be seen in a variety of other conditions such as liver disease, acute inflammatory states, disseminated intravascular coagulation, catastrophic antiphospholipid syndrome, and other disorders due to reduced synthesis or consumption of ADAMTS13 [32, 48]. Lower values can also be seen in pregnant women [48] and newborns [49]. Mild to moderate deficiency (rather than severe) or normal ADAMTS13 values can rarely be seen in TTP [32]. Even severe ADAMTS13 deficiency may not be specific for TTP as it has rarely been reported in non-TTP conditions.

8.2.4 Comparison of ADAMTS13 Testing Methods

In 2008, an international collaborative study was performed to evaluate the performance characteristics of ADAMTS13 testing methods (including activity and antigen methods) [38]. The analysis generated data on performance characteristics of 11 methods in eight laboratories, including eight methods representing the newer FRET and ELISA activity methods and antigen methods. The authors concluded that the performance characteristics of newer methods using modified vWF peptides were superior to the more historic methods, such as collagen binding, with

better ability to discriminate between ADAMTS13 levels and improved reproducibility. All methods in the study were able to detect severe deficiency. The between-method variability (imprecision), expressed as CV, was 25 %. This was improved from 40 % in a prior international study primarily using older testing methods [50]. However, between-laboratory variability was relatively high even for labs using the same methodology, suggesting laboratory-specific variables affecting the assays as a source of variation. Another study also showed that the FRETS-VWF73 assay had good concordance with other activity assays [45]. A 2009 study evaluated two commercial FRET activity methods (Technoclone and GTI (now Immucor Gamma)) and compared their assay results to a reference laboratory's method using 29 patient samples [51]. Both commercial kits showed good correlation to the reference method. For the six samples with <5 % ADAMTS13 activity by the reference method (severe deficiency), the Technoclone method identified all six as severely deficient, while the GTI method identified four of six as severely deficient with <5 % activity. The Technoclone kit demonstrated better precision (reproducibility) than the GTI kit; however, it was noted the GTI kit was subsequently modified, which could result in improved precision. In 2011, the same group evaluated two commercial FRET activity methods (Technoclone and GTI (now Immucor Gamma)) and an ELISA activity method (Technoclone) using 38 patient samples and again showed good correlation between the three methods [52]. The Technoclone FRET and ELISA methods identified 12 and 13 severely deficient plasmas (<5 % activity), respectively, while these same samples ranged from 10 to 32 % activity using the GTI kit. The findings suggest that the cutoff for severe deficiency (compatible with TTP) may be different with different kits, and laboratories should be aware of possible differences. In this study, the ELISA activity method demonstrated the best precision (CVs of <10 % across a range of values).

8.2.5 Identification of ADAMTS13 Autoantibodies

Severe ADAMTS13 deficiency is usually due to the formation of autoantibodies which are neutralizing in ~2/3 of cases (inhibit ADAMTS13 proteolytic function) or non-neutralizing in ~1/3 of cases (bind to nonfunctional regions and cause increased clearance by opsonization or other mechanisms resulting in reduced half-life or by interfering with binding to the endothelial cell surface) [53]. Both types of autoantibodies can be simultaneously detected in some TTP patients. Although ADAMTS13 autoantibodies are detected in almost all patients with severe deficiency, they have also been identified in patients with normal ADAMTS13 activity [54]. The concomitant detection of low ADAMTS13 activity and an autoantibody increases the specificity for diagnosis of idiopathic TTP. Autoantibody detection does not only differentiate between hereditary and acquired TTP, but it also can discriminate between neutralizing and non-neutralizing antibodies. Some studies have shown that the presence of autoantibodies at diagnosis with persistence in clinical remission is associated with increased risk of relapse [46]. High titers of autoantibodies have been associated with delayed response to plasma exchange, refractory disease,

and death [46]. While some studies have found no significance for ADAMTS13 IgG levels, others have shown an association between anti-ADAMTS13 IgG subclass 4 and relapse [32].

Neutralizing antibodies (inhibitors) are detected and quantified by the ADAMTS13 inhibitor assay (Bethesda assay) which is a technically difficult LDT that evaluates residual ADAMTS13 activity in a 1:1 mixture of heat-treated patient plasma and normal pooled plasma (NPP) after incubation for 2 h at 37 °C. Inhibitory antibodies present in the patient plasma inhibit ADAMTS13 activity in the NPP. A control mixture (NPP: buffer, simulates absence of an inhibitor) is also incubated for 2 h, and the residual ADAMTS13 activity in the patient:NPP mixture is compared to the residual activity in the control mixture. If an inhibitor is present, recovery of ADAMTS13 activity in the patient mixture is lower than expected. One Bethesda unit (BU) is defined as the amount of inhibitor that decreases residual NPP ADAMTS13 activity to 50 % of the expected value. Evaluation of a series of 1:1 mixtures created from different dilutions of patient sample is usually necessary to identify the reaction with residual activity closest to 50 %. Results are then corrected for the degree of dilution to determine the titer in BU. High dilutions of the patient sample are necessary to quantify high-titer inhibitors, while low dilutions quantify low-titer inhibitors. Laboratories may report a specific Bethesda titer, a semiquantitative titer, or qualitative (inhibitor positive/negative) results, depending on test design. Residual activity can be measured by any of the ADAMTS13 activity assays described previously in this chapter. Of note, heat inactivation of patient plasma is necessary because sensitivity and quantitation of titer are unreliable if the patient sample contains residual ADAMTS13 activity. Figure 8.3 illustrates the concept of the Bethesda assay for identification and quantitation of ADAMTS13 inhibitory antibodies.

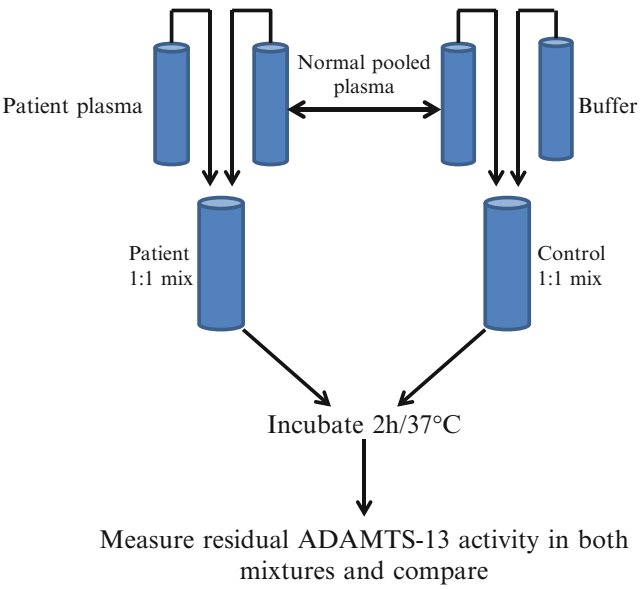

Fig. 8.3 Bethesda assay for identifying and quantifying inhibitory ADAMTS13 autoantibodies

Autoantibodies (IgG or IgM) directed against ADAMTS13 can be measured in serum or plasma samples by sandwich ELISA. In this assay, full-length recombinant ADAMTS13 is immobilized on the surface of an ELISA plate and binds to anti-ADAMTS13 antibodies from the patient sample. After wash steps, bound antibodies are detected by a labeled secondary antibody that participates in a chromogenic reaction. Color development is measured on an ELISA reader at 450 nm. The results are read from a multiple-point calibration curve created from reference plasma containing a high anti-ADAMTS13 titer. Results are expressed in units/mL or arbitrary units (AU)/mL. It is recommended that individual laboratories establish their own normal range and the history of the patient must be taken in consideration for proper interpretation of results. The ELISA for IgG autoantibodies is highly sensitive for idiopathic TTP, but less specific than the Bethesda assay. In one study, IgG antibodies were detected in 97 % of patients with clinical TTP and very low ADAMTS13 activity (<10 %), while the prevalence of IgM antibodies in this population was ~11 %. IgG or IgM antibodies were detected in 20 % of TMA patients with ADAMTS13 activity > 10 %. IgG antibodies were also found in 4 % of healthy donors and a small percentage of subjects with various causes of thrombocytopenia or with systemic lupus erythematosus or antiphospholipid antibody syndrome. Anti-ADAMTS13 IgM antibodies were prevalent in patients with systemic lupus erythematosus and antiphospholipid syndrome (~18 % of cases) and their clinical significance is not clear [54, 55]. The IgG ELISA assay is offered commercially by Technoclone (Vienna, Austria) and Sekisui Diagnostics (Stamford, CT, USA).

As described above, ADAMTS13 antibody ELISA assays are technically easy to perform, are more sensitive but less specific for acquired TTP than functional inhibitor (Bethesda) assays, and do not differentiate between inhibitory and non-inhibitory antibodies [56]. Since neutralizing antibodies are common and more specific for TTP, it is recommended to initiate autoantibody testing with a Bethesda assay. If neutralizing antibodies cannot be detected, ELISA testing is recommended. The suggested testing algorithm for use of ADAMTS13 autoantibody testing in the work-up of TTP is illustrated in Fig. 8.4.

Both Bethesda and ELISA assays detect only free antibodies not bound to ADAMTS13, but not circulating immune complexes (CICs). CICs are a pathophysiologic mechanism for many autoimmune diseases. Some studies have suggested that ADAMTS13-specific CICs may play a role in TTP pathophysiology. A recent study demonstrated CICs in one- to two-thirds of patients with TTP, and the levels of ADAMTS13-specific CICs were independent from other ADAMTS13 measurements. The same study found an association between increasing levels of ADAMTS13-specific CICs and the number of plasma exchanges needed to achieve remission [57]. An ELISA method has recently been developed and validated by an Italian group to detect ADAMTS13-specific CICs, with mean intra- and inter-assay CVs of 5.3 and 9.6 %, respectively [57]. This type of ELISA is not yet commercially available, and the potential usefulness of measuring ADAMTS13-associated CICs in clinical practice warrants further studies.

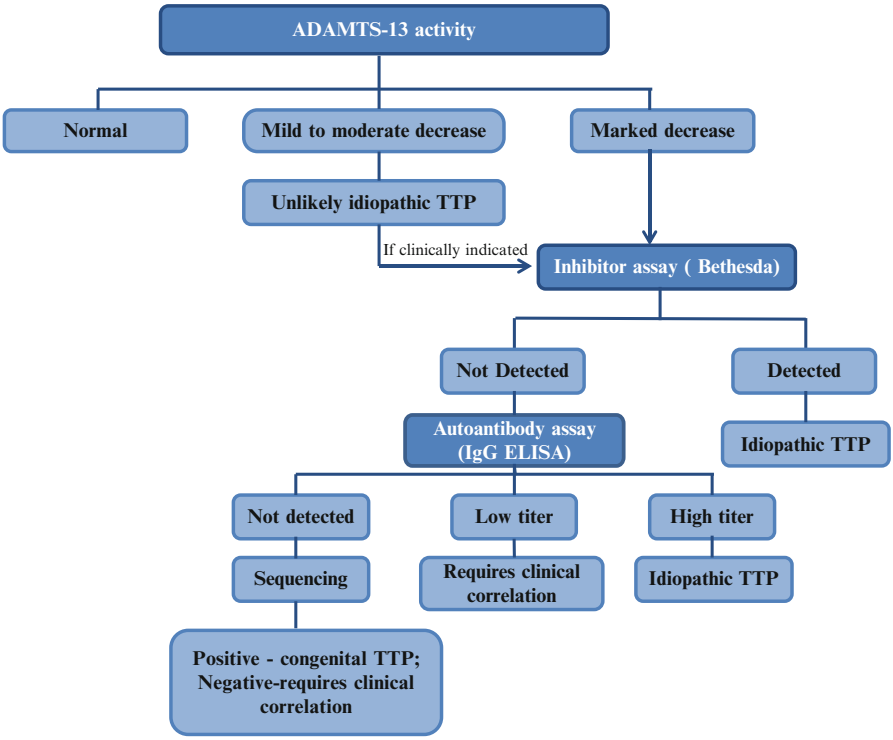

Fig. 8.4 Suggested testing algorithm for evaluating patients with suspected TTP

8.2.6 Interfering Substances

Subjects with microangiopathic hemolytic anemia, such as in TTP, may have hemolyzed or icteric plasma samples due to intravascular hemolysis and organ dysfunction, respectively. Hemoglobin has been reported to have a time- and temperature-dependent inhibitory effect on ADAMTS13 activity at concentrations of ≥2 g/L, which could be seen with marked in vivo intravascular hemolysis or induced by in vitro storage of blood samples [58]. It is important to recognize that hemolyzed samples have the potential to cause underestimation of ADAMTS13 activity and false-positive functional inhibitor studies (neutralizing antibodies identified in Bethesda assays). This does not represent assay interference and is independent of the assay method used. Hyperbilirubinemic plasmas have been reported to demonstrate underestimation of ADAMTS13 activity through interference in FRET assays using FRETS-VWF73 by acting as a quencher at the fluorescent emission wavelength [59, 60]. The interference is less pronounced with moderately elevated bilirubin concentrations, but significant at levels >100 μmol/L, which could lead to misdiagnosis of severe ADAMTS13 deficiency or false-positive Bethesda studies.

Commercial FRET assays that read ADAMTS13 activity in solution often require dilution of the patient sample prior to testing, which minimizes assay interferences from hemoglobin and bilirubin, but also limits sensitivity in activity and inhibitor assays. Recently, a novel recombinant fluorogenic substrate, FRETS-rVWF71, has been described. This substrate demonstrates little assay interference from hemolyzed or icteric samples, permitting testing on minimally diluted plasma and allowing for highly sensitive FRET assays (activity and inhibitor) [47]. Because ELISA assays (activity or antigen) and the Technoclone FRET assay involve wash steps that remove patient plasma from the reaction well prior to reading the results, colored substances such as hemoglobin and bilirubin in the patient sample should not interfere with the colorimetric results. These substances also should not interfere with ELISA testing for ADAMTS13 autoantibodies.

8.3 Clinical Utility of ADAMTS13 Testing

There is no gold standard test for ADAMTS13 activity, and TTP remains a clinical diagnosis defined as microangiopathic hemolytic anemia and thrombocytopenia without an alternative cause [46]. However, many now consider evaluation of ADAMTS13 activity to be a valuable tool in the clinicopathologic diagnosis of TTP, with absent activity having high specificity and relatively high sensitivity. For example, in one study using a FRETS-VWF73 assay, absence of ADAMTS13 activity was 89 % sensitive and 100 % specific for TTP [61]. In idiopathic TTP, severe ADAMTS13 deficiency correlates with a good response to plasma exchange therapy (~90 %), but also with an increased risk of relapsing TTP (~30 %), while relapse is uncommon in idiopathic TTP without severe deficiency [46]. Persistence of severe deficiency while in clinical remission may also be predictive of relapse [56]. Identification of ADAMTS13 autoantibodies at diagnosis also correlates with a higher risk of relapse, and antibody titer may correlate with delayed response to plasma exchange and refractory disease [46].

8.4 Genetic Testing

Genetic testing to assess for disease-causing mutations in the ADAMTS13 gene may be useful in suspected cases of congenital TTP (Upshaw–Schulman syndrome), where at least 76 mutations have been described to date [62]. The testing is available in select reference and research laboratories. Congenital TTP is rare (~5 % of TTP cases) [62] and may be suspected when recurrent TTP presents in childhood (although up to 20 % of cases do not present until adulthood) or in subjects with severe ADAMTS13 deficiency that do not have demonstrable neutralizing or non-neutralizing antibodies [32]. Affected individuals have homozygous or compound heterozygous mutations resulting in severe deficiency, while carriers have

approximately 50 % of normal activity and are asymptomatic [62]. Genetic testing entails amplification and sequencing of the ADAMTS13 gene, located on the long arm of chromosome 9 at 9q34 [32]. Genetic sequencing determines the order of nucleotides in DNA and is the best method to identify ADAMTS13 mutations, since mutations have been found throughout the gene, which has 29 exons (coding sequences) [62]. The sequence from the patient's sample is compared to a reference sequence. Any abnormalities identified are compared to mutation databases and reports in the medical literature to determine whether they are disease-causing mutations. Although the analytical sensitivity of these tests is >99 %, large deletions or duplications or mutations outside of the sequenced region will not be detected. In summary, genetic testing is useful to differentiate acquired from congenital TTP, to confirm inherited TTP, to evaluate potentially affected family members (including prenatal diagnosis), and to establish genotype–phenotype correlations.

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