George M. Rodgers *Editor*

ADAMTS13

Biology and Disease

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Editor
George M. Rodgers
Division of Hematology
University of Utah Health Sciences Center
Salt Lake City, UT, USA

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Preface

Ninety-one years ago, in 1924, Dr. Eli Moschowitz, a pathologist at Beth Israel Hospital in New York City, reported the first recognized case of thrombotic thrombocytopenic purpura (TTP). The patient developed an acute illness and on laboratory evaluation had anemia and proteinuria, but the blood urea nitrogen and creatinine tests were normal. Over a period of several days, the patient developed progressive neurologic impairment and died. At autopsy, Dr. Moschowitz noted widespread hyaline thrombosis of the terminal arterioles and capillaries. In his publication, Dr. Moschowitz concluded that death "resulted from some powerful poison which had both agglutinative and hemolytic properties." Almost 60 years elapsed before Moake and colleagues described unusually large von Willebrand factor multimers in patients with TTP, and over a decade later, Furlan et al. reported deficiency of a von Willebrand factor "depolymerase" in TTP.

Finally, in 2001 the vWF-cleaving protease was identified as ADAMTS13 (9); patients with inherited TTP have mutations in the ADAMTS13 gene, while patients with acquired TTP have antibodies to ADAMTS13. Thus, the "powerful poison" initially suggested by Dr. Moschowitz turns out to be ULvWF multimers, which in the absence of ADAMTS13 processing result in disseminated platelet thrombosis, thrombocytopenia, hemolysis, and organ dysfunction.

This book is written to provide interested readers a current overview of ADAMTS13 from perspectives of both basic sciences and clinical medicine. Drs. Lammle and von Auer summarize the history and background information leading to the discovery of ADAMTS13. Dr. Apte next provides an overview of the ADAMTS13 superfamily of proteins. Dr. Zheng then focuses on ADAMTS13 structure and function. Next, Dr. Lee provides data suggesting that ADAMTS13 may have additional biologic activities, such as regulating normal and malignant angiogenesis.

The last half of the book emphasizes the clinical importance of ADAMTS13 and its deficiency states. Dr. Fujimura and colleagues discuss the uncommon inherited disorder of ADAMTS13 deficiency (Upshaw-Schulman syndrome), and Dr. Tsai

summarizes acquired ADAMTS13 deficiency (TTP). Dr. Bentley discusses thrombotic microangiopathies and how TTP can be distinguished from related disorders. Assays of ADAMTS13 are reviewed by Drs. Smock and Heikal, and the potential role of recombinant ADAMTS13 and its variants in therapeutics is presented by Dr. Ferrari and colleagues.

I give thanks to my many contributors for their excellent chapters. I also acknowledge Aleta Kalkstein and Michael Koy of Springer for their efforts in publication of this book. Ryan Dest is acknowledged for administrative assistance.

Salt Lake City, UT, USA George M. Rodgers, MD, PhD

Contents

Contributors

Suneel S. Apte, M.B.B.S., D.Phil. Department of Biomedical Engineering (ND20), Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

Melissa J. Bentley, M.D., F.A.C.P. Ogden Internal Medicine, Ogden, UT, USA

Silvia Ferrari Baxalta Innovations GmbH, Vienna, Austria

Yoshihiro Fujimura, M.D. Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan

Nahla M. Heikal, M.D. Department of Pathology, University of Utah Health Sciences Center and ARUP Laboratories, Salt Lake City, UT, USA

Ayami Isonishi Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan

Koichi Kokame Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Osaka, Japan

Bernhard Lämmle Center for Thrombosis and Hemostasis, University Medical Center, Mainz, Germany

Former Director of University Clinic of Hematology, Emeritus Professor of Hematology, Inselspital, University Hospital and University of Bern, Bern, Switzerland

Manfai Lee, Ph.D. Division of Hematology and Hematologic Malignancies, Huntsman Cancer Institute, University of Utah Health Sciences Center, Salt Lake City, UT, USA

Masanori Matsumoto Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan

Toshiyuki Miyata Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Osaka, Japan

George M. Rodgers, M.D., Ph.D. Division of Hematology, University of Utah Health Sciences Center, Salt Lake City, UT, USA

Hanspeter Rottensteiner Baxalta Innovations GmbH, Vienna, Austria

Friedrich Scheiflinger Baxalta Innovations GmbH, Vienna, Austria

Kristi J. Smock, M.D. Department of Pathology, University of Utah Health Sciences Center and ARUP Laboratories, Salt Lake City, UT, USA

Han-Mou Tsai, M.D. iMAH Hematology Associates, New Hyde Park, USA

Charis von Auer Center for Thrombosis and Hemostasis, University Medical Center, Mainz, Germany

Hideo Yagi Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan

X. Long Zheng, M.D., Ph.D. Division of Laboratory Medicine, Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL, USA

Chapter 1
History of Thrombotic Thrombocytopenic Purpura and the von Willebrand Factor–Cleaving Protease, ADAMTS13

Bernhard Lämmle and Charis von Auer

1.1 Thrombotic Thrombocytopenic Purpura: The Initial Case Report, the Name of the Disease, and the Diagnostic Pentad of Clinical and Laboratory Findings

In 1924, Dr. Eli Moschcowitz reported on a 16-year-old previously healthy girl who died after 1 week of hospitalization for an acute illness with high fever, pallor, pain in the arms, a few petechiae on her skin, evolving paralysis of the left arm and leg, and preterminal coma [1]. The autopsy showed multiple "hyaline" thrombi in many arterioles and capillaries of the heart muscle, of the congested spleen, and of the kidneys [1]. At that time, the "hyaline" microvascular thrombi were thought to be caused by red blood cell agglutination, probably brought about by a powerful toxin with hemolytic and agglutinating properties. In an additional report on the same case [2], Moschcowitz mentioned that Dr. Max Lederer of Brooklyn had seen four similar patients and that all of them had recovered after a single blood transfusion.

Singer et al. [3], in 1947, described an 11-year-old white girl hospitalized for weakness, fever, and hemorrhagic tendency following an upper respiratory infection 2 weeks before. Her course rapidly deteriorated; she had high fever, became

B. Lämmle (✉)
Center for Thrombosis and Hemostasis, University Medical Center,
Langen Beck Strasse 1, Mainz 55131, Germany

Former Director of University Clinic of Hematology, Emeritus Professor of Hematology,
Inselspital, University Hospital and University of Bern, Bern, Switzerland
e-mail: Bernhard.laemmle@uni-mainz.de

C. von Auer
Center for Thrombosis and Hemostasis, University Medical Center,
Langen Beck Strasse 1, Mainz 55131, Germany

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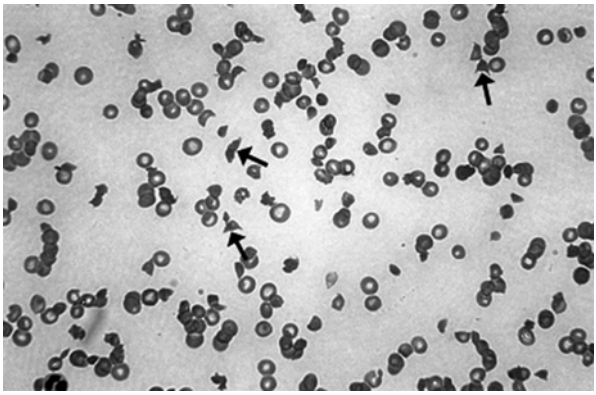

Fig. 1.1 Peripheral blood smear from a patient with acute TTP showing many fragmented erythrocytes (schistocytes) (*arrows*) and severe thrombocytopenia (reproduced with permission from B. Lämmle et al. (2005) J Thromb Haemost 3:1663–1675)

incoherent, developed transient dizziness and paresthesias, fell into coma, and died. Laboratory findings had shown severe anemia with a high reticulocyte count of 15 % and severe thrombocytopenia of 6000/μL. A sternal puncture had revealed marked erythroid hyperplasia and increased numbers of megakaryocytes, compatible with hemolysis and platelet consumption, respectively. Similar to Moschcowitz' case, the autopsy showed innumerable thrombi in capillaries, arterioles, and smaller arteries of the heart, lung, liver, spleen, kidneys, brain, and other organs [3]. In this report, the small vessel "hyaline" thrombi were suggested to consist of thrombocytes and some fibrin. Singer et al. found only 11 similar reported cases, including Moschcowitz' patient, with thrombocytopenia associated with multiple small vessel platelet thrombi. These authors suggested that they were dealing with a specific disease entity and proposed to designate it "thrombotic thrombocytopenic purpura" to highlight the presumed pathogenesis, namely, thrombocytopenia being caused by massive platelet consumption in the microvascular thrombotic process [3].

In 1966, Amorosi and Ultmann [4] reviewed 255 published patients having been diagnosed with thrombotic thrombocytopenic purpura (TTP) and added their own observations in 16 additional cases. They established a diagnostic pentad of clinical and laboratory findings: (1) hemolytic anemia with striking morphologically visible fragmentation of erythrocytes in the blood smear (schistocytes, helmet cells) (Fig. 1.1), (2) thrombocytopenia by consumption, (3) neurologic manifestations, (4) renal dysfunction, and (5) fever. They referred to the typical autopsy findings of microvascular thrombosis in any organ, especially the heart, brain, kidney, pancreas, and adrenals, and noted a striking absence of inflammatory changes in the involved vessels and rather limited areas of infarctions. As opposed to Singer et al. [3] suggesting that the "hyaline" thrombotic material consisted mainly of platelets, Amorosi and Ultmann [4] doubted on its nature. They stressed the unknown pathophysiology and dismal prognosis of TTP, affected patients usually showing a progressive disease course, the majority dying within 3 months.

1.2 A Congenital Form of Microangiopathic Hemolysis and Thrombocytopenia

Dr. Jefferson D. Upshaw, in 1978, described a 29-year-old woman who from the age of 6 months to 12 years had each year about 6–10 episodes of acute illness with fever, petechial rash, severe thrombocytopenia, and hemolytic anemia with microangiopathic changes in the red cell morphology on blood smears [5]. She had been repeatedly hospitalized and treated with antibiotics and blood transfusions with dramatic responses, usually recovering within 48 h. Corticosteroid treatment and splenectomy, performed at the age of 2 years, were not helpful. At the age of 12 years, the frequency of these acute disease episodes decreased to about 3–4 per year with completely asymptomatic intervals varying between 3 weeks and 20 months. Laboratory investigation during an acute attack showed severe thrombocytopenia (e.g., 3000/μL) and anemia (e.g., hematocrit of 19 %, marked reticulocytosis, and schistocytes on the blood smear). After transfusion of 2 units of packed red blood cells, she showed a partial response with platelet count stabilizing at ~50,000/μL and hematocrit at 32 %. One month later, she received, for a similar acute attack, 2 units of fresh whole blood which led to a complete normalization of the platelet count (278,000/μL) and hematocrit (41 %) within 48 h. This observation of a much better response to whole blood as compared to packed red cell concentrates suggested that a plasma factor was responsible for the hematologic correction. When readmitted with the next acute disease flare-up, she received 2 units of fresh platelet-poor plasma and showed a continuous rise of the platelet count, measurable at 20 h and peaking at 9 days with a value of 550,000/μL. During 11 years of consecutive follow-up, this young woman had 32 episodes of thrombocytopenia and microangiopathic hemolysis, sometimes occurring spontaneously but more often being triggered by a precipitating factor, e.g., a preceding (mild) infection, operation, pregnancy, fecal impaction, or pancreatitis. The patient always responded to fresh frozen plasma (FFP) transfusion with normalization of her platelet count and hematocrit and needed only a total of 5 units of red blood cell concentrates during 11 years of observation [5] (Fig. 1.2). Upshaw concluded that his case resembled that reported by Schulman et al. in 1960 [6] but, in contrast to these latter authors' conclusion, was not due to "thrombopoietin" deficiency but rather to a constitutional deficiency of a factor present in normal plasma that protected from microangiopathic hemolysis and thrombocytopenia by peripheral consumption. Upshaw also noted the similarity of his patient's hematologic picture with that observed in acute TTP [5].

1.3 Pathophysiological Hypotheses, Discovery of von Willebrand Factor (VWF)–Cleaving Protease, and Its Deficiency in TTP

Many different hypotheses on the etiology and pathogenesis of TTP have been proposed by various researchers over the years (for reviews, the reader is referred to Refs. [7–9]).

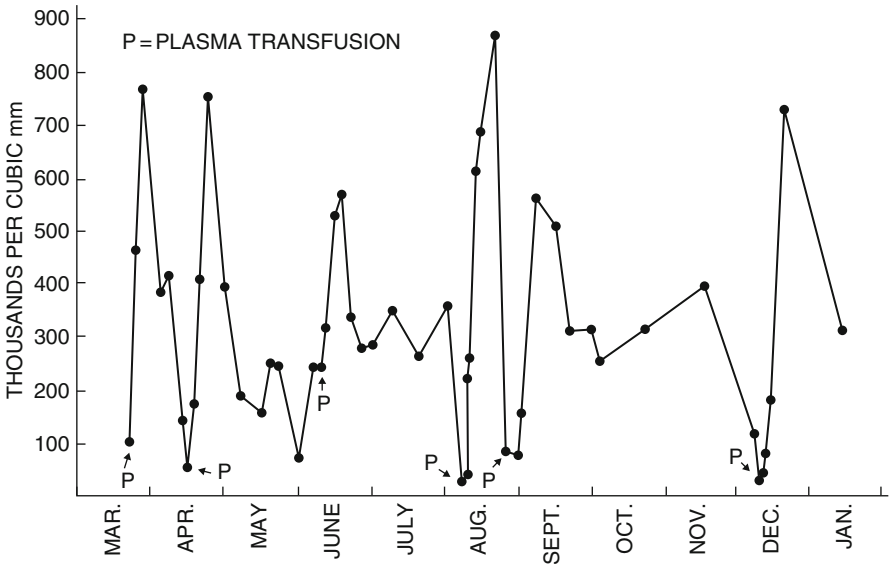

Fig. 1.2 Course of platelet count (10^{-3}/μL) over a 10-month period in the patient with chronic relapsing thrombocytopenia and hemolysis reported by J. D. Upshaw in 1978 [5]. Reproducible (often overshooting) normalization of the platelet count occurs within a few days after plasma transfusions (P). From Upshaw JD (1978) N Engl J Med 298:1350–1352, with permission

Endothelial injury, for instance, by oxidative stress, or reduced fibrinolytic activity of the vessel wall [10] has been described in TTP patients. A putative plasma factor stimulating the endothelial cell synthesis and release of prostacyclin (prostaglandin I_2), a potent inhibitor of platelet aggregation, was suggested to be missing in patients with hemolytic uremic syndrome (HUS) and TTP [11]. A 37 kDa protein agglutinating normal platelets [12] or a 59 kDa protein aggregating platelets from healthy donors in the presence of Ca^{++} and fibrinogen [13] were isolated from the plasma of a few patients with acute TTP. A calcium-dependent cysteine protease was found in the sera of 15 patients during an acute bout of TTP but not during remission and was identified to activate platelets [14]. This protease was later identified to be calpain, associated with platelet microparticles and therefore resistant to plasma inhibitors [15]. Calpain cleaves platelet glycoprotein Ib thereby impairing VWF binding to this receptor and also cleaves the VWF subunit thereby promoting VWF binding to glycoprotein IIb/IIIa on activated platelets, enhancing platelet aggregation [16]. Anti-endothelial cell and, more specifically, anti-glycoprotein IV (CD36) antibodies have been found in plasma samples of most patients diagnosed with acute TTP [17, 18]. CD36 is an integral membrane protein located on microvascular endothelial cells, platelets, as well as some other cells. It has been suggested that anti-glycoprotein IV antibodies could be pathogenetically relevant for acute TTP by causing microvascular endothelial damage as well as platelet activation [17]. A more recent study by Raife et al. [19], however, found only minimal

evidence for the presence of circulating endothelial cell- and platelet-reactive antibodies in plasma samples of TTP patients. Another group of researchers reported that plasma of patients with idiopathic TTP as well as human immunodeficiency virus-associated thrombotic microangiopathy (TMA) was able to induce apoptosis of microvascular endothelial cells but not of human umbilical vein endothelial cells [20] and circulating endothelial cells were detected in blood samples of TTP patients [21].

In 1982, Moake et al. reported the presence of unusually large (UL) VWF multimers in the plasma of four patients, including the abovementioned case reported by Upshaw in 1978 [5], with a chronic relapsing form of TTP [22]. The ULVWF multimers, similar in size to those secreted by cultured endothelial cells, were present mainly in plasma samples obtained during remission and tended to disappear during acute disease relapses (Fig. 1.3), probably by consumption of these extremely adhesive multimers during the microvascular platelet clumping process. Moake et al. suggested that the deficiency of an unknown VWF depolymerase, either a protease or a disulfide reductase, was causing the defective VWF processing leading to the relapsing course of TTP due to microvascular platelet thrombosis with ischemic organ manifestations by the extremely adhesive ULVWF multimers [22].

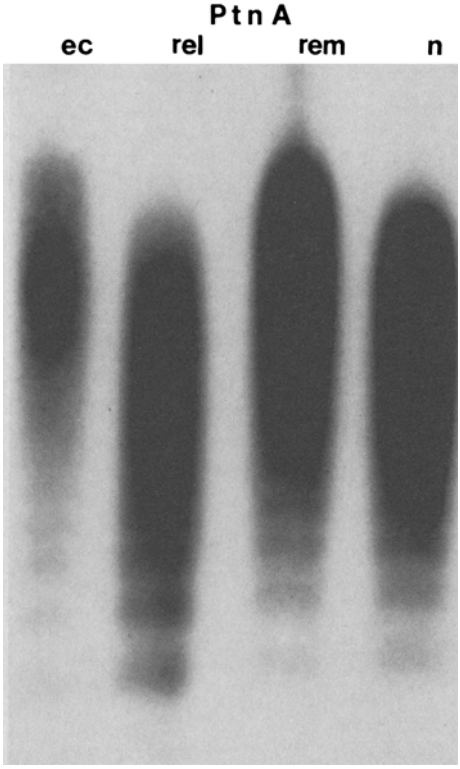

Fig. 1.3 SDS–agarose gel electrophoresis for the analysis of VWF multimer distribution using autoradiography in plasma samples of patient A with chronic relapsing TTP reported by J. L. Moake et al. (1982) N Engl J Med 307:1432–1435 with permission. Patient A displays unusually large VWF multimers during remission (rem, *upper part*), similar to the endothelial cell culture supernatant (ec). Upon acute TTP relapse (rel), the ULVWF multimers disappeared, presumably by consumption during the microvascular thrombotic process. Normal plasma (n) VWF multimers are shown for comparison

In 1996, Furlan et al. [23] and Tsai [24] simultaneously and independently described a specific VWF–cleaving protease isolated from normal plasma. This isolated protease was not inhibited by inhibitors of serine proteases, cysteine proteases, or metalloproteases. Ca^{++} or Ba^{++} ions were needed to "activate" the VWF–cleaving protease isolated from citrated plasma [23], and mild denaturation of VWF by low-ionic-strength buffer containing 1.5 M urea [23] or 1.1–1.2 M guanidinium chloride [24] made the VWF susceptible to proteolytic cleavage by this newly identified protease. Tsai and coworkers [24, 25] further demonstrated that high fluid shear stress enhanced VWF proteolysis. This newly discovered VWF–cleaving protease received considerable interest also for the fact that it cleaved the VWF subunit in vitro at the peptide bond 842 tyrosine–843 methionine (amino acid numbering including the VWF propeptide: 1605 Tyr–1606 Met) [23], the peptide bond earlier reported to be physiologically cleaved during in vivo processing of VWF [26].

This specific VWF–cleaving protease gained widespread attention when we reported four patients, including two brothers (patients A1 and A2) and two unrelated patients (B and C), with a chronic relapsing form of TTP who showed a severe, probably constitutional deficiency of the VWF–cleaving protease activity in 1997 [27] (Fig. 1.4).

Both brothers had a history of recurrent TTP bouts over many years, including severe thrombocytopenia, schistocytic hemolytic anemia, and—in the elder brother—transient severe renal insufficiency and severe neurologic involvement with multiple cerebral infarctions. Both received intermittent plasma exchange (PEX) treatments with FFP replacement which reproducibly led to a transient normalization of the hematologic parameters [27] which is reminiscent of the abovementioned patient described by Upshaw [5]. Mixing the patient plasma samples of patients A1, A2, B, and C, respectively, with normal plasma (1:1, v:v), did not reveal an inhibition of the VWF–cleaving protease in normal plasma, and given that the parents of patients A1 and A2 had a slightly decreased VWF–cleaving protease activity, we suggested that at least patients A1 and A2 might have an inherited severe deficiency of the VWF–cleaving protease and that the resulting hyperadhesive ULVWF multimers were a pathogenetically relevant factor for the recurrent TTP in these patients [27].

In the following year, we reported another patient with recurrent episodes of TTP who was followed for 400 days with repeated laboratory analyses [28]. During his first acute TTP episode, he had a severe deficiency of the VWF–cleaving protease. After prolonged treatment with daily PEX sessions, FFP replacement, and corticosteroid, vincristine, and iloprost treatment, the platelet count finally normalized after more than 1 month, and in parallel, the VWF–cleaving protease activity progressively normalized. During the further course, VWF–cleaving protease activity redisappeared completely about 4 months after disease onset, and the patient suffered from an acute TTP relapse on day 221 which necessitated resumption of PEX and corticosteroid therapy. A second relapse with resumption of plasma therapy occurred on day 330. In the meantime, we had identified a circulating inhibitor of the VWF–cleaving protease in the plasma obtained at onset of the first TTP bout and in the plasma samples obtained when the protease redisappeared 4 months after the

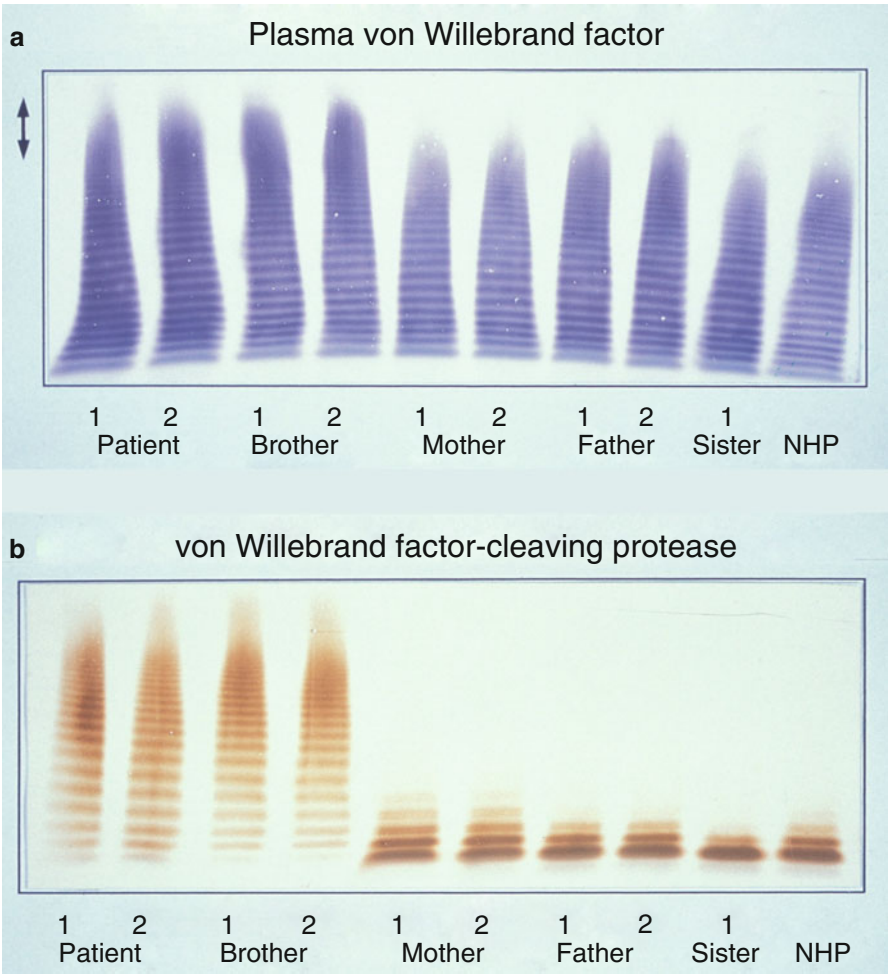

Fig. 1.4 (**a**) VWF multimer analysis by SDS–agarose gel electrophoresis and immunoblotting in two brothers with chronic relapsing TTP (patient, brother), using two plasma samples each (1, 2) obtained several weeks apart. In contrast to samples from family members (mother, father, sister) and normal human plasma (NHP), both patients showed ULVWF multimers (*double arrow*). (**b**) VWF–cleaving protease assay using purified plasma-derived VWF substrate that had been incubated overnight with diluted plasma samples of patients (patient, brother), family members (mother, father, sister), or normal plasma (NHP), each preactivated by barium chloride. VWF substrate–plasma mixtures were dialyzed overnight against low-ionic strength buffer containing 1.5 M urea before SDS–agarose gel electrophoresis and immunoblotting. Both plasma samples (1, 2) of the two patients (patient, brother) had a completely lacking VWF–cleaving protease activity; mother and father had a mildly reduced activity (barely visible for father's plasma samples), whereas sister's plasma showed normal activity. NHP denotes normal pooled human plasma. Figures (**a**) and (**b**) are modified from M. Furlan et al. (1997) Blood 89:3097–3103

initial episode. This inhibitor was identified to be an IgG autoantibody, strongly inhibiting VWF proteolysis by VWF–cleaving protease in vitro. At this time point, we decided to perform a splenectomy on day 365 which led to the disappearance of the IgG inhibitor, normalization of the VWF–cleaving protease activity, and persistent normalization of platelets and hemoglobin [28]. At the time of this writing, the patient is in continuing remission without any therapy about 18 years after his initial disease bout.

In 1998, two independent studies, published in the same issue of the *New England Journal of Medicine*, demonstrated that a severe deficiency of the VWF–cleaving protease activity was not an anecdotal finding but a rather general feature of patients diagnosed with acute idiopathic TTP [29, 30]. Furlan et al. found a severe VWF–cleaving protease deficiency in 20 of 24 patients with acute nonfamilial TTP and moderate deficiency in the remaining four subjects. An inhibitor was identified in 20 of these patients. In addition, 6 patients (3 pairs of siblings) with a familial TTP had a severe VWF–cleaving protease deficiency without inhibitor, whereas all 23 patients diagnosed with familial ($n = 10$) or nonfamilial ($n = 13$) atypical hemolytic uremic syndrome had normal (in 2 instances slightly decreased) VWF–cleaving protease activity [29]. Tsai and Lian similarly found a severe VWF–cleaving protease deficiency in all 37 patients with acute TTP, whereas no deficiency was found in remission samples and a series of hospital control samples. IgG inhibitors were detected in 26 of the 39 plasma samples obtained from 37 patients with acute TTP [30].

These findings of a severe VWF–cleaving protease deficiency in acute TTP have been heavily criticized as completely unspecific [31] or as also occurring in various inflammatory conditions, liver cirrhosis, and uremia, in later stages of pregnancy, or in newborns [32], but the methodology of measuring this enzyme was not using a proper standard curve [31], or the authors considered samples with mildly or moderately decreased VWF–cleaving protease as "deficient" samples [32]. In a prospective study on 68 hospitalized patients with thrombocytopenia of various causes (except TMAs), we found 12 of the 68 patients having VWF–cleaving protease activity $\leq$30 % of the normal, but none with a severe deficiency of <5 % [33]. Therefore, a severely deficient VWF–cleaving protease activity was considered to be a rather specific feature of TTP.

In 2001, 5 years after the initial isolation of VWF–cleaving protease from plasma [23, 24], three research teams had succeeded in purifying the protease from plasma to homogeneity and performed N-terminal amino acid sequence analysis [34–36]. Based on the partial amino acid sequence, Zheng et al. were able to identify VWF–cleaving protease as a new member of the ADAMTS (*a d*isintegrin *a*nd *m*etalloprotease with *t*hrombo*s*pondin type 1 motifs) family of metalloproteases, denoted as ADAMTS13, and to locate the respective gene to chromosome 9q34 [37]. At the same time, Levy et al. reported their genome-wide linkage analysis in four pedigrees with patients suffering from a constitutional TTP and severe VWF–cleaving protease deficiency [38]. They identified the same gene on chromosome 9q34, belonging to the ADAMTS family of zinc metalloproteases, *ADAMTS13*. In their investigated families, the authors studied patients with a severe ADAMTS13

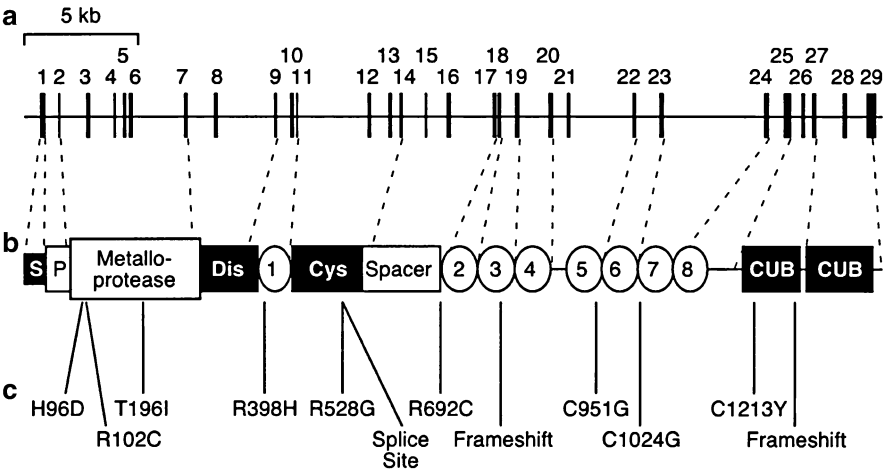

Fig. 1.5 ADAMTS13 gene structure (**a**), protein domain structure (**b**), and ADAMTS13 mutations (**c**) identified in patients with hereditary TTP by G. G. Levy et al. (2001) Nature 413: 488–494. The 29 vertical bars in panel a denote exons. In panel b, *S* denotes signal peptide, *P* propeptide, *Dis* disintegrin domain, *1* first thrombospondin type 1 domain, *Cys* cysteine-rich domain, *2–8* seven additional thrombospondin type 1 domains, *CUB* Cub domain. In panel c, missense, splice site, and frameshift mutations located over the whole gene are shown, exchanges of amino acids labeled in single-letter code. Figure reproduced by courtesy from X.L. Zheng et al. (2002) Current Opinion in Hematology 9:389–394, with permission

deficiency, asymptomatic carriers with about half-normal activity, and subjects with normal VWF–cleaving protease activity and were able to identify 12 different putative *ADAMTS13* mutations explaining 14 of the 15 disease alleles [38] (Fig. 1.5).

Thus, the missing plasma factor in Upshaw's patient suffering from chronic relapsing constitutional TTP [5] was identified as ADAMTS13 23 years later. Plaimauer et al. expressed a functionally active human ADAMTS13 in mammalian cells in 2002 [39] which corrected the lacking VWF proteolytic processing when added to the plasma of the abovementioned two brothers with severe constitutional VWF–cleaving protease deficiency identified in 1997 [27, 40].

For further insights into the fascinating discovery of the VWF proteolytic processing and its defect as a main pathogenetic factor in TTP, the reader is referred to historical sketches and personal annotations of three protagonists in the field, Drs. Miha Furlan, Han-Mou Tsai, and Joel Moake [41–43].

1.4 Therapeutic Efforts in TTP

Here, we present some important aspects on the development of the therapeutic strategies in TTP and refer the reader to some earlier reviews highlighting various aspects of treatment of this devastating disease [44–48]. Until the 1960s and early

1970s, TTP was almost universally fatal, and patients usually died out of good general health after a progressive disease course over days to a few weeks [4]. In 1959, Rubinstein et al. reported "an unusual remission in a case of thrombotic thrombocytopenic purpura syndrome following fresh blood exchange transfusions" [49]. In the late 1970s, reports on a few patients suggested a beneficial effect of exchange transfusions, PEX, and replacement using FFP [50, 51]. This largely empirical treatment was based on the assumption that either a toxic substance needed to be removed from the plasma or alternatively a missing substance was to be replaced by infusion of fresh plasma. Despite a lacking pathophysiologic basis underlying plasma therapy, the clinically evident drastically improved survival of patients with acute TTP led to a rather widespread use of PEX and/or FFP infusion. In 1987, Shepard and Bukowski reviewed the accumulated experience with exchange transfusions, FFP infusions, and PEX in TTP patients and concluded that a randomized clinical trial comparing various forms of plasma therapy would be highly desirable but difficult to perform and that at the present time they favored the initial use of PEX with FFP replacement [52]. In 1991, the Canadian Apheresis Study Group published such a randomized controlled trial comparing PEX with FFP replacement versus simple FFP infusion in TTP patients [53]. In this landmark study on 102 patients, Rock et al. demonstrated that PEX of 1.5 plasma volumes daily for 3 days, followed by PEX of 1 volume daily and FFP replacement, was superior to FFP infusion alone, response rates after the first treatment cycle and at 6 months being 47 and 78 %, respectively, for PEX/FFP versus 25 and 49 %, respectively, for FFP infusion. The mortality at 6 months was 22 % in the PEX/FFP group versus 37 % in the FFP infusion group which was significantly different [53]. These findings were supported by an accompanying single-center/single-protocol cohort study that suggested, in addition, that corticosteroid treatment may be an important adjunct to PEX/FFP and sometimes be effective if given alone without any plasma therapy in milder cases of TTP [54].

Evidently, the discovery of autoantibody-mediated severe ADAMTS13 deficiency in acquired TTP [9, 29, 30, 55] and of homozygous or double heterozygous *ADAMTS13* mutations in hereditary TTP [38, 40, 56] as the relevant pathogenetic factors gave some rationale to the various forms of plasma therapy. Thus, for the rare patients suffering from a hereditary TTP, also denoted as Upshaw–Schulman syndrome, caused by biallelic *ADAMTS13* mutations, simple FFP infusion may be effective therapeutically and prophylactically when given every 2–3 weeks [9, 57], sometimes over many years without any attacks of TTP [58]. Erroneous diagnoses of immune thrombocytopenic purpura or Evans syndrome in children with Upshaw–Schulman syndrome are common [59] and may lead to wrong therapeutic measures such as steroid therapy or even splenectomy as was already performed in the index case without any benefit [5]. Missing a timely diagnosis in a seemingly "hyperactive child" was associated with severe neurological deficits and multiple cerebral infarctions at the time of diagnosis in a 3-year-old boy [60]. In another instance, we diagnosed hereditary TTP postmortem in a 7-year-old boy who had suffered from hemolytic anemia and thrombocytopenia since birth [61].

For the more common patients with acquired TTP, large-volume PEX with replacement of FFP may both remove IgG autoantibodies and replace ADAMTS13 [46], and corticosteroids may suppress autoantibody production. Splenectomy had been empirically performed in earlier years before plasma therapy was recognized to be useful and also later in plasma-resistant or relapsing TTP patients [62]. Thirty-three patients with acquired TTP, subjected to splenectomy because of plasma refractoriness ($n=9$) or because of TTP relapse ($n=24$) from 1982 to 2002, seemed to benefit substantially from this procedure: Splenectomy generated prompt remissions in all but 5 patients, and in those with relapsing TTP the relapse rate decreased from 0.74 relapses/patient-year before to 0.10 relapses/patient-year after splenectomy with long-term observation over many years before and after the procedure [62].

Many other treatment strategies have been used, including vincristine; the anti-CD20 monoclonal antibody rituximab with the intent to inhibit autoantibody production by B-lymphocytes [63]; protein A immunoadsorption, either empirically [64] or intending to remove anti-ADAMTS13 autoantibodies [46]; cyclosporin instead of corticosteroids for autoantibody suppression [65]; an aptamer [66] or a nanobody [67] binding to the "activated" A1 domain of VWF and thereby inhibiting VWF–platelet glycoprotein Ib interaction; and, very recently, *N*-acetylcysteine, intended to disulfide-reduce ULVWF multimers [68]. It is out of the scope of this chapter to discuss these therapeutic means in more detail.

1.5 Differential Diagnosis of TTP, HUS, and Other TMAs

Today, various tentative entities of TMAs are recognized; a categorization of the most common forms is shown in Table 1.1.

Here, we do not discuss the historical aspects of all these TMAs but ask the question whether TTP and HUS, first described by Gasser et al. in 1955 [69], are different disease entities or mere variants of the same disease process (see also Refs. [45, 48]). Furthermore, we try to assess the sensitivity and specificity of the laboratory finding of a severe ADAMTS13 deficiency for the clinical diagnosis of TTP. When Furlan et al. [29] and Tsai and Lian [30] reported severe VWF–cleaving protease deficiency in 26/30 patients [29] and 37/37 patients [30] with acute TTP but normal (or mildly reduced) VWF–cleaving protease activity in 23/23 patients with a diagnosis of (atypical) HUS [29], a rather strict segregation of TTP with severe ADAMTS13 deficiency and (atypical) HUS with normal (or mildly decreased) ADAMTS13 deficiency was apparent. This was rather surprising given the often difficult clinical distinction in individual patients. Table 1.2 summarizes some earlier cohort studies from different centers on patients having been clinically diagnosed with acute TTP [29, 30, 70–76].

It is evident from these case series that only about 60–70 % (range 33–100 %) of the patients with a clinical diagnosis of TTP had a severely deficient ADAMTS13 activity [55]. Therefore, the sensitivity of finding a severely decreased ADAMTS13

Table 1.1 Overview of the most common forms of thrombotic microangiopathies

Thrombotic microangiopathies 2015
• **Hereditary TTP** with **severe constitutional ADAMTS13 deficiency** (Upshaw–Schulman syndrome)
• **Acquired idiopathic TTP** with **severe acquired ADAMTS13 deficiency**
• Acquired idiopathic TTP **without** severe ADAMTS13 deficiency
• **TMAs** associated with hematopoietic stem cell transplantation, disseminated neoplasia, drugs (e.g., mitomycin C, ticlopidine, cyclosporin), pregnancy (including HELLP), HIV infection, severe hypertension, connective tissue disease (e.g., SLE)
• **Atypical HUS** (often regulatory protein defects or hyperfunctional mutations of alternative complement pathway)
• **Typical HUS** (D+ HUS, *E. coli* O157:H7)

TTP thrombotic thrombocytopenic purpura, *ADAMTS13* a disintegrin and metalloprotease with thrombospondin type 1 domains, Nr. 13, *HELLP* hemolysis, elevated liver enzymes, low platelets syndrome, *HIV* human immunodeficiency virus, *SLE* systemic lupus erythematosus, *HUS* hemolytic uremic syndrome, *D+ HUS* diarrhea-positive HUS caused by enterohemorrhagic *E. coli* infection

Table 1.2 Proportion of patients with severely deficient ADAMTS13 activity (defined as <5 % of normal in all studies, except <10 % in Peyvandi et al. [76])

Author [reference]	Design of study	Severely deficient/total	Sensitivity (%)
Furlan et al. 1998 [29]	Retrospective, multicenter	26/30[a]	86
Tsai and Lian 1998 [30]	Retrospective	37/37[a]	100
Veyradier et al. 2001 [70]	Prospective, multicenter	47/66[a]	71
Mori et al. 2002 [71]	Retrospective?	12/18[a]	66
Vesely et al. 2003 [72]	Inception cohort, single center	16/48[b]	33
Matsumoto et al. 2004 [73]	Multicenter	56/108[b]	52
Kremer Hovinga et al. 2004 [74]	Multicenter	56/93[c]	60
Zheng et al. 2004 [75]	Single center, prospective	16/20[b]	80
Peyvandi et al. 2004 [76]	Multicenter	48/100[a]	48

Sensitivity of a severe ADAMTS13 deficiency for diagnosing acute TTP varies from 33 to 100 % in these cohort studies. Modified with permission from B. Lämmle et al. (2005) J Thromb Haemost 3:1663–1675

[a]Patients classified as having acute TTP

[b]Patients classified as having acute idiopathic TTP

[c]Patients with first attack or relapse of acute idiopathic TTP

activity for the clinical diagnosis of TTP may not be 100 %, and one has to admit that there may be cases with clinically apparent TTP without severely deficient ADAMTS13 activity using currently available in vitro assays. Tsai [77] disagreed with this statement reporting that all 127 cases of TTP investigated by him had an ADAMTS13 activity lower than 10 % and that the diagnosis of TTP should not be

made without severe ADAMTS13 deficiency. We tend to suggest that a diagnosis of TTP should not be strictly excluded in the absence of severely deficient ADAMTS13 activity. Observing a patient with recurring acquired TTP bouts over 8 years, each bout characterized by severe hemolytic anemia, thrombocytopenia, and various clinical signs suggesting TTP, we found a low normal ADAMTS13 activity using three different assays, no functional inhibitor, and only borderline-positive anti-ADAMTS13 autoantibodies by ELISA at his first disease bout [78]. During subsequent acute TTP episodes, the situation changed, and at his fifth and sixth bouts, all three ADAMTS13 activity assays (VWF multimer degradation assay, FRETS-VWF73 assay, and flow-based assay) congruently revealed a severe ADAMTS13 deficiency. In addition, a circulating ADAMTS13 inhibitor and autoantibodies directed against ADAMTS13 by ELISA were now present [78]. It is unlikely that this patient who ultimately died from another TTP bout had acute episodes of different diseases. Rather, all his acute disease attacks should be considered as "TTP," and one may question whether our currently available ADAMTS13 assays may miss an "in vivo defect" of the ADAMTS13–VWF interaction in some rare instances. From the Oklahoma TTP–HUS registry, we also learned that the response to PEX in patients with clinically diagnosed idiopathic TTP was similar in the 32 without severe ADAMTS13 deficiency as in the 16 severely deficient patients [72]. Therefore, we should probably accept that at least some patients with a "bona fide" TTP may not have a lacking ADAMTS13 activity as assessed with our currently available methodology.

Concerning the specificity of a severely decreased ADAMTS13 activity for TTP, there have been early claims that this finding was largely unspecific [31, 32] (see above, Sect. 1.3). Bianchi et al. [33], however, confirmed the specificity of a strongly depressed VWF–cleaving protease activity for a TMA most clinicians would diagnose as TTP. On the contrary, Remuzzi et al. maintained that a severely deficient VWF–cleaving protease was also found in some patients with HUS, at least in familial and recurrent cases [79], and Remuzzi concluded, therefore, that this laboratory feature was not specific for TTP [80]. Similarly, in the excellent prospective cohort study on patients with acute TMA by Veyradier et al., there were a few cases clinically diagnosed as HUS that had a lacking VWF–cleaving protease, whereas the majority of those with HUS had a normal or subnormal activity [70]. We believe that there may be clinical overlap, mainly in cases with hereditary TTP caused by biallelic *ADAMTS13* mutations, and several cases with severe constitutional ADAMTS13 deficiency, including the abovementioned index case A1 [27], had (transient) severe kidney failure making it likely that they could be diagnosed as "HUS" instead of hereditary TTP. In this context, the report by Noris et al. on two sisters with severe hereditary ADAMTS13 deficiency caused by double heterozygous *ADAMTS13* mutations is of interest [81]. Both sisters had phenotypically different disease episodes, and it was found that only the one developing chronic renal failure had, in addition, a heterozygous complement factor H mutation characteristic of atypical HUS [82]. Thus, modifying genetic and possibly environmental factors may alter the phenotype of acute disease episodes in hereditary and possibly also in acquired TTP which may then preclude to clearly categorize these cases as TTP or atypical HUS.

Tsai and collaborators carefully investigated 16 children with typical HUS after *Escherichia coli* O157:H7 infection and noted normal VWF–cleaving protease activity and a loss of large VWF multimers in all of them [83]. They also highlighted the different composition of microthrombi in postmortem histologic preparations. Glomerular microthrombi in typical HUS were fibrin rich but did not contain VWF. In contrast, postmortem brain sections of a TTP patient revealed microthrombi that were mainly composed of platelets and VWF [83]. Hosler et al. performed a retrospective autopsy review on 56 cases and concluded that TTP and HUS were distinct pathologic entities. The 25 patients diagnosed with TTP demonstrated platelet-rich thrombi in the heart, pancreas, kidney, adrenal gland, and brain, whereas the 31 with a diagnosis of HUS had fibrin-/red cell-rich thrombi, largely confined to the kidney [84]. Most physicians are reluctant, however, to perform diagnostic biopsies in patients admitted with acute TMA, having severe thrombocytopenia. Therefore, in most centers, no diagnostic biopsies are performed. Similar to Tsai et al. [83], we performed a study in 29 children diagnosed with enterohemorrhagic *E. coli*-associated HUS diagnosed at two centers in London [85]. Whereas 28/29 children had a normal (in 2 instances subnormal) VWF–cleaving protease activity, one 18-month-old toddler had a severely decreased protease activity and an inhibitor. Stool cultures obtained on admission had grown *E. coli* O157. Follow-up investigations 3 years later showed normal renal function, and VWF–cleaving protease assay gave a normal value, also in his parents and his sister [85]. It is difficult to interpret this situation in retrospect, but one may hypothesize that this toddler in fact had a transient autoantibody-mediated ADAMTS13 deficiency and the superimposed enterohemorrhagic *E. coli* infection triggered an acute TMA disease bout which is somewhat reminiscent to the animal TTP model reported by Motto et al. in which Shiga toxin injection was the best identified trigger to bring about an acute TTP-like disease in susceptible *ADAMTS13* knockout mice [86].

In conclusion, severe ADAMTS13 deficiency is a rather specific finding for a TMA which most clinicians tend to diagnose as TTP, but there may be some cases clinically presenting as classical TTP in which our current diagnostic tests do not show a lacking ADAMTS13.

1.6 Conclusions

These historical considerations show the fascinating evolution of our knowledge on TTP and other TMAs during the past decades. Even though new insights into disease mechanisms were obtained and a defective proteolytic regulation of the newly synthesized and secreted extremely adhesive unusually large VWF multimers has been recognized as an important pathogenetic factor, many questions remain and new ones are raised. We refer to the other chapters of this book highlighting various state-of-the-art aspects of TTP and ADAMTS13. We believe that these historical annotations should be kept in mind, also for further research, because it is not excluded that some earlier pathophysiologic hypotheses may be shown to contribute to the complex pathogenesis of the various forms of TMAs.

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Chapter 2
Overview of the ADAMTS Superfamily

Suneel S. Apte

2.1 What Are ADAMTS Proteases, and How Are They Related to Other Metalloproteases?

ADAMTS proteases were unknown until 1997, when Kuno et al. [1] identified a novel metalloprotease with a catalytic domain containing a reprolysin (or snake venom-like) active-site sequence motif found in ADAM (a disintegrin and metalloprotease) proteases [2]. Among the predicted features of this new protease that set it apart from the ADAMs were the presence of thrombospondin type 1 repeats (TSRs) and the absence of a membrane-spanning domain, which is present in all ADAMs. Subsequently, sequencing of the human and mouse genomes enabled discovery of additional mammalian gene products that shared the characteristics of ADAMTS1. These 19 gene products resembled each other more closely in domain composition and primary structure than they did other metalloproteases, leading to designation of a new protease family [3, 4]. The protease named as ADAMTS5 [4] was also subsequently named ADAMTS11 [5]; therefore, the designation ADAMTS11 is no longer used. ADAMTS proteases have two functional domains, namely, a protease domain and an adjoining domain comprised of multiple modules, including TSRs, which is termed the ancillary domain (Fig. 2.1) [6]. ADAMTS proteases belong to a superfamily of ADAMTS proteins, which also includes 7 ADAMTS-like (ADAMTSL) proteins in mammals [6]. ADAMTSLs lack a propeptide, catalytic module, and disintegrin-like module, i.e., they lack the regions comprising the protease domain of ADAMTS proteases (Fig. 2.1), and, therefore, are not proteases [7]. ADAMTSLs are encoded by a distinct set of genes and do not result from alternative splicing of,

S.S. Apte, M.B.B.S., D.Phil. (✉)
Department of Biomedical Engineering (ND20), Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA
e-mail: aptes@ccf.org

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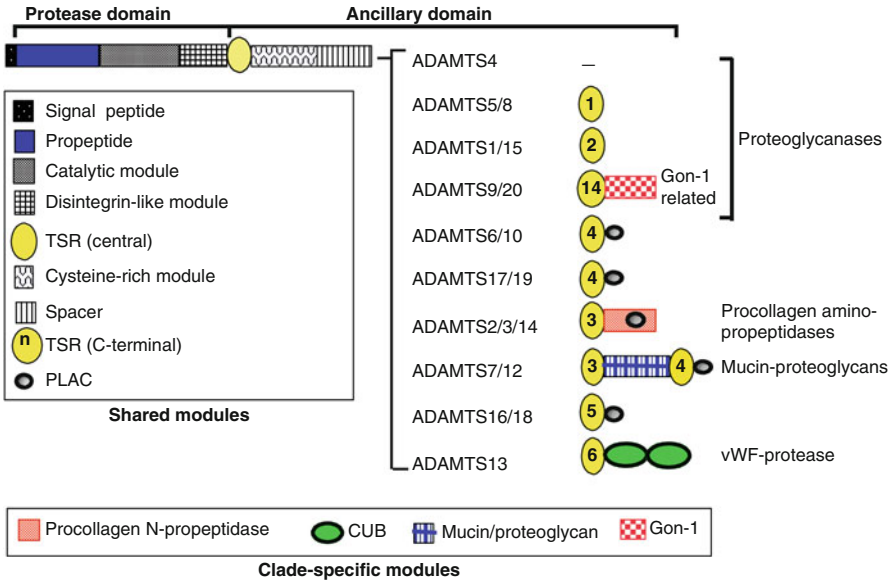

Fig. 2.1 Structure of ADAMTS proteases. The domain backbone shared by each ADAMTS protease is shown at the *top*, and modules present in every ADAMTS are shown in the box on the *left*. The modular organization of specialized ADAMTS clades is indicated on the *right*, and the key to these modules is located at the *bottom* of the figure. The clades are named according to structural or functional characteristics that best define them. Domain structures are based on reference sequences obtained from GenBank. CUB, complement C1r/C1s, Uegf, Bmp1 domain; PLAC, protease and lacunin domain. Reproduced from Apte, S.S., 2009. A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif (ADAMTS) superfamily: Functions and mechanisms. Journal of Biological Chemistry 284, 31493–31497

or the use of, alternative promoters within ADAMTS genes. Notably, MMPs and ADAMs do not have such non-protease relatives.

Identification of ADAMTS orthologs in invertebrate genomes, such as those of the roundworm *C. elegans*, fruit fly *D. melanogaster*, and sea squirt *Ciona intestinalis*, allowed determination of the evolutionary relationships within the family [8]. This phylogenetic analysis suggested that mammalian ADAMTS proteases arose from duplication and divergence of a small number of related proteases encoded by ancestral genomes [8]. As a consequence of gene duplication, most ADAMTS proteases (ADAMTS13 as exception) have one or more homologous proteases (Fig. 2.1), and thus the mammalian ADAMTS family is divided into several subfamilies [8, 9]. ADAMTS13 is likely to be a relatively recent, chordate innovation, possibly related to evolution of a closed circulation and the need for hemostasis, since none of the invertebrate ADAMTS proteins resemble its primary structure or contain CUB domains. Instead, the most ancient ADAMTS genealogical relationship appears to be between a nematode protein GON-1, *Drosophila* ADAMTS-A, and two mammalian orthologs, ADAMTS9 and ADAMTS20 [10].

Although ADAMTS genes are dispersed throughout the human and mouse genomes, three pairs of probable tandem duplications are known [11]. Two of the tightly linked pairs involve functionally related ADAMTS proteases, i.e., ADAMTS1/ADAMTS5 and ADAMTS8/ADAMTS15 on human chromosomes 21 and 11, respectively; the corresponding mouse loci are linked on mouse chromosomes 16 and 9, respectively. The third genetic linkage involves ADAMTS13, whose locus is linked to that of ADAMTSL2, on human chromosome 9q34 and mouse chromosome 2. These two genes are 120 and 100 kb apart in the human and mouse genomes, respectively, with *CACFD1* intervening in the human genome, along with two additional intervening genes, *Slc2a6* and *Tmem8c* in mouse. There is no evidence for functional interaction between ADAMTSL2 and ADAMTS13, and on the basis of the degenerate synteny in the region, ADAMTS13 and ADAMTSL2 genes probably do not share regulatory regions. ADAMTSL2 is implicated in an inherited connective tissue disorder named geleophysic dysplasia [12, 13], which has no clinical overlap with thrombocytopenic purpura.

Like ADAMs and MMPs, ADAMTS proteases are synthesized as zymogens, which undergo proteolytic excision of the N-terminal propeptide by proprotein convertases such as furin. The similarity of the ADAMTS catalytic domain structure and mechanism to ADAMs and MMPs renders them accessible to the same endogenous inhibitors, namely, tissue inhibitor of metalloproteinases-3 (TIMP3) and α2-macroglobulin [14–16]. These similarities contrast with a number of marked distinctions between the structures and biological roles of ADAMTS proteases and ADAM. In contrast to ADAMs, which are all cell membrane-anchored, as are several membrane-bound MMPs, all ADAMTS proteases are secreted. Several have been shown to bind close to the cell surface through interactions with pericellular matrix components such as proteoglycans [10, 17–19]. Thus, the majority of ADAMTS proteases may be operational cell surface proteases that could have a role in modifying pericellular matrix, modulating signaling molecules, or influencing cell adhesion and migration. ADAMTS1 was shown to shed syndecan-4 [20] and the epidermal growth factor (EGF) receptor ligands heparin-binding EGF and amphiregulin [21]. However, there is little evidence that ADAMTS proteases have a major role in ectodomain shedding, which remains the principal function of ADAMs, a contention supported by strong genetic and biochemical evidence [2]. Paradoxically, while the overall structure and active-site sequences of ADAMTS proteases are closer to ADAMs than MMPs, ADAMTS proteases share with MMPs, but not ADAMs, a propensity for cleavage of extracellular matrix/secreted molecules. This property is attributable to the affinity of the ancillary domain for extracellular matrix.

2.2 Biosynthesis, Posttranslational Modification, and Regulation of ADAMTS Activity

As secreted proteins, all ADAMTS proteases have a signal peptide, which directs them to the endoplasmic reticulum (ER), where their folding and posttranslational modification are initiated. There, disulfide bond formation occurs between pairs of

cysteine residues brought together by energetically stable states of the newly folded protein, a process that is assisted by chaperones [22]. The ADAMTS9 propeptide provides an intramolecular chaperone required for its secretion, whereas the ADAMTS13 propeptide is not similarly required [23, 24]. Most ADAMTS proteases (ADAMTS4 being an exception) have consensus motifs for N-glycosylation, which occurs co-translationally and is likely to assist folding, thus constituting a potential quality control mechanism. For instance, ADAMTS9 and a nematode ADAMTS named *mig-17* are not secreted if they lack N-glycosylation [23, 25, 26].

TSRs undergo two uncommon posttranslational modifications in the ER, namely, protein *O*-fucosylation [27] and *C*-mannosylation [28]. *O*-fucosylation leads to addition of either a mono- or disaccharide to TSRs. The initiating modification is the addition of fucose to a TSR containing the consensus sequence $C^1XX(\underline{S/T})C^2XXG$ by protein O-fucosyltransferase 2 (POFUT2) [27]. Such motifs are present in only 49 proteins encoded by the human genome, of which half belong to the ADAMTS superfamily. This *O*-linked fucose then acts as the recipient for a glucose residue, a step mediated by β3-glucosyltransferase (B3GLCT), leading to the formation of a glucoseβ1–3fucose disaccharide [29]. *O*-fucosylation appears to be a quality control step for secretion, since POFUT2 does not modify unfolded peptides containing the consensus sequence, and neither ADAMTS13 nor ADAMTSL2 are secreted in the absence of *O*-fucosylation [30, 31]. POFUT2, acting via modification of one or more crucial substrates, possibly ADAMTS proteins, is essential for survival, since *Pofut2*-deficient embryos do not survive past early development [32]. A probable candidate for this severe phenotype is ADAMTS9, since *Adamts9-deficient* embryos do not survive past gastrulation [33]. Human *POFUT2* mutations have not been identified, presumably because they would be lethal. However, *B3GLCT* mutations lead to a specific disorder named Peters plus syndrome, comprising several ocular and non-ocular manifestations, but not a hemostatic abnormality [34]. From this, it can be surmised that B3GLCT is required for secretion and/or function of some, but not all, ADAMTS proteins and, specifically, not for ADAMTS13 activity.

Protein *C*-mannosylation occurs on Trp (W^0) residues within W^0XXW^{+3} or W^0XXC^{+3} motifs, which lie just upstream of the *O*-fucosylation consensus sequence in TSRs; this modification has been shown to occur on ADAMTSL1 and is predicted in other superfamily members [35, 36]. Since *C*-mannosylation occurs on unfolded peptides, it is unlikely to be a quality control mechanism or a prerequisite for folding, and its precise function in ADAMTS proteins is not known. In addition to these three forms of glycosylation, ADAMTS7 and ADAMTS12, which are orthologous proteins, undergo extensive O-glycosylation in regions having the sequence attributes of mucin domains, namely, an abundance of Pro, Ser, and Thr residues [19]. Furthermore, their mucin domains contain sequons for attachment of glycosaminoglycan (GAG) chains. Indeed, ADAMTS7 is modified by attachment of the GAG chondroitin sulfate [19], which makes ADAMTS7 the only protease that is also a proteoglycan, and supports the likelihood of similar modification occurring at sequons present in ADAMTS12 [19].

Most ADAMTS propeptides are over 200 amino acids (aa) in length and contain three Cys residues, whereas the ADAMTS13 propeptide is only 40-residue long and contains two Cys residues [24]. ADAMTS propeptides are excised by proprotein

convertases, e.g., furin, in the trans-Golgi or extracellularly, i.e., at the cell surface or in extracellular matrix [10, 14, 18, 19, 37–41]. A typical furin processing site (Arg-Xaa-Arg/Lys-Arg) is present in all ADAMTS proteases but ADAMTS10 at the junction of the propeptide and catalytic module. ADAMTS10 has a suboptimal (Gly-Leu-Lys-Arg) site at the propeptide–catalytic module junction, but contains additional consensus sites within the propeptide [42]. Whereas propeptide excision was not required for activity of ADAMTS9 and ADAMTS13 [23, 24], it is essential for activity of ADAMTS1, ADAMTS4, ADAMTS5, and ADAMTS15 [38–40, 43].

A structure of the ADAMTS13 catalytic domain is presently unavailable. However, three-dimensional structures of the catalytic and disintegrin-like modules of ADAMTS1, ADAMTS4, and ADAMTS5 were obtained by X-ray crystallography and showed a very similar fold [44–46]. These structures demonstrated that the ADAMTS disintegrin-like module did not resemble snake venom disintegrins, but formed a unique conserved fold whose N-terminal half resembled ADAM cysteine-rich domains. These structures also showed that the disintegrin-like module was closely juxtaposed to the catalytic module and formed part of the interacting surface with inhibitors, i.e., it provided a functional extension of the catalytic domain. Indeed, exclusion of the disintegrin-like module from ADAMTS-like proteins [6] further suggests that the ADAMTS protease domain comprises both the catalytic domain and disintegrin-like modules.

ADAMTS protease domains without attached ancillary domains generally lack activity toward native substrates, since the ancillary domains constitute a major substrate-binding region, as shown by several studies employing recombinant ADAMTS proteases, including ADAMTS13 [17, 47–49]. A crystal structure obtained for the ADAMTS13 ancillary domain (excluding the C-terminal TSRs and CUB modules) [50] has been invaluable in understanding the three-dimensional topography and mechanisms of the ADAMTS family and is the only available ancillary domain structure to date. Together with site-directed mutagenesis of the ADAMTS13 ancillary domain and localization of epitopes for ADAMTS13 autoantibodies, the structure demonstrated the importance of exosites that mediate its activity against vWF [51, 52].

There are several examples of posttranslational modification of ADAMTS substrates being crucial determinants of their proteolytic activity. For example, proteolysis of the proteoglycans aggrecan and versican by ADAMTS1, ADAMTS4, and ADAMTS5 requires the chondroitin sulfate side chains on these substrates, since the deglycosylated substrates are poorly cleaved [53, 54]. Indeed, recent work identified two chondroitin sulfate attachment sites on versican V1 that lie in greatest proximity to the cleaved Glu^{441}–Ala^{442} bond as specific and essential determinants of ADAMTS1 and ADAMTS5 activity [53]. O-linked glycans of vWF have been shown to influence its proteolysis by ADAMTS13 [55]. ADAMTS2 activity against procollagen I requires that this substrate have a triple helical conformation [56]. In the case of ADAMTS13 processing of vWF, stretching of the A1–A3 domains flanking the scissile bond is required, constituting an unusual, shear force-mediated "posttranslational" substrate modification [57, 58]. This requirement may explain why partial vWF denaturation using urea is required for efficient processing by ADAMTS13 in vitro. ADAMTS activity can also be regulated by cofactors or

inhibited by interactions with other molecules. For example, ADAMTS13 processing of vWF can be accelerated by factor VIII [52]. Fibulin-1 is a cofactor for ADAMTS1 and ADAMTS5 [59–61], whereas fibronectin inhibits ADAMTS4 [62]. The ADAMTS-like protein papilin was shown to be a noncompetitive inhibitor of ADAMTS2 [63].

2.3 Biological and Disease Pathways Involving ADAMTS Proteases

This chapter highlights ADAMTS proteases that are unequivocally implicated in biological pathways through identification of mutations in Mendelian disorders or via engineered animal mutations.

ADAMTS1 deficiency in mice leads to considerable lethality at birth, together with a high frequency of genitourinary anomalies such as hydronephrosis [64, 65]. Surviving female *Adamts1* null mice are infertile because ADAMTS1 is required for versican proteolysis during maturation and rupture of the ovarian follicle [66–68]. ADAMTS1 is also required for versican proteolysis during myocardial compaction, a morphogenetic process in which cardiac myocytes are brought together during the embryonic period to form a functional myocardium [69]. The implication of ADAMTS2 in collagen maturation via a bovine genetic disorder named dermatosparaxis predated the association of ADAMTS13 with von Willebrand factor. Dermatosparaxis results from accumulation of unprocessed cutaneous procollagen having a "hieroglyphic" ultrastructural appearance, leading to extreme skin fragility that is the hallmark of this disorder [70]. The underlying mechanism was identified as the lack of an enzymatic activity essential for the removal of the N-propeptide of procollagen I, the major collagen type in the dermis of the skin [71]. Later, a human connective tissue disorder, Ehlers–Danlos syndrome type VIIc (or dermatosparactic type), having similar skin fragility and collagen fibril anomalies was identified [72] (Table 2.1), and both the bovine and human conditions were attributed to ADAMTS2 mutations in the respective species [73]. ADAMTS3, an ADAMTS2 ortholog, processes procollagen II and procollagen III and is highly expressed in cartilage, where collagen II is a major component [74, 75]. In addition, this enzyme was recently shown to proteolytically activate the pro-angiogenic and pro-lymphangiogenic factor VEGF-C. Proteolysis of VEGF-C is enhanced by the binding of ADAMTS3 to a cofactor, collagen- and calcium-binding epidermal growth factor domain 1 (CCBE1) [76]. The cleavage site in VEGF-C is similar to that in procollagens, and CCBE1 has a C-terminal domain with collagenous repeats, which may provide the basis for its interaction with ADAMTS3.

ADAMTS4 and ADAMTS5 (termed aggrecanase-1 and aggrecanase-2, respectively) are implicated in proteolytic destruction and loss of aggrecan from joint cartilage in osteoarthritis [77]. Aggrecan is a heavily modified chondroitin sulfate proteoglycan that interacts with hyaluronan in cartilage extracellular matrix.

Table 2.1 Human Mendelian disorders resulting from ADAMTS mutations

Mendelian disorder	MIM number	Gene name and chromosomal locus	Mode of inheritance
Ehlers–Danlos syndrome (EDS), dermatosparaxis type or (VIIC)	225410	ADAMTS2, 5q35.3 [73]	Autosomal recessive
Weill–Marchesani syndrome 1/Weill–Marchesani syndrome, autosomal recessive/mesodermal dysmorphodystrophy, congenital	277600	ADAMTS10/19p13.2 [84]	Autosomal recessive
Thrombotic thrombocytopenic purpura, congenital/Upshaw–Schulman syndrome	274150	ADAMTS13, 9q34.2 [109]	Autosomal recessive
Weill–Marchesani-like syndrome	613195	ADAMTS17, 15q26.3 [87]	Autosomal recessive
Microcornea, myopic chorioretinal atrophy, and telecanthus (MMCAT)	615458	ADAMTS18, 16q23.1 [91]	Autosomal recessive

The large aggregates thus formed are highly hydrated and endow cartilage with its shock-absorbing properties. Proteolysis of aggrecan is thought to be a major initiating mechanism of arthritis, since it exposes other cartilage components such as collagen II to subsequent destruction by MMPs and other proteases. Aggrecanases are considered to be a major drug target in arthritis, and many small-molecule active-site inhibitors and function-blocking antibodies have been generated and investigated preclinically [77, 78]. ADAMTS5 is strongly expressed in cardiac outflow tract endocardial cushions, where it is required for versican proteolysis during the sculpting of cushions to form thin valve leaflets, and is implicated in TGFβ signaling [79, 80]. *Adamts5*-deficient mice had reduced sculpting of pulmonic valves during embryogenesis and myxomatous mitral valves in adult hearts [79].

ADAMTS9 is crucial for early mouse development, since embryos lacking this protease do not survive past gastrulation [81]. This highly conserved protease has significant roles in mammalian development gleaned from analysis of single and combinatorial mouse mutants. For example, ADAMTS9 haploinsufficiency leads to cardiac and aortic defects and to a highly penetrant ocular anterior segment dysgenesis [82, 83]. In combination with the *Adamts*20^{bt} homozygous mutant, *Adamts9* haploinsufficiency leads to death at birth from cleft palate [33]. *ADAMTS10* mutations lead to Weill–Marchesani syndrome, with short stature, brachydactyly and ectopia lentis (dislocation of the lens) being the major clinical features (Table 2.1) [84]. Since WMS is also caused by fibrillin-1 mutations [85], a functional relationship between ADAMTS10 and fibrillin-1 has emerged and is validated by studies showing that ADAMTS10 binds fibrillin-1 and enhances microfibril biogenesis [42]. ADAMTS10 cleaves fibrillin-1 poorly [42], and ectopia lentis in WMS suggests that ADAMTS10 is primarily required for the formation of the zonule, a microfibril-comprised structure that suspends the lens in the optic path. A WMS-like phenotype in humans, and ectopia lentis in dogs, results from *ADAMTS17* mutations [86, 87], suggesting it may function similarly to ADAMTS10 (Table 2.1).

Recently, rats with a targeted mutation of *Adamts16* identified its potential role in regulation of blood pressure and male fertility [88, 89], and other works have suggested a connection between *Adamts16* and renal development [90]. The related protease ADAMTS18 is implicated in a syndrome comprising microcornea, myopic chorioretinal atrophy, and telecanthus (Table 2.1) [91, 92]. The *Adamts*20^{bt} mutant has a white spotting phenotype, with the spotting confined to the mid-torso, and results from failure of neural crest-derived melanoblasts to properly colonize hair follicles in that region (hair follicles, but not the intervening skin, are the exclusive domain of melanoblasts in mice) [93, 94]. ADAMTS20 is not required for neural crest cell migration, but for the proliferation and survival of neural crest cells once they reach the hair follicles [94].

As further evidence of cooperativity of ADAMTS proteases, mice with combined *Adamts5* and *Adamts20* deficiency have soft tissue syndactyly, which results from failure of interdigital web regression in the embryo [59]. Interdigital webs are present not only in aquatic birds and bats but also during embryogenesis in humans, mice, and other mammals, where they participate in the development of digits. They regress by rapid sculpting after digit formation is complete, i.e., by massive apoptosis coupled with matrix proteolysis. ADAMTS proteolysis of versican in the interdigit matrix is required for apoptosis of interdigit mesenchyme, suggesting that these ADAMTS proteases couple matrix proteolysis to cell death during web regression [59]. A similar role for *Adamts9* in web regression was elucidated first in combination with *Adamts5* or *Adamts20* and, more recently, by its limb-specific conditional inactivation [59, 81].

An interesting contrast between ADAMTS13 and other family members relates to substrate specificity. The biology of thrombotic thrombocytopenic purpura suggests an exclusive protease–substrate relationship between ADAMTS13 and von Willebrand factor, whereas other ADAMTS proteases appear not to be as exquisitely specific. For example, numerous substrates have been identified for the prototypic ADAMTS protease, ADAMTS1, including chondroitin sulfate proteoglycans such as aggrecan and versican, collagen I, nidogen-1 and nidogen-2, the matricellular proteins thrombospondin-1 and thrombospondin-2, and the cell-anchored EGFR ligands HB-EGF and amphiregulin [20, 21, 95–99]. Of these, however, a significant biological impact has hitherto been established mostly for proteolysis of versican [67–69], which is also targeted by ADAMTS4, ADAMTS5, ADAMTS9, ADAMTS15, and ADAMTS20 [10, 39, 43, 94, 99, 100].

Genome-wide association studies and transcriptome analysis have identified associations of ADAMTS loci with several common disorders [101–105], but these associations remain suggestive until functionally validated. This is because the single nucleotide polymorphisms used in GWAS map only to the vicinity of the gene locus, i.e., with few exceptions, they are not within the exons of the gene, and do not introduce amino acid changes in the proteins. Furthermore, proximity of the SNP to the intergenic or intronic regions of an ADAMTS gene locus does not imply that it is necessarily in a regulatory region of that gene, since the SNP may actually affect regulation of another locus in the general region or, sometimes, even further away if it lies within an enhancer. Thus, SNPs do not immediately implicate the ADAMTS protease in that disease unless additional conditions are met, for which there are

currently few examples. One SNP for which this burden of proof has been partially met is ADAMTS7, which was associated with coronary artery disease [106]. One of the SNPs led to a $Ser^{214}Pro$ substitution in the propeptide. Biochemical analysis following expression of the Ser and Pro variants suggested that the Pro variant quantitatively impaired ADAMTS7 propeptide excision by furin [107], which is thought to be a prerequisite for proteolytic activity. Thus, the Pro variant potentially has lower activity, and individuals with the Pro/Pro ADAMTS7 protein are predicted to have reduced protease activity compared to those with Ser/Ser variants [107].

2.4 Summary and Conclusions

This chapter provides the reader with a concise background on the general molecular aspects of ADAMTS proteases and demonstrates their considerable diversity of structure and function. It is clear that ADAMTS13 is something of an outlier among the 19-member ADAMTS family. ADAMTS13 structural biology, enzyme–substrate interactions, posttranslational modification, biochemical assays, and roles in genetic and acquired disease are better understood than any other ADAMTS protease. It is the only family member presently for which enzymatic replacement via production of recombinant enzyme is sought [108], whereas a specific blockade is sought for ADAMTS4 and ADAMTS5 in osteoarthritis. Although ADAMTS13 has been arguably more extensively studied for longer than most other family members, there is much about it remaining to be investigated. For instance, little is known about its transcriptional regulation, intermolecular interactions, and turnover. Continuing research on ADAMTS13, with the state of the art represented in this volume, as well as other ADAMTS proteases, will continue to elucidate the shared principles and individual distinctions of this remarkable protease family.

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Chapter 3
ADAMTS13: Structure and Function

X. Long Zheng

Abbreviations

ADAMTS13	A disintegrin and metalloprotease with thrombospondin type 1 repeats, 13
CUB	For the complement C1r/C1s, Uegf, Bmp1
DTCS	Disintegrin, first thrombospondin type 1 repeat, Cys-rich, and spacer domains
ELISA	Enzyme-linked immunosorbent assay
HNPs	Human neutrophil peptides
HSCs	Hepatic stellate cells
K_D	Dissociation constant
MMPIs	Matrix metalloprotease inhibitors
TTP	Thrombotic thrombocytopenic purpura
ULVWF	Ultra-large VWF
VWF	von Willebrand factor
VWF73	A VWF peptide containing 73 amino acids from D1596 to R1668

3.1 Introduction

In 1996, Tsai [1] and Furlan et al. [2] first described a metalloprotease that has the ability to cleave von Willebrand factor (VWF). Both groups attempted to purify this enzyme without full success. However, these investigators learned quite a lot about its biochemical characteristics of this metalloprotease with plasma and partially

X.L. Zheng, M.D., Ph.D. (✉)
Division of Laboratory Medicine, Department of Pathology, The University of Alabama at Birmingham, WP P230, 619 19th Street South, Birmingham, AL 35233, USA
e-mail: zhengl@uab.edu; longzheng01@gmail.com

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concentrated materials. This led to the discovery of hereditary [3] and acquired [4, 5] deficiencies of this metalloprotease (known as VWFCP) activity, the primary cause of chronic and relapsing thrombotic thrombocytopenic purpura (TTP). In 2001, VWFCP was finally purified to near homogeneity and its N-terminal sequence was obtained, which led to the identification of VWFCP as a member of ADAMTS (*a d*isintegrin *a*nd *m*etalloprotease with *t*hrombo*s*pondin *t*ype 1 repeats) family of proteases [6, 7]. It was named ADAMTS13. The complete cDNA and protein sequences of ADAMTS13 were rapidly determined and reported [8, 9]. Soon, various mutations in the *ADAMTS13* gene were identified in cases of hereditary TTP [10, 11]. Most mutations cause abnormalities in ADAMTS13 protein folding and secretion, resulting in severe deficiency of ADAMTS13 activity in plasma [11]. Also, autoantibodies against ADAMTS13 protease [5, 12] were found in nearly all acquired (idiopathic) TTP patients with ADAMTS13 less than 5 % of normal.

The success in identification and cloning of ADAMTS13 has greatly enhanced our ability to understand the mechanism of TTP and other thrombotic microangiopathies. This has also provided us with a valuable tool for investigation of the basic biology of ADAMTS13, including biosynthesis and secretion, structure–function relationships [13], and cofactor-dependent regulation of ADAMTS13 activity [14, 15]. This chapter will focus on an overview of recent progress on the structure–function analyses and regulation of ADAMTS13 function to catalyze VWF substrate and inhibit arterial thrombosis.

3.2 ADAMTS13 Domain Organization

The ADAMTS family belongs to the metzincin superfamily of zinc-containing metalloproteases [16, 17]. ADAMTS13 shares similarities in domain structure with other ADAMTS proteases, but has significant differences that make it the most divergent member of the group [18]. All ADAMTS proteases are organized in such a way that they start with a short signal peptide and a prodomain, followed by the zinc-containing metalloprotease domain. The signal peptide and prodomain are removed in the endoplasmic reticulum and Golgi apparatus, respectively, during the biosynthesis and secretion processes. The mature ADAMTS13 contains a metalloprotease domain, a disintegrin domain, the first thrombospondin type 1 (TSP1) repeat, a cysteine-rich domain, and a spacer domain in addition to several more TSP1 repeats and two CUB domains (Fig. 3.1). These C-terminal domains in ADAMTS family proteases are required for a wide range of biological functions, including processing of procollagen [19, 20], cleavage of extracellular matrix components [21, 22], organogenesis and reorganization of tissues during development and angiogenesis [23], and regulation of hemostasis and inflammation [24, 25].

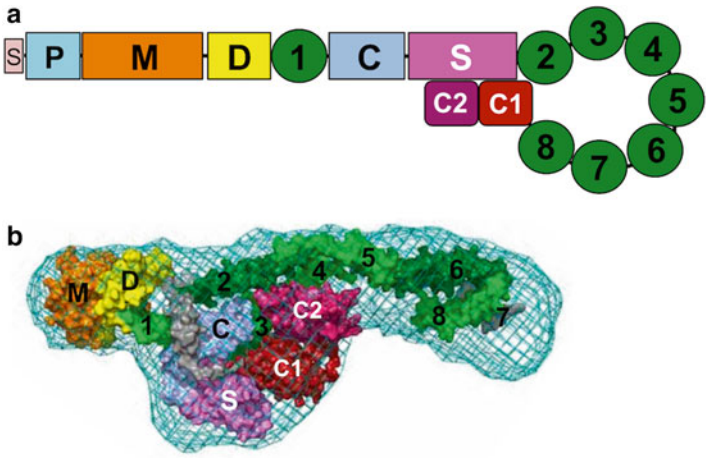

Fig. 3.1 ADAMTS13 domain organization. (**a**) Schematic representation of human full-length ADAMTS13 protein; (**b**) envelopes calculated from SAX scattering profiles of ADAMTS13, suggesting a folded or condensed conformation with the CUB domain in close proximity to the Cys-rich and spacer domains (Adapted from Muia et al. PNAS, 2014; 111:18584–89) [54]

3.3 ADAMTS13 Domain Function

ADAMTS13 is primarily synthesized in hepatic stellate cells (HSCs) and secreted as an active enzyme into the bloodstream [26–28]. Plasma ADAMTS13 binds and cleaves newly released ultra-large forms of VWF (ULVWF) usually anchored on the endothelial surface as strings/bundles [29–31]. The cleavage of cell-bound ULVWF and soluble VWF by ADAMTS13 occurs specifically at the Tyr1605–Met1606 site to reduce VWF size and adhesiveness [29–31], thereby preventing excessive platelet adhesion, aggregation, and thrombus formation after injury. Inability to cleave ULVWF and large soluble VWF by ADAMTS13 results in the potentially fatal syndrome TTP [24, 32, 33]. Therefore, investigation of structure–function relationships and cofactor-dependent regulation of ADAMTS13 activity is pivotal for our understanding of molecular mechanisms of TTP and other thrombotic disorders.

3.3.1 Prodomain

The prodomain, also known as propeptide, is an important regulatory domain in ADAMTS proteases and acts as a chaperone to aid in protein folding and maintains the latency of the metalloenzyme [23]. A “cysteine switch” mechanism coordinates with the zinc atom such that the access to the active site is essentially blocked [34, 35].

The dissociation of the cysteine residue from the zinc atom results in activation of ADAMTS proteases. While the cysteine switch is one of the most conserved features in the reprolysin family of zinc metalloproteases, ADAMTS13 lacks the conserved cysteine residue to keep its protease domain in its latency [8]. Unlike other ADAMTS proteases that possess an ~200-residue prodomain, ADAMTS13 has a short one, consisting of only 41 residues [8, 36]. Recombinant human ADAMTS13 expressed in transfected cells with or without the prodomain is normally secreted and able to cleave VWF substrate with similar efficiency [36], confirming that the prodomain in ADAMTS13 is not required for its folding and maintenance of its latency.

3.3.2 Metalloprotease Domain

The business center of the ADAMTS molecule resides in the metalloprotease domain, which is the most conserved domain in the reprolysin or adamalysin family of proteases [8, 37]. A sequence of three histidine residues coordinates an essential zinc (Zn^{2+}) ion in this highly conserved motif (HEXXHXXGXXHD). In conjunction with the active site at Glu225, the Zn^{2+} ion coordinates a water molecule that drives hydrolysis of the scissile bond (Tyr1605–Met1606) in the central A2 domain of VWF [37, 38]. A high-affinity Ca^{2+}-binding site close to the active site of the metalloprotease domain is also found to be essential for efficient proteolysis to occur. Three other putative Ca^{2+}-binding sites have been proposed from modeling the protease domain of ADAMTS4 and 5. The first site is Glu83, Asp173, Cys281, and Asp284; this is broadly conserved among ADAMTS proteases and mediates low-affinity Ca^{2+} binding. The second site comprises Glu164 and Asp166 with one or more of the residues Asn162, Asp165, and/or Asp168. Mutations in this site, however, seem to have no effect on Ca^{2+}-dependent ADAMTS13 activity; the third site includes Asp187 and Glu212 with Asp182 or Glu184 [37, 38]. Mutations at this site dramatically reduce Ca^{2+}-dependent ADAMTS13 activity, which indicates a high-affinity Ca^{2+}-binding site [37, 38]. While the metalloprotease domain is essential for catalysis to occur, this protease domain alone has little to no activity toward VWF substrates, suggesting other ancillary domains in the C-terminus of ADAMTS13 are important for ADAMTS13 functions.

3.3.3 Disintegrin-Like Domain

Immediately adjacent to the metalloprotease domain is the disintegrin-like domain [8, 39]. Disintegrins are a cluster of proteins found in viper venom that act to counter blood clotting by inhibiting platelet aggregation and integrin-dependent cell adhesion. Disintegrin-like proteins or proteins with disintegrin-like domains are found across a wide range of species. Modeling of the crystal structure of the DTCS

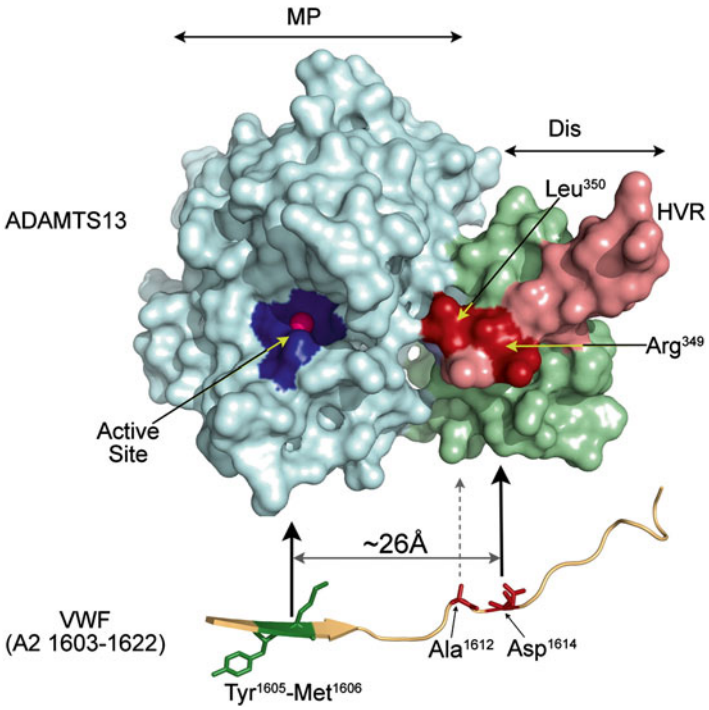

Fig. 3.2 Model depicting the proposed role of the ADAMTS13 disintegrin-like domain. Homology model of the ADAMTS13 MP-Dis. The metalloprotease domain is shown in *light blue* showing the 3 active site His and catalytic residues Glu (*dark blue*) that coordinate a catalytic Zn^{2+} ion (*pink*). The disintegrin-like domain is depicted in *light green*, *light pink*, and *red*. The hypervariable region (HVR) is highlighted in *light pink* with Arg349 and Leu350 highlighted in *red* (de Groof R. et al. Blood 2009; 113:5609)

fragment together with the metalloprotease domain of ADAMTS1, 4, and 5 demonstrates that the metalloprotease and the disintegrin-like domains are an inseparable functional unit [13, 39] (Fig. 3.2). Functional analysis indicates that an addition of the disintegrin-like domain to the metalloprotease enhances the cleavage efficiency and substrate specificity [40–42]. An ADAMTS13 variant lacking the disintegrin-like domain [40, 42] or carrying a point mutation in the variable regions of the disintegrin-like domain (i.e., Arg349, Leu350, Val352) (Fig. 3.2) [43] exhibits dramatically reduced proteolytic activity toward small peptide substrates and macromolecular VWF substrate. These amino acids lie adjacent to the active site cleft. Arg349 is located approximately 26 Å from the active site Zn^{2+}. The catalytic efficiency of ADAMTS13 is reduced 10–20-fold when either of these residues is mutated [43]. The study suggests that Asp1614 in the VWF A2 domain (located 26 Å from the Tyr1605–Met1606 scissile bond) may interact with Arg349 in ADAMTS13 (Fig. 3.2). This in turn helps position the scissile bond into the active site cleft. In addition, the residues Leu198, Val195, and Arg349 in the disintegrin

domains also make direct contact with the residues Leu1603, Tyr1605, and Asp1614 in the A2 domain [13, 43], respectively. Therefore, the disintegrin-like domain works in concert with other non-catalytic ancillary domains to ensure that the scissile bond is brought into the active center of the metalloprotease domain for proteolysis [44].

3.3.4 Cysteine-Rich Domain

A cysteine-rich (or Cys-rich) region immediately follows the disintegrin-like domain [13, 39]. Cysteine-rich domains are found in many different proteins with similar yet distinct functions. This Cys-rich domain is found to play a critical role for ADAMTS13 function as mutations in this region result in dramatically reduced activity toward VWF peptide substrates [40], cell-bound ULVWF [45], and soluble VWF under various assay conditions [40, 42, 46]. More recently, a hydrophobic pocket in the Cys-rich domain of ADAMTS13 is shown to directly interact with the hydrophobic pocket in the central A2 domain of VWF [47]. The modification of several potential glycan-attaching sites in this region results in reduced binding of ADAMTS13 variant to VWF and its activity, suggesting that the glycan attachment site and perhaps its vicinity are important for ADAMTS13 function. Further analysis by swapping ADAMTS13 Cys-rich domain with that of ADAMTS1, a closely related member of ADAMTS family, demonstrates the hydrophobic pocket involving residues Gly471-Val474 critical for VWF binding and proteolysis (Fig. 3.3). In the reverse experiment, the hydrophobic pocket comprising residues Ile1642, Trp1644, Ile1649, Leu1650, and Ile1651 in the central A2 domain of VWF is shown to be the complementary site for interaction with the hydrophobic pocket involving residues Gly471-Val474 in the Cys-rich domain of ADAMTS13 (Fig. 3.3) [47]. Together, these studies provide clear evidence supporting the critical role of the Cys-rich domain in substrate recognition and proteolysis.

3.3.5 Spacer Domain

The spacer domain got its name because of its diversity among the members of ADAMTS family [13] and has been traditionally thought to function as a connection between highly structured N-terminal domains and more flexible C-terminal domains (Fig. 3.1). Recent studies demonstrate that the spacer domain is rather conserved in ADAMTS13 from zebra fish to mammals, although divergent from that in other ADAMS family members [13]. This suggests that the spacer domain may provide specificity in substrate recognition.

Earlier studies have shown that a deletion of the C-terminal TSP1 2–8 repeats and CUB domains does not significantly affect ADAMTS13 activity [48, 49]. However, further deletion of the spacer domain nearly abolishes ADAMTS13's

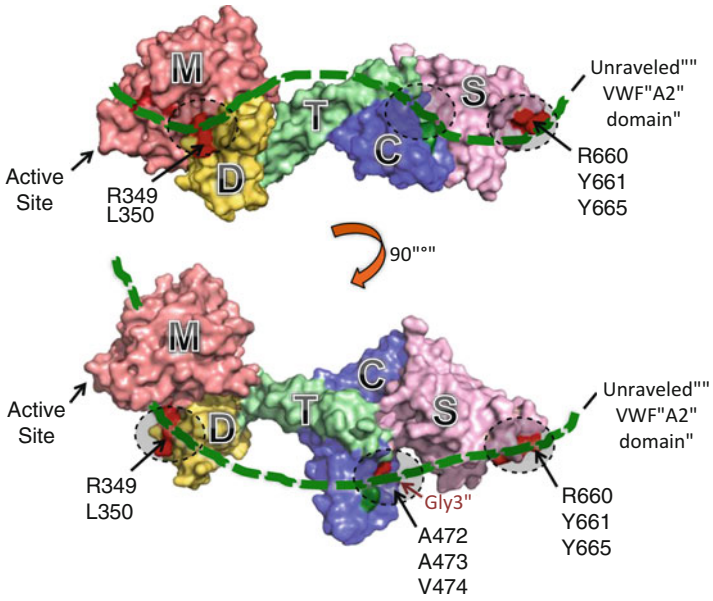

Fig. 3.3 Proposed model of interactions between ADAMTS13 and VWF peptide. Two different views of a 3-D structure of the MDTCS fragment. Metalloprotease domain (M) was not determined in the crystal structure; the disintegrin domain (D), first thrombospondin type 1 repeat (T), Cys-rich domain (C), and spacer domain (S) are present in the crystal structure described by Akiyama et al. in PNAS, 2009;106:19274–9. A part of unraveled central A2 domain of VWF (*dotted green line*) may interact with MDTCS at three distinct exosites (*circled*) consisting of surface-charged residues R660, Y661, and Y665 in the spacer domain; a hydrophobic pocket involving A472, A473, V474, and H476 (Gly3) in the Cys-rich domain; and a non-conserved region involving R349 and L350 in the disintegrin domain (Adapted from de Groot et al, Blood, 2015) [47]

ability to cleave VWF despite its low residual activity toward small peptide substrates [40, 50]. Recent studies demonstrate that the surface-charged residues in the flexible loops of the spacer domain are critical for substrate recognition and proteolysis [51]. For instance, an alanine substitution at residues R659, R660, and Y661 in the spacer domain significantly impairs its proteolytic activity (Fig. 3.3). Interestingly, conserved substitutions of these residues (i.e., R659K, R660K, and Y661F) plus several other adjacent residues (i.e., F592Y or R568K) appear to increase ADAMTS13 activity [52]. This is because the mutations in the spacer domain disrupt its potential interactions with the C-terminal tail (such as the CUB domains), thereby releasing the autoinhibition [53, 54]. The importance of the spacer domain in substrate specificity also comes from the domain swap experiments, in which the replacement of the first TSP1 repeat, Cys-rich, and spacer (TCS) domains of ADAMTS5 with those of ADAMTS13 alters ADAMTS5's substrate specificity [55]. In the presence of TCS of ADAMTS13, ADAMTS5/13 chimera cleaves the Glu1615–Ile1616 bond in the peptidyl substrate and multimeric VWF under shear stress [55].

3.3.6 Thrombospondin Type 1 Repeats

ADAMTS13 contains eight TSP1 repeats (Fig. 3.1). The TSP1 repeats in other ADAMTS family proteases play a role in cellular localization and substrate recognition [56]. All TSP1 repeats contain the consensus WXXW motif [8], and mutations in this motif in the central TSP1 repeat (i.e., W387A) [57] significantly reduce ADAMTS13 secretion and binding affinity to VWF. Also, this WXXW motif is often modified by the attachment of an α-mannosyl group to the C2 atom of the first tryptophan. Moreover, seven of the eight TSP1 repeats also contain the conserved sequence CSX(S/T)CG, in which the hydroxyamino acid at position four is usually modified by the disaccharide Glc-Fuc-O-Ser/Thr [8, 58]. It is still not clear, however, whether posttranslational modifications play a role in ADAMTS13 function in vivo. In ADAMTS13, each individual TSP1 repeat may contribute differentially to function. The first TSP1 repeat has been shown to bind directly to the central A2 domain (i.e., the VWF73 peptide) with a dissociation constant (K_D) of 136 nM [40], but the other TSP1 repeats may also bind VWF at a more distal location such as the D4 domain [59]. The more distal C-terminal TSP1 repeats have also been shown to interact with the endothelial cell surface via a receptor, CD36 [60]. This binding may facilitate the interactions between ADAMTS13 and endothelial cell membrane, thereby enhancing the availability of ADAMTS13 for cleaving VWF. However, binding of ADAMTS13 to the CD36 receptor does not appear to affect ADAMTS13 activity in vitro. In this scenario, the TSP1 repeats follow their canonical role as a cellular localization domain. More recently, the TSP1 repeats of ADAMTS13 are shown to contain unpaired free cysteine residues [61, 62] that may interact with the free cysteine exposed under fluid shear to prevent lateral association of the VWF molecules or function as a disulfide bond reductase to attack a preformed disulfide bond in VWF multimers, thereby reducing the formation of ULVWF strings in vivo.

3.3.7 CUB Domains

Unique to ADAMTS13 are two CUB domains (Fig. 3.1), but their function remains to be elusive. CUB is a structural domain found in several extracellular or membrane-bound proteins with a wide range of functions. On the one hand, the CUB domains can be removed without affecting ADAMTS13 function [48, 49]; on the other hand, an isolated CUB domain or peptides derived from CUB domain inhibit the cleavage of ULVWF by a full-length ADAMTS13, suggesting that the CUB domains may function as a docking site to endothelial ULVWF [30]. In vivo, human ADAMTS13 variants lacking CUB domains cleave newly released ULVWF strings normally and are sufficient to inhibit thrombus growth in murine models using the mesenteric arteriolar occlusion assay [46]. In contrast, murine ADAMTS13 lacking the CUB domains was reported to be moderately defective in cleaving platelet-decorated ULVWF strings [63]. The reasons for the discrepancy are not fully understood and are likely confronted by the sensitivity of various assays.

More recent studies demonstrate that the CUB domains, through their interactions with the Cys-rich and/or spacer domain, may function as a negative regulator of ADAMTS13 activity [53, 54]. Kinetic studies show that an ADAMTS13 variant lacking either the CUB domains or the TSP1 2–8 repeats plus CUB domains or mutations in the spacer domain that disrupt the spacer–CUB interaction exhibit increased proteolytic cleavage activity compared with full-length ADAMTS13 toward both peptidyl and multimeric VWF substrates. The CUB–spacer interaction is also proposed based on the data obtained from low-resolution small-angle X-ray, which shows that CUB domains fold back to touch the spacer domain (Fig. 3.1) [54]. However, a crystal structure of full-length ADAMTS13 is still necessary to confirm this observation. Additional evidence to support this hypothesis is that a monoclonal antibody that binds to the CUB domains increases ADAMTS13 activity [53], presumably by interfering with the CUB–spacer interaction that releases autoinhibition.

3.4 Regulation of ADAMTS13 Activity

3.4.1 Natural Inhibitors

There has been no natural inhibitor of ADAMTS13 identified to date. ADAMTS13 is secreted as an active protease at the concentrations of ~0.5–1.0 μg/ml [64]. ADAMTS13 activity is primarily regulated at the substrate level. VWF itself is a potential natural inhibitor as it is present in plasma at the concentration (~10 μg/ml) [65], approximately ten times higher than plasma ADAMTS13. In healthy subjects, ADAMTS13 and VWF complexes are identified [66]. Challenge with DDAVP (1-desamino-8-D-arginine vasopressin) in healthy individuals triggers acute release of endothelial VWF and reduces plasma ADAMTS13 activity [67], likely due to consumption. Serine protease inhibitors, cysteine protease inhibitors, and matrix metalloprotease inhibitors (MMPIs) do not seem to inhibit ADAMTS13 activity [48]. Serum α-2 macroglobulin that efficiently inhibits matrix metalloproteases (MMPs) and other ADAMTS proteases including ADAMTS4, 5, 7, and 12 does not inhibit ADAMTS13 activity either. Inflammatory cytokines such as interleukin-6 (IL-6) are shown to inhibit proteolytic cleavage of ULVWF by ADAMTS13 in cultured endothelial cells [29]. Immunoglobulin (Ig) G-type anti-ADAMTS13 antibody, which is present at low levels in healthy individuals [68], may somehow regulate ADAMTS13 activity under physiological conditions. Other potential inhibitors including human neutrophil peptide-1 (HNP1) (or α-defensin-1) [69], α-thrombin [70], and plasmin [70, 71] may diminish ADAMTS13 activity by various mechanisms.

3.4.2 Divalent Metal Ions

Without a doubt, zinc ion is the key component for ADAMTS13 activity, although ADAMTS13 activity in citrated plasma can be activated with other metal ions. Activation by divalent metal ions is found to increase in the following order: $Zn^{2+}\approx$

$Cu^{2+} \approx Cd^{2+} \approx Ni^{2+} \approx Co^{2+} < Mn^{2+} < Mg^{2+} < Ca^{2+} \sim Sr^{2+} < Zn^{2+}$ [2]. Zinc and calcium are two metal ions that cooperatively enhance ADAMTS13 activity in citrated plasma and purified recombinant products [72, 73]. Barium, on the other hand, which appears to activate ADAMTS13 to the greatest extent in citrated plasma, does not have a role in citrate-free plasma when zinc and calcium are present at the physiological concentrations [72]. These results suggest that barium does not necessarily activate ADAMTS13, but rather reflects its ability to release zinc and calcium ions from citrated plasma.

3.4.3 Shear Stress

Newly released ULVWF that is anchored on the endothelial cell surface is in the "open" conformations; therefore, the cleavage by ADAMTS13 does not necessarily require fluid shear stress [45, 74, 75]. It is unknown how the cell-bound ULVWF exposes the central A2 domain that confers its sensitivity to ADAMTS13 proteolysis. Once released into solution, ULVWF rapidly adopts the "closed" conformations because it becomes highly resistant to proteolysis by ADAMTS13 in the absence of flow or denaturants. Soluble ULVWF regains its sensitivity to ADAMTS13 after being exposed to arterial shear stress (20–100 dyne/cm^2) [1, 76, 77], which presumably unfolds the central A2 domain of VWF and exposes the cleavage site. In an in vitro experiment, fluid shear stress can be generated by passing the reaction mixture containing VWF through a long (~3 m) metal capillary tube [1] or using a cone plate viscometer [78] or a benchtop mini-mixer [15, 46, 66, 76, 77] or flowing the materials through microfluidic channels [62]. In any case, the proteolytic cleavage of soluble VWF by ADAMTS13 increases as a function of increasing shear stress, incubation time, and concentrations of ADAMTS13 enzyme [15, 76]. In vivo, high shear conditions are found in the narrowed area, at the branching points, in small arterioles, and in the microvascular circulation. These areas are prone to form thrombosis after injury; it is not surprising that ADAMTS13 has the greatest effect in cleaving VWF when shear stress is the greatest [62]. In fact, increased VWF proteolysis and reduction of the plasma ratio of VWF activity to VWF antigen correlates with the severity of aortic stenosis in patients [79, 80]. Surgical correction of the stenosis normalizes plasma VWF multimer distribution, further illustrating the importance of fluid shear stress on ADAMTS13 and VWF interaction.

3.4.4 Coagulation Factor VIII

Coagulation factor VIII (FVIII) binds VWF with high affinity (K_D, ~0.5 nM) [81, 82]. Such a binding interaction is important for prolongation of the half-life of circulating FVIII [83]. Recent studies have demonstrated that binding of FVIII to

VWF also enhances its proteolysis by ADAMTS13 under fluid shear stress [14, 15, 77]. This effect is not observed when urea or guanidine is present [77, 84]. These results suggest that FVIII and VWF interactions facilitate conformational changes of VWF under shear stress. Additional experiments are needed to confirm this hypothesis.

The cleavage of naturally occurring VWF variants with type 2N mutations that exhibit defective FVIII binding is not enhanced by FVIII under the same conditions [85]. In addition, recombinant FVIII variants (such as 2RKR or FVIII-HC) that do not bind VWF also show no enhancing effect on VWF proteolysis by ADAMTS13 under shear [14, 77]. Interestingly, a light chain FVIII alone, despite having a ten-fold reduction in VWF binding, is still sufficient for enhancing the cleavage of VWF by ADAMTS13 [14]. These results suggest that FVIII binding with VWF is required the rate-enhancing effect on VWF proteolysis by ADAMTS13. The in vitro biochemical effects are also observed in the murine hemophilia A model. Reconstitution by a hydrodynamic injection of a plasmid encoding either a B-domainless FVIII or a light chain FVIII in FVIII-deficient mice prevents the accumulation of ULVWF multimers in plasma [14]. However, whether hemophilia A patients who have less than 1 % circulating FVIII activity exhibit reduced VWF proteolysis and increased multimers is yet to be determined.

3.4.5 Platelets and Their Surface Receptors

Platelets bind VWF with high affinity through its surface receptor glycoprotein Ib (GPIb) α subunit [86–88]. Such an interaction is important for platelets to adhere to injured endothelium for tissue repair. Recent studies have demonstrated that platelet–VWF interaction may facilitate conformational changes of VWF under shear, thereby enhancing proteolysis by ADAMTS13 [15, 88]. In this case, platelet activation is not required as similar effects are observed with addition of formalin-fixed and lyophilized platelets or fresh live platelets. Platelet integrity is also not required because addition of soluble GPIbα to VWF increases its cleavage by ADAMTS13 under both denaturing conditions [88] and fluid shear [15, 78]. These results indicate that cofactor activity of platelets or platelet receptors in accelerating VWF proteolysis does not solely depend on the mechanical force exerted on VWF by two platelets bound on either side as suggested [89]. Ristocetin, an antibiotic that binds the A1 domain close to the site that GPIbα binds, also enhances the cleavage of VWF by ADAMTS13 without shear stress [15, 90]. Therefore, it appears that the association of platelet GPIbα or ristocetin with the A1 domain may affect the accessibility of the A2 domain where the ADAMTS13 cleavage bond resides. Binding of platelets, soluble GPIb, and FVIII to VWF can synergistically enhance VWF proteolysis by ADAMTS13 under fluid shear. However, ristocetin may alleviate the requirement of FVIII to enhance the cleavage [15]. These results indicate that the mechanism underlying the rate-enhancing effects of platelets, GPIb, and FVIII or ristocetin may be different. It is known that FVIII binds to the D'D3 region of VWF

[91], but platelets, GPIb, and ristocetin all interact with the A1 domain or its neighboring regions. Further investigation in this area may shed light on the mechanism of cofactor-dependent regulation of ADAMTS13 activity.

3.5 Anti-ADAMTS13 Autoantibody

Acquired (idiopathic) TTP is primarily caused by immunoglobulin G (IgG)-type autoantibodies that bind and block plasma ADAMTS13 activity. While traditional functional assays are not sensitive enough to detect all autoantibodies, ranging from 44 to 95 % [5, 92], an enzyme-linked immunosorbent assay (ELISA) [93] or a newly developed flow cytometric assay [94] has greatly improved sensitivity for autoantibody detection to nearly 100 % in cases with severe ADAMTS13 deficiency.

Antibody profiling reveals that IgG1 and IgG4 are the predominant subclasses of anti-ADAMTS13 antibodies seen in TTP [95]. Nearly all anti-ADAMTS13 antibodies bind to the Cys-rich and spacer domains, particularly the spacer domain [12, 52, 96–100]. Less frequently, IgG autoantibodies bind to the other domains including propeptide, metalloprotease domain, disintegrin domain, first TSP1 repeat, more distal TSP1 repeats, and CUB domains [12, 101]. The significance of antibodies that bind distal portion of ADAMTS13 remains to be determined. Antibody mapping study reveals that the antigenic epitopes of anti-ADAMTS13 autoantibodies are more restricted to the loop region in the spacer domain comprising residues Y572-N579, V657-G666, and G662-V687 [98], in particular, the residues R658, F592, R660, Y661, and Y665, which form exosite 3 that is important for binding to the VWF substrate [52, 99]. Deletion or substitution of these residues nearly abolishes anti-ADAMTS13 autoantibody binding in a majority of acquired TTP patients [99, 100].

The mechanism underlying the production of anti-ADAMTS13 autoantibodies is not understood. Female predominance and autoantibodies in identical twin sisters suggest a genetic predisposition. An overrepresentation of the HLA-DRB1*11 allele in acquired TTP patients supports this notion [102]. ADAMTS13 is taken up by immature dendritic cells from HLA-DRB1*11 donors through their macrophage mannose receptor [103]. This receptor-mediated endocytosis may contribute to the priming and stimulation of low-affinity self-reactive CD4+ T cells and subsequent production of autoantibodies by B cells. Bacterial or viral infection has been shown to trigger the onset of hereditary and acquired TTP, partly mediated by increased production of autoantibodies and/or VWF release through activation of immune system and activation of endothelium, respectively. There is also the possibility of a bystander or molecular mimic hypothesis in which antibodies against microbes may cross-react with ADAMTS13.

3.6 Reengineering ADAMTS13 for Therapy

Replacement of residues in exosite 3 with alanine residues nearly completely abolishes anti-ADAMTS13 IgG binding, but also significantly impairs proteolytic activity of ADAMTS13. Loss-of-function ADAMTS13 variants have no therapeutic value. Jian et al. have modified residues by subtle changes in surface-charged residues such as changing arginines to lysines or vice versa in hope of eliminating autoantibody binding, but retaining A13 activity. Two variants are identified with such a property. ADAMTS13-M4 (R660K/F592Y/R568K/Y661F) and ADAMTS13-M5 (R660K/F592Y/R568K/Y661F/Y665F) exhibit an increased specific activity toward VWF but reduced sensitivity to inhibition by anti-ADAMTS13 antibodies [52], resulting from reduced antibody binding. Recent studies suggest that mutations in the spacer domain may disrupt the inhibitory activity of the distal C-terminal tail function [53], providing an explanation for the gain-of-function of these ADAMTS13 variants. These novel ADAMTS13 variants may be explored as novel therapeutics for patients with acquired TTP due to inhibitors.

3.7 Beyond TTP

The function of the ADAMTS13 protease is complex and may extend beyond the realm of TTP. Apart from its role as the VWF-cleaving enzyme, ADAMTS13 is involved in other blood-related disorders and inflammation. Reduced plasma ADAMTS13 activity and increased plasma VWF levels are associated with the development of myocardial infarction [104–107], ischemic stroke [108–112], pre-eclampsia [113, 114], and malignant or cerebral malaria [13, 115–118]. In a mouse model, *Adamts13*$^{-/-}$ mice on an *ApoE*$^{-/-}$ background exhibited larger atherosclerotic lesions and increased macrophage infiltration as compared to *ApoE*$^{-/-}$ mice with normal circulating ADAMTS13 activity [25, 119, 120]. Additionally, other studies have noted that *Adamts13*$^{-/-}$ mice exhibited increased infarct sizes in the myocardium [106, 107, 121] and brain [109, 122] after ischemic/reperfusion injury. Increased VWF concentrations and reduced ADAMTS13 activity in a pregnant woman are associated with an increased risk of developing preeclampsia [113, 114]. Ultra-large VWF released from activated endothelial cells may be the result of *Plasmodium falciparum* infection but also serve as a binding vehicle for the parasites [116, 123], resulting in severe cerebral malaria. ADAMTS13 may function as systemic anti-inflammatory agent [25, 109, 119, 120], which reduces both acute and chronic neutrophil, monocyte, and macrophage infiltration in various model systems.

In summary, ADAMTS13 was discovered when studying the pathogenesis of TTP. Its biological importance may extend far beyond its involvement with TTP. Both antithrombotic and anti-inflammatory activities make ADAMTS13 quite special. Deficiency of ADAMTS13 activity is associated with many other pathologies, including myocardial/cerebral infarction, malignant malaria, preeclampsia,

and acute and chronic inflammation. While the N-terminal MDTCS domains of ADAMTS13 appear to be sufficient for cleaving VWF in vitro and in vivo, the role of more distal C-terminal domains remains to be determined. Further understandings of the autoantibody binding sites may help redesign ADAMTS13 variants for therapeutics of acquired TTP with inhibitors.

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Chapter 4
ADAMTS13 and Angiogenesis

Manfai Lee and George M. Rodgers

4.1 Introduction

Nineteen members of the ADAMTS protein family have been identified to date, and they all share common domains at the N-terminus: a signal peptide, prodomain, metalloproteinase domain, disintegrin-like domain, thrombospondin type 1 motif, cysteine-rich domain, and spacer domain. The variations in the C-terminal domains distinguish these ADAMTS family members. Extensive research has revealed biological functions of most ADAMTS members. ADAMTS2, ADAMTS3, and ADAMTS14 are known to process several types of procollagen proteins [1]. Another subgroup, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS11, ADAMTS15, and ADAMTS20, is known to cleave aggrecan and proteoglycans [2–7]. ADAMTS7 and ADAMTS12 are known to degrade cartilage oligomeric matrix proteins [8]. ADAMTS13 is the only metalloproteinase known to cleave ultra-large von Willebrand factor (vWF) polymers [9]. The roles of the other ADAMTS family members, ADAMTS6, ADAMTS16, ADAMTS17, and ADAMTS19, remain to be determined.

Ten of the nineteen ADAMTS family members—ADAMTS1, ADAMTS2, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS12, ADAMTS13, ADAMTS15, and ADAMTS18—have been reported to exhibit pro- or antiangiogenic activities with different activation pathways. ADAMTS13 is the only member in the

M. Lee, Ph.D. • (✉)
Division of Hematology and Hematologic Malignancies,
Huntsman Cancer Institute, University of Utah Health Sciences Center,
30 North 1900 East, Salt Lake City, UT 84132, USA
e-mail: manfai.lee@hsc.utah.edu

G.M. Rodgers, M.D., Ph.D.
Division of Hematology, University of Utah Health Sciences Center,
30 North 1900 East, Salt Lake City, UT 84132, USA
e-mail: george.rodgers@hsc.utah.edu

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family that possesses dual functions, both proangiogenic and antiangiogenic activities. Herein, we will focus our discussion on the angiogenic roles of ADAMTS13.

4.2 Endothelial Cell Secretion of ADAMTS13

Under normal physiological conditions, the vascular endothelium functions as a barrier between underlying somatic cells and the circulating blood. The endothelium controls angiogenesis, vasodilation and vasoconstriction, permeability, response to inflammation, and hemostasis. In the quiescent, unperturbed state, confluent endothelial cells (ECs) inhibit blood coagulation and platelet function to ensure a healthy vascular system. However, in response to vascular injury, endothelial cells secrete ultra-large vWF [10]. Plasma ADAMTS13 cleaves the ultra-large vWF, and the resulting vWF binds to the exposed basement membrane and platelets, thereby initiating platelet thrombus formation.

Using Northern blots, Levy et al. detected transcripts for ADAMTS13 in many different tissue types [11]. Levels of ADAMTS13 are higher in hepatic and ovarian tissue compared to levels in kidney, spleen, and pancreatic tissue. Subsequently, Plaimauer et al. reported the detection of high levels of ADAMTS13 in hepatic tissues and lower levels in brain, heart, testis, and placental tissues [12].

Turner et al. [13] reported that ECs, including human umbilical vein endothelial cells (HUVECs), constitutively express ADAMTS13. Cultured normal human venous and arterial endothelial cells contain ADAMTS13. The enzyme is functionally active in endothelial cell lysates and is located in cell cytoplasm separately from vWF (in Weibel–Palade bodies). Compared to the 5–8 % of stellate cells in the human liver, the constitutive release of ADAMTS13 by the vast number of ECs throughout the vasculature may be an important source of the circulating plasma ADAMTS13.

Kling et al. [14] demonstrated an increase in transcriptional regulation and expression of ADAMTS13 in HUVECs. In experiments using an enzyme-linked immunosorbent assay (ELISA) to measure total ADAMTS13 protein expression in supernatants and cell lysates 24 h after passage, the supernatants from subconfluent HUVECs contained ~6 pg/mL of ADAMTS13, as compared to only 2 pg/mL measured in the confluent group. Forty-six to ninety-six hours after cell passage, the concentration of ADAMTS13 decreased to 4 pg/mL in the subconfluent group, while the expression of ADAMTS13 in the confluent group remained at 2 pg/mL. Subconfluent HUVECs expressed ADAMTS13 protein at a level three- to fourfold higher than confluent cells during the initial 24 h of measurement and steadily decreased to a steady-state level of a twofold increase at subsequent time points. The consistent amount of ADAMTS13 protein expressed by confluent HUVECs over time may merely reflect the quiescent state of the cells producing a constitutive protein. On the other hand, the increased ADAMTS13 expression observed in subconfluent HUVECs may reflect their proliferative state due to their higher metabolic activity. With a relatively long ADAMTS13 half-life of 2–3 days, significant alterations in ADAMTS13 protein could persist for days in subconfluent cells; expression diminishes as the cells approach confluence.

4.3 ADAMTS13 and Normal Angiogenesis In Vitro

Angiogenesis is the growth of blood vessels from the existing vasculature. Under normal conditions, angiogenesis is a highly regulated physiological process and is necessary for normal wound healing and embryonic development. Two types of angiogenesis are known today: (1) sprouting angiogenesis and (2) intussusceptive angiogenesis.

Sprouting angiogenesis is initiated by the release of proteases from activated endothelial cells followed by the degradation of the underlying basement membrane (Fig. 4.1). Consequently, endothelial cells begin to migrate into the stroma of the neighboring tissues and initiate cell proliferation and differentiation into mature blood vessels [15]. Sprouting angiogenesis is initiated in hypoxic conditions where the poorly perfused tissues induce the formation of blood vessels to satisfy the metabolic requirements of parenchymal cells.

Intussusceptive angiogenesis, also known as splitting angiogenesis, occurs when the vessel wall extends into the lumen causing a single vessel to split into two. The process occurs faster than sprouting angiogenesis and does not rely on immediate endothelial proliferation and migration.

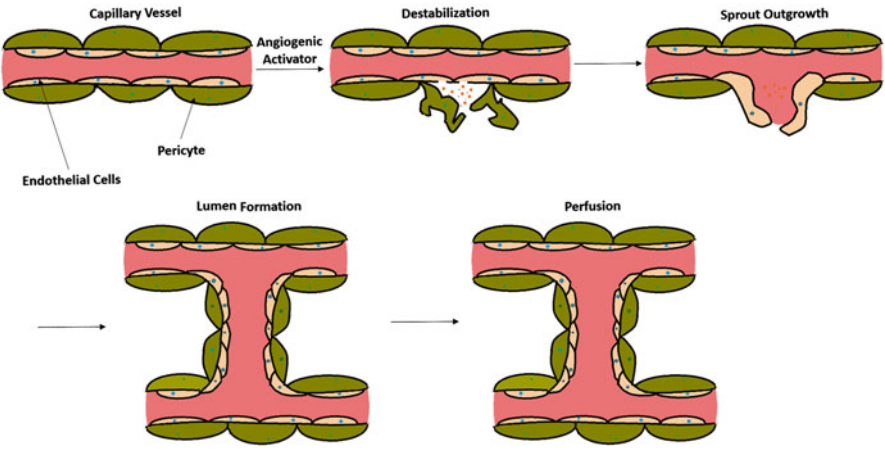

Fig. 4.1 Sprouting angiogenesis: the internal walls of capillary vessels are lined with endothelial cells and are separated from the exterior pericyte by a basement membrane. To induce angiogenesis, different signaling agonists, such as VEGF or nitric oxide, interact with the extracellular matrix proteases. This process leads to an increase in capillary permeability (leaky capillary) and destabilizes the integrity of the capillary structure. Release of signaling chemicals (depicted as *orange dots*) triggers the endothelial cells to migrate and protrude outward. In the presence of VEGF and other angiogenic promoters, these endothelial cells proliferate and form tubules. Eventually, they connect with and join adjacent leaky capillary vessels and establish a new capillary scaffold

Each of the angiogenic processes is tightly regulated with the regulation of endogenous factors that promote and inhibit angiogenesis. In the normal state, endothelial cells are usually quiescent and divide approximately every 7 years [16]. In the malignant state, when the angiogenic switch is dysregulated, endothelial cell growth rate is accelerated and replication can occur as often as every 7–10 days [17]. The lack of integrity of these newly formed neoplastic capillaries and smooth muscle wall and the irregular leaky basement membrane facilitate tumor cell leakage into the circulation, thereby leading to metastatic disease [18–20].

Until recently, the possible role(s) of ADAMTS13 secreted by the vasculature have not been defined. It is generally believed that liver-secreted ADAMTS13 is responsible for cleaving ultra-large vWF [21, 22]. Whether EC-secreted ADAMTS13 plays a role in regulating hemostasis is unclear, however, its physiological roles are an active subject of research [23–28].

ADAMTS13 shares similar domain architecture with members of the ADAM and ADAMTS protein families. Many ADAMS and ADAMTS family members are multifunctional proteins and have been shown to serve as regulators of proteolytic and non-proteolytic events. For example, the active metalloproteinase domain has been demonstrated to degrade extracellular matrix components, leading to the release of growth factors and cytokines, and subsequently promote cell proliferation, migration, and angiogenesis. The disintegrin or cysteine-rich domains on the other hand can regulate adhesion and migration. The thrombospondin motif has been shown to inhibit angiogenesis by modulating VEGF activity. In the case of ADAMTS13, recent studies have shown that it can (1) promote angiogenesis and (2) inhibit VEGF-induced angiogenesis.

Lee et al. [29] used different cell models to study the angiogenic roles of ADAMTS13 in ECs. Incubation of HUVECs with recombinant human (rh)-ADAMTS13 resulted in an increase in cell-tube formation (Fig. 4.2), cell proliferation, and cell migration across either a polycarbonate membrane (Boyden chamber assay) or a scratched wound (scratch wound assay) [29] (Fig. 4.3). Figure 4.2 illustrates the formation of networks of branched EC tubules within the extracellular matrix in a Matrigel assay model when 200 ng/mL ADAMTS13 (1.4 nM) was incubated with subconfluent ECs. As compared with the negative control group, ADAMTS13 increased tube formation by 65 %.

It is possible that the constitutive release of ADAMTS13 by endothelial cells throughout the vasculature may be playing an important role in regulating angiogenesis. In a recent publication, Starke et al. reported that an increase in angiogenesis was observed in vWF-deficient mice [30]. Using three different model systems, they showed that (1) loss of endothelial vWF results in increased in vitro angiogenesis; (2) neo-angiogenesis and vascularization are increased in the vWF-deficient mouse; and (3) the phenotypes can be recapitulated in ECs from patients with vWF. These findings suggest that endothelial cell-secreted ADAMTS13 and vWF regulate angiogenesis.

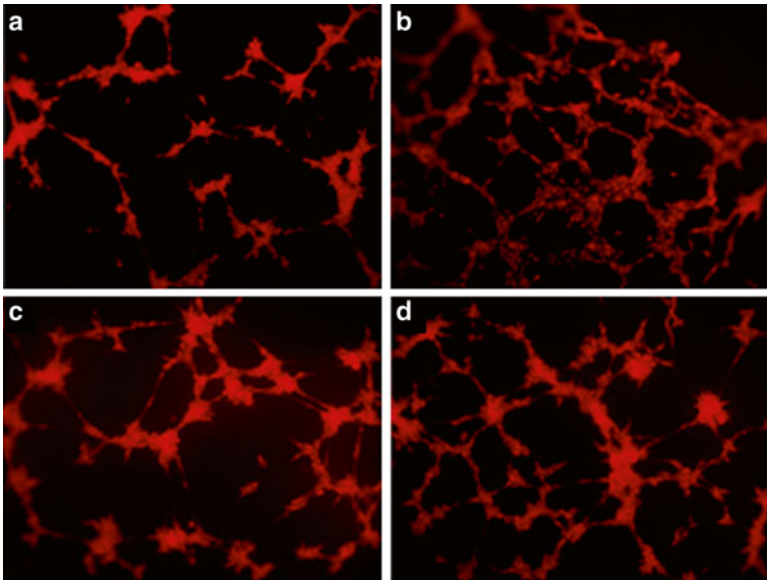

Fig. 4.2 Subconfluent HUVECs (70 % confluence) with a seeding density of 15,000 cells/well were added to a thin layer of Matrigel and incubated for 5 h. The cells were supplemented with (**a**) endothelial basal media-2 (EBM-2), (**b**) EBM-2 with 20 % fetal bovine serum, (**c**) 30 ng/mL VEGF, and (**d**) 200 ng/mL ADAMTS13. The cells were fixed with 3.7 % paraformaldehyde and permeabilized with 0.5 % Triton X100 for 5 min and finally stained with 50 μg/mL CellMask™. Fluorescent micrographs (x10) were obtained using a fluorescent microscope. ADAMTS13 treatment increased EC cell-tube formation to a similar extent as VEGF. From Lee M, et al. Microvascular Res 2012; 84: 109–115, with permission

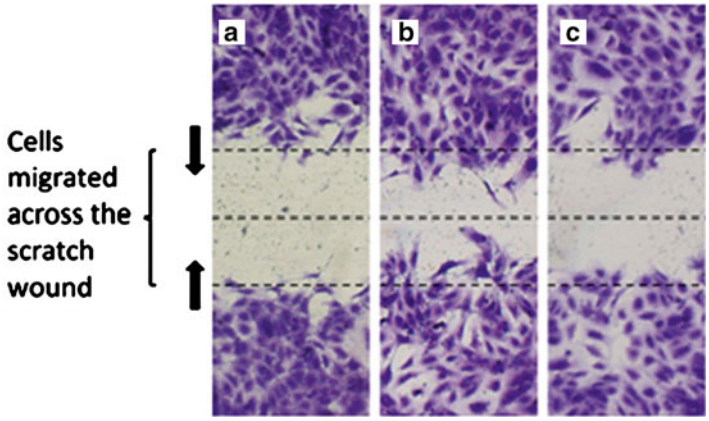

Fig. 4.3 Migration of HUVECs across a scratch wound. HUVECs grown to 85 % confluence in a 24-well plate were scratched using a sterile P-200 pipette tip. The cells were replenished with EBM-2 media containing: (**a**) 0.1 % BSA, (**b**) 6.2 ng/mL $VEGF_{165}$, and (**c**) 10 ng/mL ADAMTS13. The cells were further incubated for 6 h and finally fixed with 3.7 % paraformaldehyde and stained with 0.5 % crystal violet for cell counting. Cell migration was increased following EC treatment with ADAMTS13 and VEGF. From Lee M, et al. Microvascular Res 2012; 84: 109–115, with permission

4.4 ADAMTS13 Promotes Blood Vessel Migration/ Formation in the Chicken Chorioallantoic Membrane Model

Chick chorioallantoic membrane (CAM) assay is a commonly used model to study angiogenesis [31, 32] and tumor invasion [33–37]. As discussed by Lokman et al. [37], the CAM model has many distinct advantages, i.e., (a) CAM highly vascularized, (b) simplicity and cost-effectiveness, (c) high reproducibility, and (d) increase in half-life of test compounds due to its closed system as compared to animal models.

Deryugina and Quigley [33] developed a protocol to insert onplants supplemented with proteins onto the CAM. Following this published protocol, our laboratory successfully inserted onplants consisting of either $VEGF_{165}$ or ADAMTS13 (premixed with rat-tail type 1 collagen) to day 8 CAM. Vascular networks around the onplants developed rapidly, and by day 11, the vasculature were imaged using a stereomicroscope. As illustrated in Fig. 4.4, a significant increase in blood vessel formation was observed around the onplants in the presence of VEGF or ADAMTS13, as opposed to the absence of new vessel development in the negative control group. Using the quantification method developed by Deryugina, we compared the number of grids with new capillary formation, and we observed that both VEGF and ADAMTS13 stimulated an eightfold increase in vessels compared to the EBM-2 media control.

4.5 Antiangiogenic Roles of ADAMTS13

ADAMTS13 was also found to bind and inhibit $VEGF_{165}$ activity and abrogate VEGF-induced angiogenesis in vitro. Biological entities that possess dual proangiogenic and antiangiogenic characteristics are not uncommon. Thrombospondin-1, a known potent antiangiogenic factor, has been shown to induce angiogenesis under different conditions [38]. It was reported that the heparin-binding portion (25 kDa) of thrombospondin-1 promotes angiogenesis by inducing matrix metalloproteinase MMP-9 expression in bovine aortic endothelial cells, whereas the other fragment (140 kDa) suppresses fibroblast growth factor-induced angiogenesis [38].

Lee et al. [29] observed a dose–response profile when HUVECs were co-incubated with 2 ng/mL $VEGF_{165}$ (104 pM) and 1–1000 ng/mL ADAMTS13 (6.8 pM–6.8 nM) (Fig. 4.5). A significant decrease in the formation of tube junctions was observed when the ADAMTS13 concentration exceeded 68 pM. The effectiveness of ADAMTS13 to neutralize VEGF-induced angiogenesis in HUVEC was clearly illustrated in a BrdU proliferation assay (Fig. 4.6). While either $VEGF_{165}$ (2.08 nM) or ADAMTS13 (4.08 nM) induced HUVEC proliferation significantly, co-incubation of VEGF and ADAMTS13 reversed VEGF-induced proliferation over a time course of 72 h.

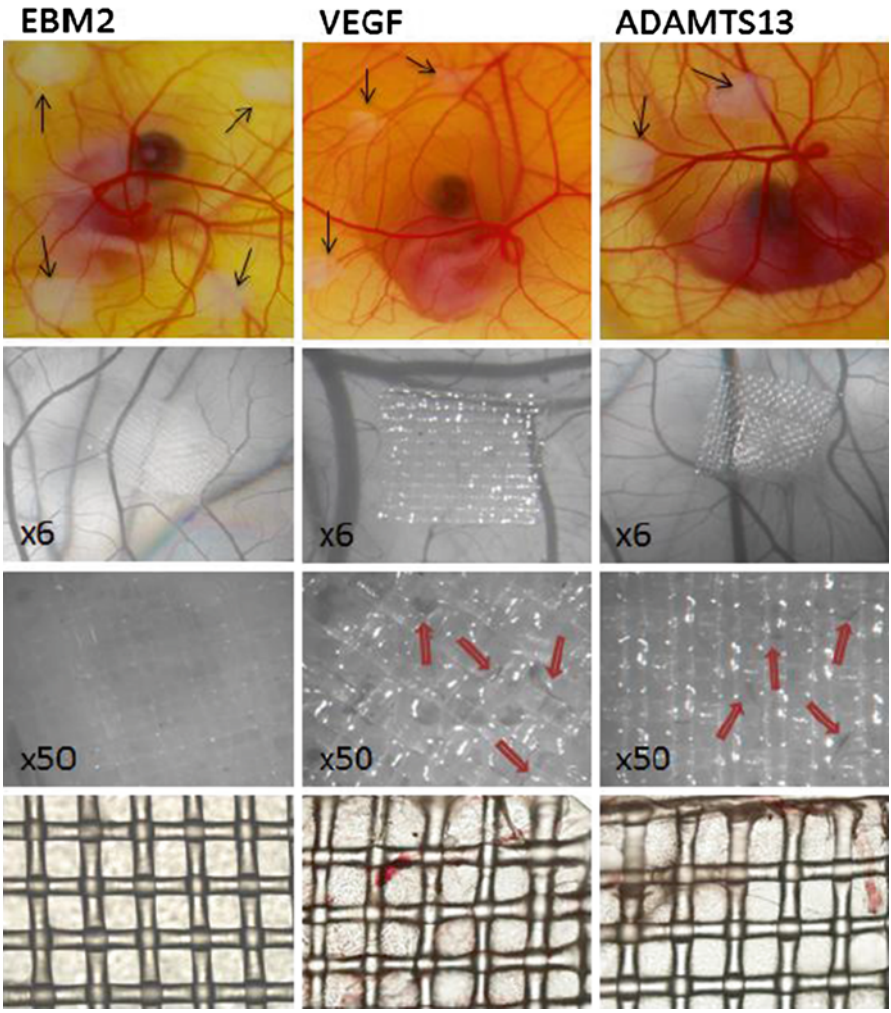

Fig. 4.4 ADAMTS13 promotes angiogenesis in the chick chorioallantoic membrane model. VEGF (40 ng/mL) and full-length ADAMTS13 (306 ng/mL) promoted blood vessel migration (*single arrows*). The presence of blood vessels is shown in the x6 and x50 magnification images, and they are clearly visible after dissecting the onplants (lower 2 rows of images). *Double arrows* indicate where new blood vessels migrated from underneath and into the onplants

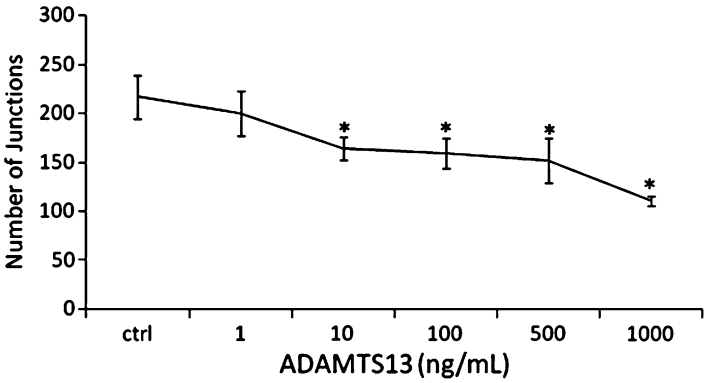

Fig. 4.5 ADAMTS13 inhibits VEGF-stimulated HUVEC tube formation in vitro. HUVEC (20,000 cells/well) were co-incubated with 104 pM $VEGF_{165}$ and ADAMTS13 (6.8 pM–6.8 nM) for 24 h. The number of tube junctions are proportional to the number of tubes formed in the Matrigel and were quantified using Angioquant software. Mean ± SD values are shown. Ctrl = VEGF alone. *Asterisks* indicate values statistically different from the control ($p < 0.05$). From Lee M, et al. Microvascular Res. 2012; 84; 109–115, with permission

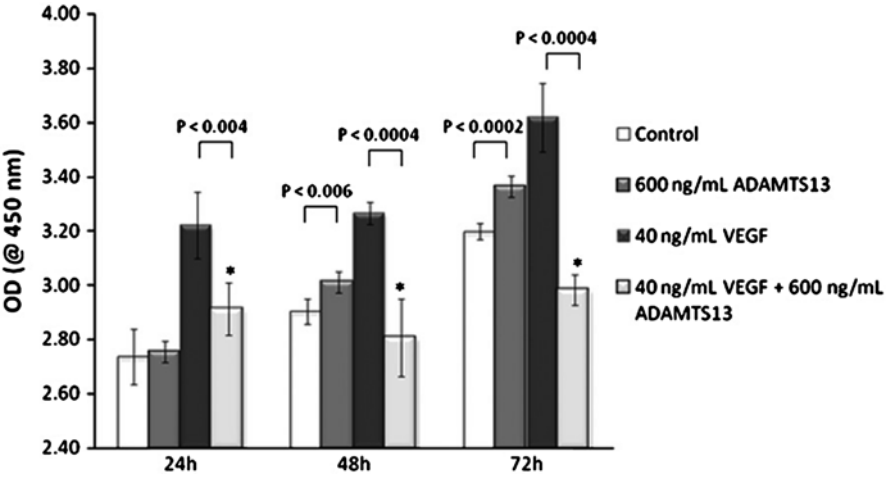

Fig. 4.6 Effects of FL-ADAMTS13 on angiogenesis over a 72 h time course in a BrdU proliferation assay. HUVEC cultured in media containing 4.08 nM ADAMTS13 proliferated faster than the negative control after 48 and 72 h. VEGF-induced proliferation was suppressed in the presence of ADAMTS13. Mean ± SD values are shown in the figure. ANOVA test indicated that the proliferation of HUVECs was suppressed over 72 h, as indicated by the *asterisks* ($p > 0.37$). From Lee M, et al. Microvascular Res. 2012; 84; 109–115, with permission

4.6 Studies of Domain–Function Relationships

ADAMTS13 is known to cleave ultra-large vWF. Using monoclonal antibodies that recognize epitopes on ADAMTS13, Igari et al. reported that catalytic activity is strongly correlated with the metalloprotease and disintegrin-like domains [39, 40]. In all cases, the C-terminal TSP1 repeats domain was not found to be involved in the binding or cleaving ultra-large vWF. However, previous studies provide some insights into the possible role(s) of the TSP1 repeats. TSP1, a secreted glycoprotein, has been found to possess angiogenic activity. Its role to inhibit angiogenesis is well recognized by its binding to CD47. Early studies with animal models demonstrated that inhibiting TSP1 protein increases cell migration, adhesion, and proliferation [41, 42]. Multiple clinical studies are completed or ongoing globally to investigate whether tumor growth and metastasis can be controlled by regulating TSP1 proteins [43, 44]. Interestingly, under specific conditions, TSP1 glycoprotein can also exhibit proangiogenic activity [38].

To understand the domain(s) of ADAMTS13 responsible for the observed angiogenic activities, Lee et al. used five ADAMTS13 variants, i.e., MDT, MDTCS, TSP1 2-8, TSP1 2-8+CUB, and TSP1 5-8+CUB, and analyzed their function in inducing angiogenesis in vitro [45]. ADAMTS13 TSP1 2-8, TSP1 2-8+CUB, and TSP1 5-8+CUB induced HUVEC proliferation and migration significantly higher than the EBM-2 control. The efficacy of these variants was comparable to that of full-length ADAMTS13. When ADAMTS13 MDT and MDTCS variants were tested, they exhibited much weaker mitogenic and chemotactic effects, suggesting that the C-terminal TSP1 repeats domain is primarily involved in the observed proangiogenic activities.

One hypothetical mechanism of ADAMTS13 involvement in EC angiogenesis is that ADAMTS13 interacts with an unidentified receptor on HUVEC which alters VEGFR2 phosphorylation and VEGF expression. Whether ADAMTS13 can bind permanently to the receptor or compete with other growth factors or signaling molecules remains unclear.

Incubation of HUVEC with 1 nM full-length ADAMTS13 or ADAMTS13 variants with the TSP1 repeats domain resulted in an upregulation of VEGF expression in HUVEC lysates. The response occurred within 10 min after the addition of ADAMTS13, with VEGF levels in the cell lysate rising to 7.9 ng/mL. Over a time course of 2 h, the VEGF levels then steadily decreased to 6.6 ng/mL (Fig. 4.7a). Simultaneously, the phosphorylation level of VEGFR2 increased by 1.9-fold within 10 min after the addition of ADAMTS13 (Fig. 4.7b).

The findings that ADAMTS13 is secreted by HUVEC and can modulate angiogenesis may have important implications. Perhaps ADAMTS13 is playing a critical role in regulating VEGF expression in cells; (1) endothelial cell-secreted ADAMTS13 may bind VEGFR2 and result in the phosphorylation of VEGFR2, thereby inducing VEGF expression leading to angiogenesis; and (2) ADAMTS13 may function as an angiogenic inhibitor when VEGF is abundant or overexpressed.

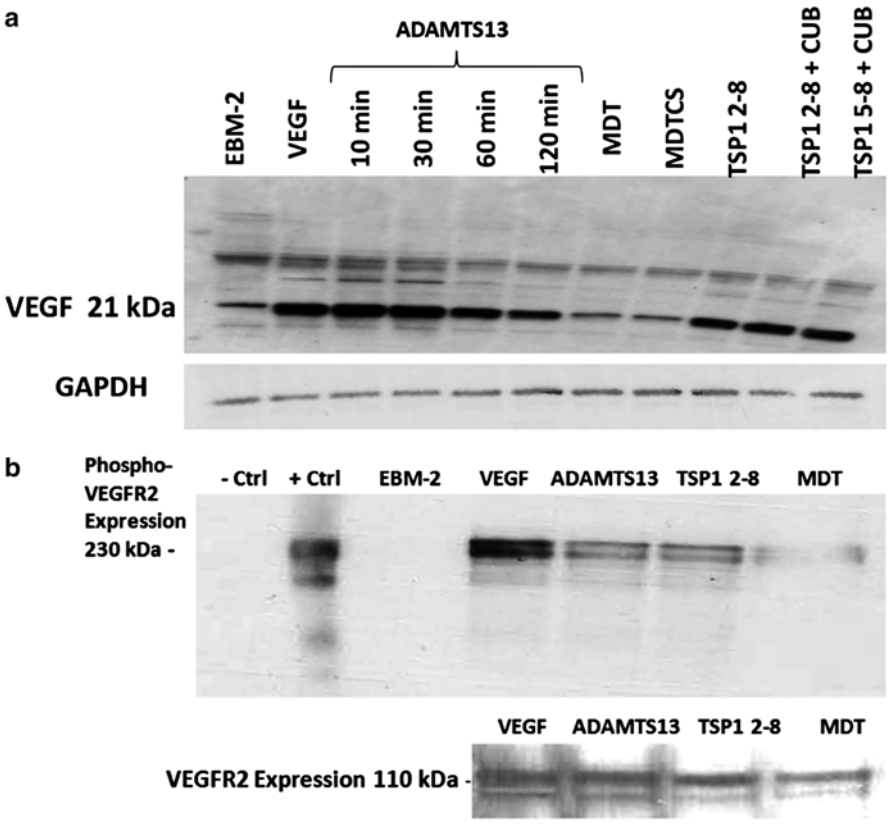

Fig. 4.7 (**a**) Immunoblot of VEGF and GAPDH (internal control) under reducing conditions in a time-dependent study when HUVEC were incubated with 1.0 nM ADAMTS13. (**b**) Immunoblot of the phosphorylation of VEGFR2 when HUVEC were incubated with 1 nM full-length ADAMTS13. ADAMTS13 increased VEGF expression, and this was associated with increased phosphorylation of VEGFR2. From Lee M, et al. Cell Molec Life Sci 2015; 72: 349–356

4.7 Expression of ADAMTS13 in Brain-Tumor Cells: Implications for Tumor Angiogenesis

ADAMTS13 activity has been associated with tumor metastasis. Olekoswicz et al. [46] reported that ADAMTS13 activity was ≤15 % of normal in 20 patients with metastatic solid tumors without thrombotic microangiopathy (TMA). Several investigators have proposed mechanisms for cancer-associated TMA that include endothelial cell injury and increased adhesion of tumor cells to the endothelium [47]. In addition, tumor cells have been found to synthesize a protein similar to platelet glycoprotein Ibα that can bind to vWF to promote platelet aggregation [48]. Interestingly, in one clinical case, a patient who underwent a surgical resection of a

glioblastoma multiforme tumor almost spontaneously developed autoimmune acquired TTP and severe depletion of ADAMTS13 soon after the surgery [49].

Several reports, including tumor cell line screening for ADAMTS13 activity performed in our laboratory (LN-229 and U-87 glioblastoma cells) [50], have shown that central nervous system-derived tumor cell lines, including microglia, astroglioma, neuroblastoma, and adult human brain endothelial cells, express ADAMTS13 [51, 52]. In addition, ADAMTS13-mRNA expression in astroglioma cells can be inhibited by interleukin (IL)-Iβ in a dose-dependent manner [51], suggesting that brain-tumor-secreted ADAMTS13 may have a role regulating hemostasis of the local microenvironment under inflammatory conditions. Mechanisms involved in the reduced functional activity of ADAMTS13 in metastatic tumors and the involvement in local hemostasis are unclear; however, based on our understanding with ADAMTS13 and EC, it is possible that brain-tumor-secreted ADAMTS13 may affect tumor vasculature.

While current studies have been limited to brain tumors, it is possible that ADAMTS13 may be overexpressed in other tumor cell lines. Understanding the roles of ADAMTS13 in tumor angiogenesis is important, as the treatment of cancer is difficult, and no contemporary treatments other than the surgery are curative for most solid tumors. Anti-VEGF/VEGFR therapies have been shown to prevent tumor angiogenesis and reduce tumor vasculature density in vitro, but their clinical effectiveness is incomplete. A number of explanations have been offered to explain the modest effectiveness of anti-VEGF/VEGFR therapy, from overwhelming secretion of other growth factors and cytokines to differential maturation of tumor vasculatures [53].

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Chapter 5
Hereditary Deficiency of ADAMTS13 Activity: Upshaw–Schulman Syndrome

Yoshihiro Fujimura, Koichi Kokame, Hideo Yagi, Ayami Isonishi, Masanori Matsumoto, and Toshiyuki Miyata

5.1 Introduction

Upshaw–Schulman syndrome (USS) is a hereditary deficiency in the activity of von Willebrand factor-cleaving protease (VWF-CP) [1], termed ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13) [2–5]. The inheritance mode is autosomal recessive, and parents of patients are usually asymptomatic carriers with one disease-causing mutation (DCM) in the gene. USS is caused by severe congenital deficiency of plasma ADAMTS13 activity due to homozygous or compound heterozygous mutations in the *ADAMTS13* gene.

When ADAMTS13 activity is deficient, unusually large VWF multimers (UL-VWFM) released from vascular endothelial cells are not cleaved appropriately, leading to platelet hyperagglutination under high shear stress [6]. Therefore, USS is also called congenital thrombotic thrombocytopenic purpura (TTP). The classic hallmarks of USS are severe neonatal jaundice with negative Coombs test necessitating an exchange blood transfusion; patients experience repeated childhood episodes of thrombocytopenia and microangiopathic hemolytic anemia (MAHA) that respond to infusion of fresh frozen plasma (FFP) (Fig. 5.1) [7]. However, recent studies have indicated that during childhood, the clinical features of the patients with USS may be milder than expected and often manifest as isolated thrombocytopenia.

Conflict of Interests YF, KK, HY, AI, MM, and TM: Nothing to declare.

Y. Fujimura, M.D. (✉) • H. Yagi • A. Isonishi • M. Matsumoto
Department of Blood Transfusion Medicine, Nara Medical University, Shijyo-cho 840, Kashihara, Nara 634-8522, Japan
e-mail: yoshifuji325@naramed-u.ac.jp

K. Kokame • T. Miyata
Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Osaka, Japan

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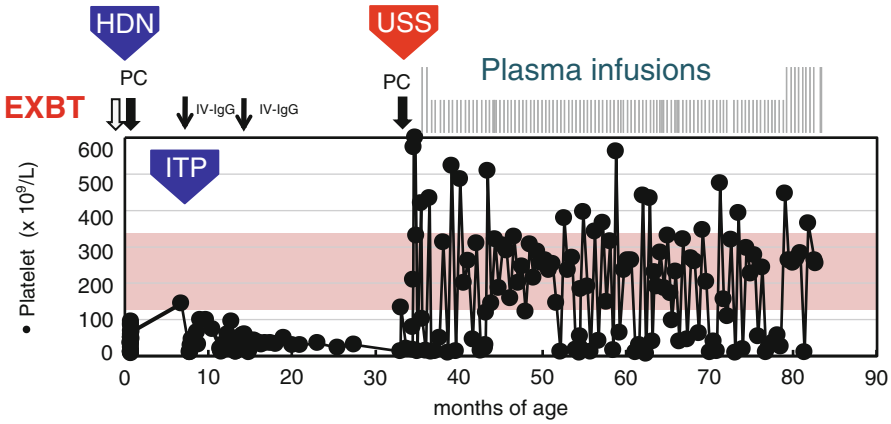

Fig. 5.1 Clinical course in a male USS patient with the early-onset phenotype. The patient was born in 2004. Soon after birth, he developed severe jaundice, thrombocytopenia, and petechiae. He received exchange blood transfusion (*EXBT*) twice, together with phototherapy, and then received transfusions of platelet concentrate (*PC*) twice. At 7 months of age, he was incorrectly diagnosed as having chronic immune thrombocytopenic purpura (*ITP*), and high-dose intravenous IgG therapy (*IV-IgG*) was instituted, but without a remarkable increase in platelet counts ($20–50 \times 10^9$/l). At the age of 2 years and 9 months, he was diagnosed as having USS with severe deficiency of both ADAMTS13 activity (<0.5 % of normal) and ADAMTS13 antigen (<0.1 % of normal), but no ADAMTS13 inhibitor (<0.5 Bethesda U/ml). *ADAMTS13* gene analysis revealed that he is a compound heterozygote of p.Q723K and p.R398C. Since then, he has received prophylactic FFP infusions every 2 weeks, with dramatic but transient increment of platelet counts 2–3 days after each plasma infusion [7]

These results suggest that a differential diagnosis of USS from primary immune thrombocytopenia or Evans syndrome would not be easy without determination of ADAMTS13 activity.

5.2 Historical Background

The first published description of USS is presumed to be the report of Dacie et al. [8] in 1953, who reviewed 12 patients with atypical congenital hemolytic anemia. One was a 6-year-old girl who had experienced repeated episodes of severe jaundice, thrombocytopenia, hemolytic anemia, and schistocytes since birth. Before visiting the hospital where Dacie's review was performed, she had already received a splenectomy at another hospital without any clinical improvement, and she ultimately died of renal failure at 7 years of age after a long history of illness. This patient was the third of four children, the first of whom was jaundiced at birth and died of hemorrhage at age 2. The second child, also jaundiced at birth, died on the fourth day of life as a result of bleeding from the bowel. The fourth child and their parents were

asymptomatic and apparently healthy. Consequently, the authors concluded that the first three children must have had a hitherto unrecognized hereditary blood disease.

In 1960, Schulman et al. [9] reported an 8-year-old girl born in Germany who experienced repeated bleeding episodes associated with chronic thrombocytopenia and MAHA, but had no specific clotting factor abnormalities. The onset of her bleeding episodes could be traced back to the postnatal period, when she had a large ecchymosis on the dorsum of one hand. These symptoms were dramatically but transiently improved by infusions of FFP, suggesting that this patient had a congenital deficiency in a "platelet-stimulating factor" in her plasma. Subsequently, in 1967, Monnens et al. [10] reported the case of a female infant who had symptoms similar to Dacie's patient and died at 9 months after illness; autopsy revealed a solitary case of TTP. Furthermore, in 1975, Wallace et al. [11] reported a family in which four out of seven siblings suffered from TTP.

In 1978, Upshaw [12] reported the case of a 29-year-old female who experienced repeated episodes of thrombocytopenia and MAHA six to seven times a year since the age of 6 months, but had been successfully treated with plasma infusions. This case was very similar to Schulman's, except that Upshaw's patient did not exhibit neonatal jaundice. Rennard and Abe [13] revealed a slightly reduced plasma level of cold-insoluble globulin (fibronectin) during the acute phase in Upshaw's original case and proposed the designation of USS for these types of patients. However, two groups of investigators, Koizumi et al. [14] and Goodnough et al. [15], reported no correlation between plasma fibronectin levels and disease activity in two USS patients, including Schulman's original case. After thrombopoietin was cloned and a sensitive assay for its detection was established, Miura et al. [16] reported five USS Japanese patients with normal plasma levels of thrombopoietin.

In clinical practice, Miura et al. [17] extensively evaluated the efficacy of several plasma components in a young boy with USS. Those authors demonstrated that whole blood, fresh or fresh frozen plasma (FFP), cryosupernatant, cryoprecipitate, and FVIII/VWF concentrate were effective in treatment of USS. By contrast, albumin, recombinant globulin, fibronectin, fibrinogen, and factor IX concentrate were ineffective. Hara et al. [18] and Karpman et al. [19] confirmed the clinical efficacy of FVIII/VWF concentrates in patients with USS. Hara et al. also reported clinical exacerbation, with reappearance of the acute-phase symptoms, within 1 h after infusion of 1-desamino-8-D-arginine vasopressin (DDAVP) in a patient with USS. Although unique clinical features and its suitable treatment were gradually discovered, the pathophysiology of USS has remained unclear, and the term USS has been almost forgotten among clinicians and researchers. Instead, the practical diagnostic term "chronic relapsing TTP" (CR-TTP) has been widely used to describe this disease of unknown etiology. This term was coined by Moake et al. [20], who had insight into four CR-TTP patients, including Upshaw's case: UL-VWFM was present in the plasma of these patients during the remission phase, but disappeared in the acute phase after infusion of FFP. He speculated that these patients had some defect in the processing of very large VWF multimers synthesized and secreted by endothelial cells. Retrospectively, however, it was subsequently shown that two of the patients had congenital or acquired TTP (one patient with each type).

In 1997, Furlan et al. [21] showed that four other CR-TTP patients lacked VWF-CP activity; however, they did not check for the presence of inhibitors of VWF-CP; indeed, it was later shown that two of their CR-TTP patients had congenital TTP, and the others had acquired TTP [22]. In 2001, using the VWFM assay described by Furlan et al., we discovered severely deficient VWF-CP activity in the absence of any inhibitor in three Japanese patients with USS. In each of these cases, the parents were asymptomatic with mild to moderate decreased levels of VWF-CP, except for one carrier who had 5.6 % of the normal level of VWF-CP [23]. Later, this carrier was shown to have a unique single nucleotide polymorphism (SNP) in one allele of the *ADAMTS13* gene, the p.P475S mutation, which is very common in the Japanese population (roughly 10 % of healthy individuals) [24]. A direct link between *ADAMTS13* gene mutations and USS was provided by Levy et al. [4], who performed positional cloning of the disease-linked gene in seven USS families. Almost simultaneously, several groups of investigators succeeded in either protein purification of this enzyme, cDNA cloning of the gene, or both [2, 3, 25, 26]. ADAMTS13 consists of 1427 amino acid residues with the following multi-domain structure: signal peptide (S)–propeptide (P)–metalloproteinase (M)–disintegrin (D)–cysteine rich (C)–spacer (S)–first thrombospondin-1(T1)–T2–T3–TY4–T5–T6–T7–T8–CUB1–CUB2. To date, more than 140 ADAMTS13 gene mutations responsible for USS have been reported worldwide; of these, 54 were from Japan (Fig. 5.2) [27].

5.3 Diagnostic Criteria for USS

Persistent deficiency of ADAMTS13 activity (<10 % of normal) with no evidence of inhibitory anti-ADAMTS13 autoantibodies (inhibitor titer <0.5 Bethesda U/ml) is essential for the diagnosis of USS. For this purpose, FRETS-VWF73 (sensitivity: ~3 % of normal) [28] is the gold standard assay. However, to predict early- or late-onset phenotypes (see below), more sensitive assays (sensitivity: ~0.5 % of normal), such as SELDI-TOF-MS [29] or chromogenic act-ELISA [30], are often needed. Further, USS patients who received plasma therapy or experienced pregnancy may have non-neutralizing IgG autoantibodies, which can be detected by commercial ELISA kits using ADAMTS13-coated plates.

A confident diagnosis of USS can be made by analyzing the *ADAMTS13* gene for DCMs with homozygosity or compound heterozygosity. Further, because USS is inherited in an autosomal recessive fashion, each asymptomatic parent of a patient will carry one DCM and will consequently have reduced plasma ADAMTS13 activity (20–50 % of normal).

5.4 Mechanism of TTP Episodes in USS

The mechanisms underlying TTP episodes in USS patients have not been entirely elucidated. However, our studies clearly showed that during remission, the plasma of USS patients promotes the aggregation of normal platelets under high shear stress [31].

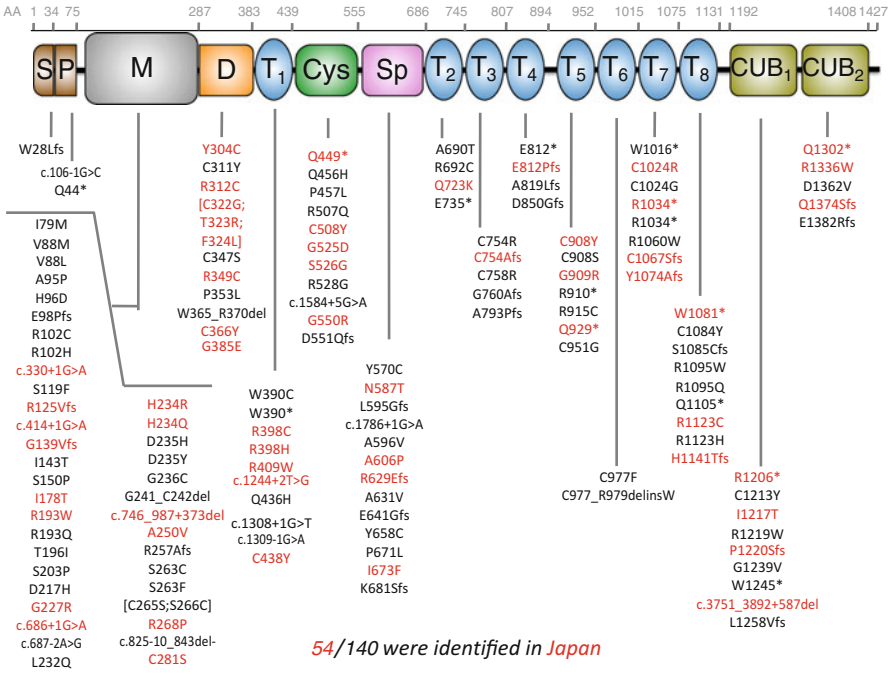

Fig. 5.2 ADAMTS13 gene mutations in USS patients. The ADAMTS13 protein consists of 1427 amino acid residues with the following multi-domain structure: signal peptide (S)–propeptide (P)–metalloproteinase (M)–disintegrin-like (D)–cysteine rich (C)–spacer (S)–first thrombospondin-1(T1)–T2–T3–TY4–T5–T6–T7–T8–CUB1–CUB2. To date, 140 *ADAMTS13* mutations responsible for USS have been reported, of which 54 were from Japan (shown in *red letters*). Note the quite different sets of mutations found in Caucasians (mostly) vs. Japanese patients, presumably due to founder effects. The descriptions of protein-sequence mutations follow the recommendations of the Human Genome Variation Society

UL-VWFM is mainly produced in vascular endothelial cells, stored in Weibel–Palade bodies, released into circulation upon stimulation, and then transported to peripheral arterioles where high shear stress is generated. Under high-shear-stress conditions, the molecular conformation of UL-VWFM changes from the quiescent globular form to the activated extended form [32], increasing accessibility for ADAMTS13. In USS patients, plasma ADAMTS13 activity is usually severely deficient; consequently, the activated UL-VWFM accumulated in the circulation interacts more intensively with platelet glycoprotein Ib, thereby generating signals that activate platelets, including Ca^{2+} influx into platelets and adenosine diphosphate (ADP) release from platelets [33–36]. A series of these reactions leads to the formation of platelet microaggregates with VWF and fibrinogen, resulting in thrombocytopenia. Furthermore, schistocytes are assumed to arise due to red cell shearing by fibrin strands, which are formed around platelet thrombi in TTP.

Although USS patients always lack ADAMTS13 activity, they do not always exhibit TTP. Instead, several aggravating factors are involved, as described in Table 5.1. Unlike USS patients, *ADAMTS13* knockout mice are born without TTP

Table 5.1 Summary of history of 55 USS patients in Japan

Family:
• 48 families • Autosomal recessive inheritance, but female predominance (33 F and 22 M) • Up to 79 years of age
Exchange blood transfusion for severe neonatal jaundice: 21/55 patients (38%). Hemodialysis for chronic kidney disease: 6 patients (4 are deceased)
Aggravating factors:
• Severe infections such as influenza • Pregnancy: 28 occasions in 15 female patients (1 patient was diagnosed after death) • DDAVP • Interferon • Heavy drinking of alcohol • Aging
ADAMTS13 gene analysis in 53 patients: 9 homozygotes 41 compound heterozygotes

signs and exhibit normal survival. Further, female *ADAMTS13* knockout mice do not exhibit TTP symptoms during pregnancy or after delivery [37, 38]. Therefore, Motto et al. showed that Shiga toxin produced by pathogenic hemorrhagic *Escherichia coli* could induce TTP-like syndromes in *ADAMTS13* knockout mice. On the basis of these observations, they postulated that the *ADAMTS13* knockout is not sufficient to evoke TTP and that a "second hit" is required. Later, however, Huang et al. showed [39] that the B-subunit of Shiga toxin binds to its receptor, globotriaosyl ceramide (Gb3), on the surface of vascular endothelial cells and mediates VWF release, suggesting that elevated plasma levels of HMW-VWFMs are physiologically important. This speculation was supported by subsequent observations that high-dose infusion of human recombinant (r) VWF preparations containing HMW-VWFMs could induce TTP-like symptoms in *ADAMTS13* knockout mice without any additives [40]. These results strongly suggest that a sudden enormous increase in the plasma ratio of HMW-VWFM to ADAMTS13 activity may induce TTP episodes. We assume that this phenomenon could be induced by sepsis or transplantation, even in normal individuals, because some cytokines are strong inducers of UL-VWFMs from vascular endothelial cells [41].

5.5 USS Registry in Japan

USS is a rare disease, so its prevalence has not been accurately determined. In Europe, based on the number of diagnosed patients, estimates of prevalence range from 0.5 to 4 cases/million/year. However, a much higher prevalence has been reported in certain areas, including Scandinavia and countries around the Baltic Sea, than in Switzerland [42].

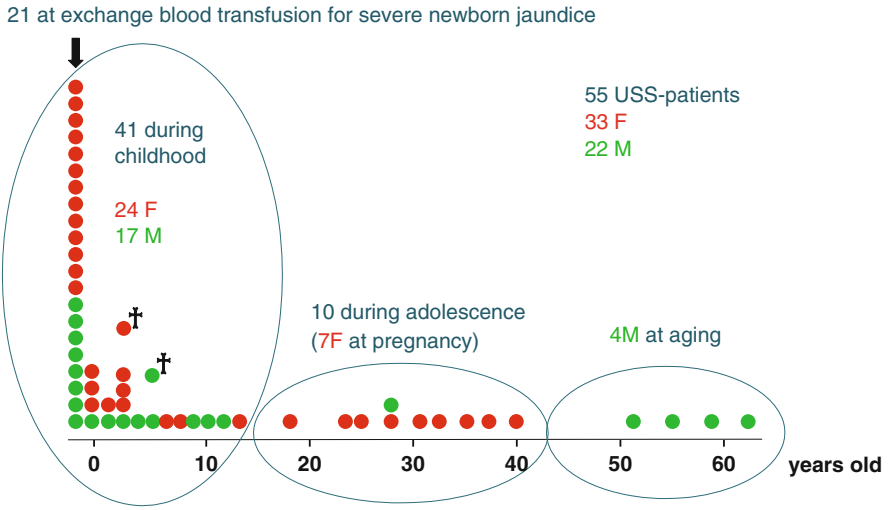

Fig. 5.3 Patient's age at the first thrombocytopenic episode in 55 USS patients in Japan. USS patients in our registry were classified into three groups according to the age of their first episode of thrombocytopenia: during childhood (41 patients, 24 females and 17 males), aged 15–45 years (10 patients, 9 females and one male), and aged >45 years (4 patients, all males). Among the patients, 21 (21/55, 38 %) had a classic hallmark of USS, severe neonatal jaundice necessitating exchange blood transfusion. Furthermore, seven out of nine females aged 15–45 years developed thrombocytopenia at pregnancy. Note that four male patients were all diagnosed after the age of 50 years. Crosses (*plus*) indicate patients who were diagnosed as having USS after their deaths, based on ADAMTS13 analyses

Our institute, Nara Medical University, has functioned as a thrombotic microangiopathy (TMA) referral center in Japan since 1998. As of 2013, we have collected a large dataset of 1251 TMA patients (unpublished). Through this cohort study, we identified 55 USS patients (33 females and 22 males) belonging to 48 different families, aged from early childhood to 79 years.

Clinical signs of USS in childhood are milder than expected. In fact, patients often exhibit isolated thrombocytopenia, which pediatricians often overlook or misdiagnose as primary immune thrombocytopenia or Evans syndrome. In Fig. 5.3, we summarize when our USS patients experienced their first reported episodes of thrombocytopenia: during childhood (41 patients, 24 females and 17 males), aged 15–45 years (10 patients, 9 females and 1 male), and aged >45 years (4 patients, all males). Further, a high relapse rate has been reported in patients with neonatal onset, who required more intensive treatment. Indeed, brain strokes occurred in one-third of such patients, of whom roughly half suffered from neurologic complications, and end-stage renal disease was also seen in 20 % of the patients [43, 44].

Among our 55 patients, 21 (21/55, 38 %) had the classic hallmark of USS, severe neonatal jaundice requiring exchange blood transfusion. In fact, 29 patients (15 females and 14 males) were correctly diagnosed during childhood, termed the "early-onset phenotype" (below 15 years old), but the remaining 26 patients

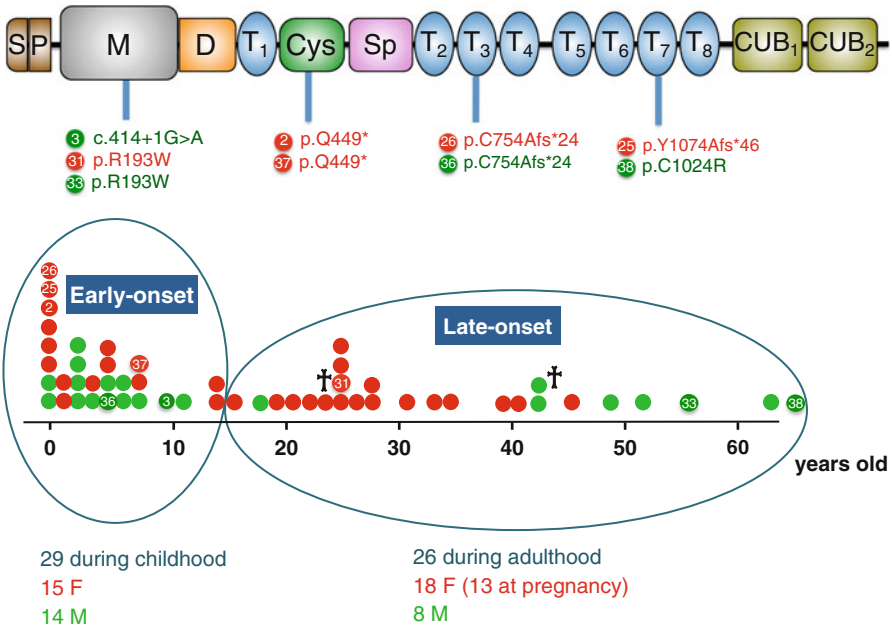

Fig. 5.4 Patient's age at correct diagnosis of USS or TMAs in 55 USS patients in Japan and genotype–phenotype relationships in nine Japanese USS patients in our registry with homozygous *ADAMTS13* gene mutations. Although USS patients are sometimes incorrectly diagnosed as having primary immune thrombocytopenia or Evans syndrome, the majority of patients are correctly diagnosed as having USS or TMAs according to their clinical signs, with or without ADAMTS13 assays. Therefore, this figure shows patient's age at correct diagnosis of USS or TMAs, among 55 USS patients in Japan. Note that 29 patients (15 females and 14 males) were correctly diagnosed during childhood (<15 years old), termed the "early-onset phenotype," and the remaining 26 patients (18 females and 8 males) were diagnosed during adulthood, termed the "late-onset phenotype." In the latter group, 13 out of 18 female patients were correctly diagnosed during pregnancy. Crosses (*plus*) indicate the patients who were diagnosed as having USS after their deaths, based on ADAMTS13 analyses. In our registry, we identified nine unrelated USS patients carrying homozygous gene mutations: p.R193W ($n=2$), c.414+1G>A ($n=1$), p.Q449* ($n=2$), p.C754Afs*24 ($n=2$), p.Y1074Afs*46 ($n=1$), and p.C1024R ($n=1$). All patients carrying these mutations had plasma ADAMTS13 activity less than 0.5 % of normal, except for one male patient carrying the p.C1024R mutation (3.4 %) who experienced his first TTP episode at the age of 63. These patients developed TTP as indicated in the figure, suggesting that residual plasma ADAMTS13 activity is in part a reflection of the late-onset phenotype, rather than the location of gene mutations

(18 females and 8 males) were diagnosed during adulthood, termed the "late-onset phenotype" (Fig. 5.4, bottom). Fifty-three patients were subjected to *ADAMTS13* gene analysis, and a pair of DCMs was identified in 50 patients: 9 homozygotes and 41 compound heterozygotes. Among 26 patients with the late-onset phenotype, 18 were females, of whom 13 (13/18, 72 %) developed their first TTP episodes during pregnancy. The remaining eight male patients, with one exception, were diagnosed after 42 years of age.

5.6 Genotype–Phenotype Relationship

Kokame et al. [45] analyzed the *ADAMTS13* gene in the Japanese general population and identified six common missense SNPs: p.T339R, p.Q448E, p.P475S, p.P618A, p.S903L, and p.G1181R. The allele frequencies of these SNPs correlated with ABO blood type in the population. p.Q448E and p.P475S were significantly associated with plasma ADAMTS13 activity. In addition, the authors speculated that one individual in 1.1×10^6 should be homozygous or compound heterozygous for USS-causative mutations and estimated that 110 individuals in Japan might have hereditary ADAMTS13 deficiency or USS. Camilleri et al. [46] reported that the most prevalent SNP in the United Kingdom was p.Q448E, followed by p.R7W and p.A900V.

Since 2001, more than 140 ADAMTS13 gene mutations have been reported worldwide. In 2010, Lotta et al. [47] analyzed 76 *ADAMTS13* mutations in USS patients, including 45 missense (59 % of total), 10 nonsense (13 %), 10 deletions (13 %), 4 insertions (6 %), and 7 splice-site mutations (9 %). Of those patients, 64 % were compound heterozygous and 36 % were homozygous for ADAMTS13 mutations. Of 45 missense mutations, 33 localized between the M and S domains (inclusive), which are functionally essential for ADAMTS13 activity. USS patients have been reported on all continents, and the most frequent mutation in Central Europe, c.4143insA, was assumed with common genetic background, around Baltic Sea coast [48]. They also investigated the relationships between specific *ADAMTS13* mutations and the clinical features of USS and reported that patients with homozygous mutations at the N-terminal domains tended to exhibit lower residual ADAMTS13 activity and earlier age of onset (specifically, first TTP episode requiring FFP) than patients with homozygous C-terminal domain mutations [47]. However, these findings differ slightly from ours.

In our cohort study, we identified nine unrelated USS patients carrying homozygous gene mutations: p.R193W ($n=2$), c.414+1G>A ($n=1$), p.Q449* ($n=2$), p.C754Afs*24 ($n=2$), p.Y1074Afs*46 ($n=1$), and p.C1024R ($n=1$) (Fig. 5.4, top). These mutations localized in the domains of M, Cys-rich, third TSP-1, and seventh TSP-1 domains. Among them, patients with the early-onset phenotype carried c.414+1G>A ($n=1$), p.Q449* ($n=2$), p.C754Afs*24 ($n=2$), and p.Y1074Afs*46 ($n=1$), whereas those with the late-onset phenotype carried p.R193W ($n=2$) and p.C1024R ($n=1$). Most patients carrying these mutations had plasma ADAMTS13 activity less than 0.5 % of normal, although the patient carrying the p.C1024R mutation had some activity (3.4 %). The latter patient, whose history we describe in detail elsewhere [7], developed his first TTP episode at the age of 63. This observation clearly indicated that residual plasma ADAMTS13 activity is in part a reflection of the late-onset phenotype, rather than the location of the gene mutations. To further support this observation, Lotta et al. [49] recently reported the associations between residual plasma ADAMTS13 activity and the phenotypic features of USS.

Consequently, we identified 54 mutations in Japanese USS patients. Strikingly, these mutations were very different from those reported in the United States and Europe. However, p.R349C mutation was previously reported in a Chinese USS patient in Hong Kong [50], and c.330+1G>A was identified in a Korean patient [51]. The *ADAMTS13* mutations with the highest frequency in Japan are as follows (sorted in order of decreasing prevalence): p.R193W, p.C908Y, p.Q449*, and p.C754Afs*24. The p.Q449* mutation is localized to the northern part of Japan, p.C908Y is localized to the western part, and p.R193W is relatively widespread across Japan. The p.C754Afs*24 mutation is localized to the southern island [52].

5.7 Pregnancy

Pregnancy-associated TMAs occur at a frequency ranging from 1 in 25,000 to 1 in 100,000 pregnancies worldwide. Among them, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome is typical, but pregnancy-associated TTP is assumed to represent approximately 10–30 % of all adult TTP cases in the North American, European, and Japanese TMA registries [53–58]. Differential diagnosis of these serious diseases is prerequisite for choosing the best therapeutic approach.

In 2008, we reported the first series of pregnancy-associated TTP caused by hereditary deficiency of ADAMTS13 activity in nine USS patients from six unrelated Japanese families, whose diagnoses were made during their first pregnancy [59]. Six out of the nine patients had episodes of thrombocytopenia during childhood that were incorrectly diagnosed as primary immune thrombocytopenia. They manifested thrombocytopenia during the second or third trimesters in each of their 15 pregnancies, often followed by TTP episodes. Of the 16 babies (one twin pregnancy), eight were stillborn or died soon after birth, and the remaining seven were all premature except one, who was born naturally following FFP infusions to the mother starting at 8 weeks of gestation. All nine USS patients had severely deficient ADAMTS13 activity (less than 0.5 % of the normal), but lacked ADAMTS13 inhibitors. *ADAMTS13* gene analyses in these patients demonstrated that eight of these women were compound heterozygous for p.Y304C and p.G525D (two siblings), p.R125Vfs*6 and p.Q1302* (two siblings), p.R193W and p.R349C (two siblings), p.I178T and p.Q929*, or p.R193W and p.A606P; the remaining woman was homozygous for p.R193W. Among the nine USS patients, seven had *ADAMTS13* gene mutations in the M domain, and two had mutations within the D/C domains.

In 2012, Camilleri et al. [46] described pregnancy-associated TTP in six USS patients in the United Kingdom, of whom three were compound heterozygotes for c.C265S(+)S266C and p.R1060W, p.A596V and p.R1095Q, or c.G241_C242del and p.A690T. The remaining three were homozygotes for p.R1060W, p.R102H, or p.C977F. The p.R1060W missense mutation in the seventh TSP-1 domain is associated with late-onset adult TTP [60]. Furthermore, Kentouche et al. [61] reported

three USS patients with pregnancy-associated TTP: two compound heterozygotes for 763-769dupl7 and p.R1060w and p.R1034* and c.4143insA and one homozygote for c.4143insA. More recently, Calderazzo et al. [62] described one such patient with a novel homozygous mutation of p.D1362V.

Although the clinical observations have obviously demonstrated that pregnant women with USS are prone to develop TTP without replenishment of ADAMTS13, prophylactic plasma infusions are apparently beneficial for both mother and baby [63]. For this purpose, FFP at a single dose of 10 ml/kg every 3–4 weeks is effective, but more frequent FFP infusions (10 ml/kg for 2–5 consecutive days) are required at each relapse. Based on our experience, we propose that for uneventful delivery, the infusion of FFP at a dose of 10 ml/kg or 400–480 ml/week to the mother should start in the early stage of pregnancy (8 weeks of gestation). However, the best plasma therapy protocol for pregnant USS patients remains to be established in future studies.

Moatti-Cohen et al. [63] recently reported that the proportion of USS patients with pregnancy-associated TTP appears to be much higher than that of acquired ADAMTS13-deficient TTP, contrary to previous expectations. This observation emphasizes the necessity of measuring ADAMTS13 activity and inhibitor titers in thrombocytopenic patients of unknown etiology during pregnancy, as well as during childhood.

5.8 Renal Involvement

Prophylactic plasma infusions may control TTP episodes in USS patients, but may not effectively prevent the development of some renal impairment. Therefore, severe chronic renal failure (CRF) requiring hemodialysis is one of the serious complications that arise in USS patients. In our registry, 6 out of 55 patients fell into severe CRF despite receiving plasma therapy over time; four of them are now deceased (Table 5.1).

Noris et al. [64] reported differences in the clinical presentations of USS patients with severe deficiency of ADAMTS13 activity in a family carrying the compound heterozygous mutations p.G1239V and p.V88M. One male patient was asymptomatic, but a female patient had experienced several TTP episodes since her first pregnancy. Furthermore, a female patient carrying an additional heterozygous p.S890I mutation in complement factor H (CFH) continued to have relapses and severe clinical manifestations, including neurologic signs and renal insufficiency. She progressively developed end-stage renal failure despite hemodialysis and eventually died of cerebrovascular events. Because the CFH-p.S890I mutation has been implicated as a DCM for atypical hemolytic uremic syndrome (aHUS), it might have significantly influenced the clinical phenotype of this patient. Recently, Mise et al. [65] also reported a similar patient who carried the compound heterozygous mutations p.I1217T and p.C908Y in *ADAMTS13* in association with low serum levels of C3 and normal C4, suggesting selective activation of the alternative pathway.

These results indicated that gene abnormalities in the complement regulatory system might influence the clinical features and disease severity of USS. Conversely, Feng et al. [66] reported that half of aHUS patients exhibit partial deficiency of ADAMTS13 activity (50 % of normal), although the etiology was not addressed. Thus, the potential linkage between ADAMTS13 and complement regulatory system represents the next target in efforts to better understand the pathophysiology of USS.

5.9 Treatment of USS

FFP is still predominantly used for prophylactic and therapeutic purposes in USS patients. In 1999, Furlan et al. [67] measured the half-lives of plasma ADAMTS13 activity in two patients with relapsing thrombotic thrombocytopenic purpura (TTP) after plasma exchange, using a VWFM assay (sensitivity: ~3 % of normal activity). They found that the half-lives in the two patients were 3.3 and 2.1 days, respectively.

In 2001, we performed consecutive analyses of plasma ADAMTS13 activity, platelet counts, plasma VWFM patterns, and high-shear-stress-induced platelet aggregation (H-SIPA) generated by a mixture of patient plasmas and washed normal platelets, following infusion of FFP to two USS patients [31]. The maximal level in ADAMTS13 activity was observed 30 min after FFP infusion and then gradually decreased over a week. The platelet count began to rise 24 h after FFP infusion, plateaued from days 7 to 11, and then decreased. UL-VWFMs present in patient plasmas before FFP infusion began to disappear 1 h after FFP infusion and were totally absent 24 h later. However, the UL-VWFMs began to reappear 2 days later and subsequently returned to pre-infusion levels by day 14 (Fig. 5.5, left). We found that H-SIPA, measured using a mixture of patient plasmas and washed normal platelets, was remarkably enhanced before FFP infusion. The enhanced H-SIPA began to normalize soon after FFP infusion and totally disappeared at 24 h later. Two days later, however, the enhanced H-SIPA reappeared, gradually increased, and then returned to the pre-infusion level 14 days later. Thus, the reduction in H-SIPA was closely related to the reduction in plasma UL-VWMFs.

Most recently, we determined the recoveries and half-lives of plasma ADAMTS13 in four Japanese patients with USS who received regular prophylactic FFP infusions, using a highly sensitive chromogenic activity-ELISA [30] and an antigen-ELISA developed in-house (sensitivity: 0.1 % of normal) [68]. ADAMTS13 in FFP prepared in the Japan Red Cross Blood Center contained 81 ± 16 % of the activity and 124 ± 46 % of the antigen [69]. FFP infusion (3.1–5.6 ml/kg) was administered to the four USS patients, who weighed between 49 and 57 kg, over a period of no more than 2 h. Under these circumstances, the recovery of both ADAMTS13 activity and antigen 30 min after infusion was 71 ± 9.6 % and 70 ± 15.2 %, and the average maximum values of ADAMTS13 activity and antigen by single FFP infusion were 5.8 ± 0.6 % and 5.8 ± 2.6 %, respectively. The average half-lives (mean ± 2SD) of ADAMTS13 activity and antigen were 2.8 ± 0.6 and 2.7 ± 1.6 days, respectively (Fig. 5.5, right).

These observations suggested that 2-week intervals might be suitable for preventive FFP infusion, and it seems that 5 ml/kg biweekly FFP infusion is a reasonable

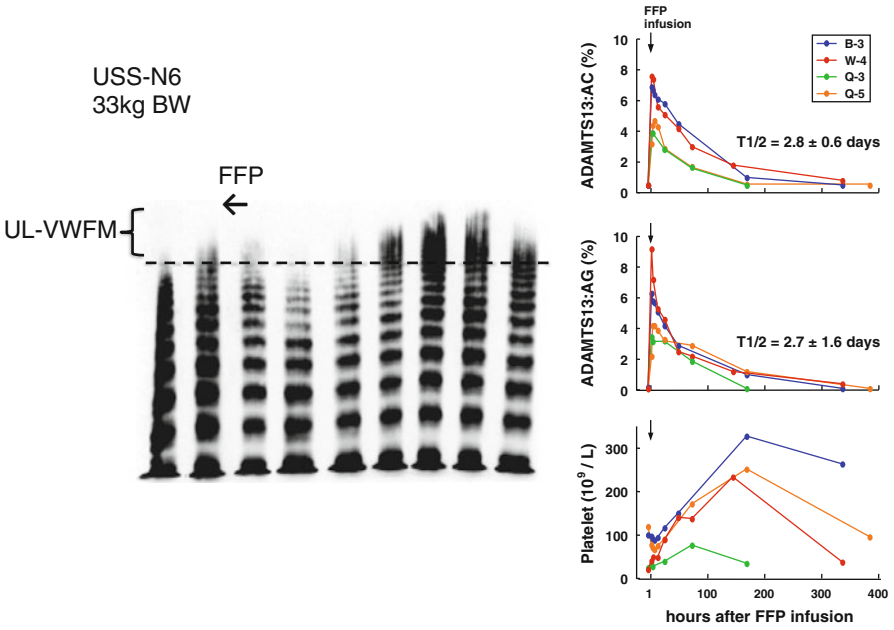

Fig. 5.5 Effect of FFP infusion on UL-VWFMs in a USS patient and half-life determinations of ADAMTS13 activity and antigen in four USS patients. A USS patient (N6, body weight 33 kg) received fresh frozen plasma (*FFP*) infusion at a dose of 5 ml/kg, and changes in VWF multimers were analyzed by SDS–1.2 % agarose gel electrophoresis. UL-VWFM was clearly detected in the plasma before FFP infusion, but decreased 1 h after the infusion and almost completely disappeared 24 h after infusion. However, UL-VWFMs reappeared 2 days after infusion and subsequently increased (*left*) [31]. The half-lives of plasma ADAMTS13 were determined in four Japanese USS patients (B-3, W-4, Q-3, and Q-5) who received regular prophylactic FFP infusions. The four patients weighed between 49 and 57 kg, allowing infusion of FFP (3.1–5.6 ml/kg) to be completed within 2 h. At 30 min after the infusions, the average values of ADAMTS13 activity and antigen were 5.8 ± 0.6 % and 5.8 ± 2.6 %, respectively, and the half-lives of ADAMTS13 activity and antigen were 2.8 ± 0.6 and 2.7 ± 1.6 days, respectively (*right*)

protocol for preventing TTP episodes. In sharp contrast with prophylactic use of FFP, intensified FFP infusion was required when the patients with USS developed TTP episodes triggered by pregnancy or influenza. In these acute phases, 10 mg/kg FFP infusion every 1–2 weeks was thought to be necessary for treatment. Based on our analyses, intensified FFP infusion (10 mg/kg) had sufficient impact to raise plasma ADAMTS13 activity beyond 10 % of normal in USS patients. Recently, 10 % of plasma ADAMTS13 activity was widely accepted as the lower limit of the normal range in the criteria for USS diagnosis, suggesting that 10 ml/kg FFP infusion is quite reasonable for treatment in the acute phase. Recently, recombinant ADAMTS13 (rADAMTS13) was developed. In *ADAMTS13* knockout mice, pre-infusion of rADAMTS13 could prevent TTP episodes induced by rVWFM preparation [40]. Thus, it is conceivable that sterilized plasma-derived purified ADAMTS13 [70] or rADAMTS13 preparation could be efficiently substituted for FFP in the near future.

5.10 Conclusion

During the last decade, substantial progress has been made in diagnosis and treatment of USS, and the development of rapid assays for ADAMTS13 activity will facilitate identification of new patients whose illnesses previously masqueraded as other conditions. Furthermore, it would be desirable to smoothly introduce rADAMTS13 preparation into clinical use, because this may avoid some adverse reactions resulting from the use of FFP.

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Chapter 6
Acquired Thrombotic Thrombocytopenic Purpura

A Disease due to Inhibitors of ADAMTS13

Han-Mou Tsai

Abbreviations

ADAMTS13	A disintegrin and metalloprotease with thrombospondin type 1 repeat, member 13
aHUS	Atypical hemolytic-uremic syndrome
CUB	Complement C1r/C1s, Uegf, Bmp1
Cys	Cysteine-rich region
DIC	Disseminated intravascular coagulopathy
Dis	Disintegrin
ELISA	Enzyme-linked immunosorbent assay
FRET	Fluorescence resonance energy transfer
GPI	Glycosylphosphatidylinositol
HELLP	Hemolysis, elevated liver enzymes, and low platelet counts
IFN-γ	Interferon-gamma
IL-4	Interleukin-4
ITP	Idiopathic thrombocytopenic purpura
MAHA	Microangiopathic hemolytic anemia
MP	Metalloprotease
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Spa	Spacer domain
TMA	Thrombotic microangiopathy
TNF-α	Tumor necrosis factor-alpha
TSR	Thrombospondin type 1 repeat
TTP	Thrombotic thrombocytopenic purpura
VWF	von Willebrand factor

H.-M. Tsai, M.D. (✉)
iMAH Hematology Associates, New Hyde Park, USA
e-mail: hmtsai@gmail.com

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Thrombotic thrombocytopenic purpura (TTP), first described by Eli Moschcowitz in 1924 as a fatal acute febrile illness presenting with fleeting focal neurological deficits, changes in mental status, and microangiopathic hemolytic anemia (MAHA), is characterized pathologically with widespread hyaline thrombi affecting the arterioles and capillaries of multiple organs [1]. Although not mentioned in the original case report, thrombocytopenia was later recognized to be a leading indicator of the disease.

For antemortem diagnosis of TTP, the disorder has been defined as a clinical syndrome of pentad (thrombocytopenia, MAHA, neurological deficits, fever, and renal abnormalities), triad (thrombocytopenia, MAHA, neurological deficits), or diad (thrombocytopenia and MAHA). In addition to this uncertainty, there was also no consensus on whether patients with prominent renal failure or comorbid conditions should be excluded.

6.1 From a Syndrome to a Disease

The difficulty of defining TTP as a clinical syndrome arises from two facts: more than one disorder may cause the syndrome of pentad, triad, or diad and some patients with TTP do not meet the criteria of pentad, triad, or even diad.

Clinically, some patients present with thrombocytopenia alone and are mistaken to have idiopathic thrombocytopenic purpura (ITP); others present with transient ischemic attack or stroke. A correct diagnosis of TTP is not possible for such patients until the disease evolves to also cause MAHA and thrombocytopenia. This delay often results in unnecessary morbidity and even mortality.

On the other hand, analysis of patients referred for investigation of MAHA reveals that in the absence of vascular devices such as left ventricular assist devices (LVAD), extracorporeal membrane oxygenator, or prosthetic heart valves, MAHA and thrombocytopenia may result from at least five different types of pathology (Table 6.1), each of which in turn may result from one or more etiologic mechanisms. Thus, there are multiple causes of the syndrome of thrombocytopenia and MAHA that often overlap in their clinical features. In this scheme, TTP is merely one of the many causes. TTP is considered distinct from the category of thrombotic microangiopathy (TMA) because its pathology comprises intravascular VWF-platelet thrombosis but not endothelial injury.

The pathological changes in association with MAHA share the common feature of thrombosis or stenosis in arterioles and capillaries (Fig. 6.1). Arteriolar stenosis generates abnormally high levels of shear stress, which is at its highest level at the endothelial boundary of the arterioles. Narrowing of vascular lumen increases the shear stress by the third order. Fragmentation of red blood cells occurs when they are entrapped in the narrowed arteriolar lumens and constantly exposed to abnormal shear stress.

In arterial stenosis due to thrombosis, thrombocytopenia reflects the consumption of platelets in the process of thrombosis. Ischemia in association with arteriolar

Table 6.1 A comparison of pathological lesions associated with the syndrome of MAHA and thrombocytopenia

Pathology	VWF thrombosis	Fibrin thrombosis	Thrombotic microangiopathy	Vasculitis/ vasculopathy	Intravascular tumor cells
Primary event	VWF-platelet thrombosis	Activation of coagulation system	Endothelial cell injury	Infection or autoimmunity	Metastatic neoplasm
Vessel components involved	Luminal	Luminal	Endothelial cells Intima	Intima, media, adventitia	Luminal
Thrombosis	Yes	Yes	Variable	Not prominent	Minimal
Fibrinoid necrosis	No	No	No	Yes (early stage)	No
Inflammatory cells	No	No	No	Vasculitis: yes Vasculopathy: minimal	No
Internal elastic lamina	Not affected	Not affected	Not affected	Affected	Not affected
Example	TTP	DIC, HELLP syndrome Uncommon: CAPS, HIT, PNH	Stx-HUS Neu-HUS Anti-VEGF Other drugs aHUS *DGKE* mutations *MMACHC* mutations	Renal scleroderma Lupus vasculitis *R. rickettsii* Viremia Fungemia	Metastatic neoplasm

Abbreviations: *aHUS* atypical hemolytic-uremic syndrome, *CAPS* catastrophic antiphospholipid antibody syndrome, *EC* endothelial cells, *DGKE* diacylglycerol kinase epsilon, *DIC* disseminated intravascular coagulopathy, *HELLP* hemolysis, elevated liver enzymes and low platelets, *HIT* heparin-induced thrombocytopenia, *MMACHC* methylmalonic aciduria and type C homocystinuria, *Neu-HUS* hemolytic-uremic syndrome due to infection with neuraminidase-producing microorganisms, *stx-HU* Shiga toxin-associated hemolytic-uremic syndrome

thrombosis or stenosis causes organ dysfunction. Therefore, any disorder that causes arteriolar thrombosis will be associated with the syndrome of MAHA, thrombocytopenia, and organ dysfunction. Any scheme that defines TTP as a syndrome of thrombocytopenia and MAHA will invariably include patients with the other types of pathology yet exclude some patients with the disease.

6.1.1 A Mechanistic Definition of TTP

TTP is mechanistically defined as a disease with a propensity to arteriolar thrombosis due to genetic mutations or autoimmune inhibitors of ADAMTS13 (Table 6.2).

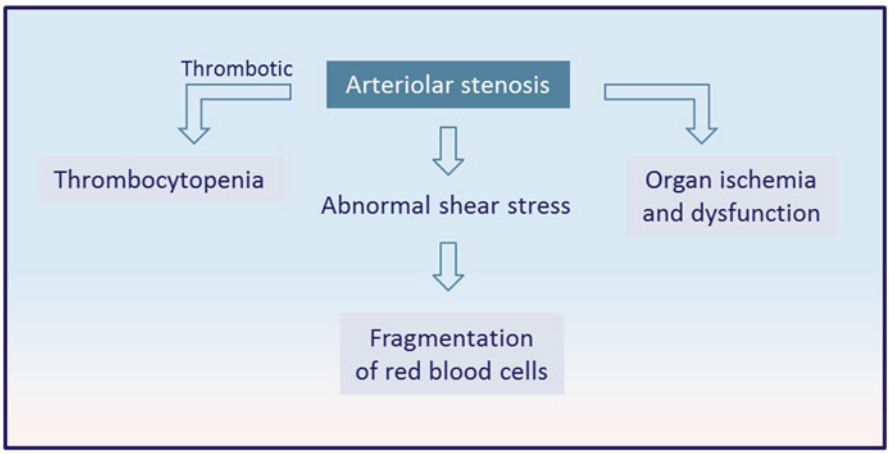

Fig. 6.1 Pathogenesis of the syndrome of thrombocytopenia and microangiopathic hemolytic anemia (MAHA). Microangiopathic hemolytic anemia (MAHA) signifies arteriolar stenosis in patients without a vascular device such as a ventricular assist device, extracorporeal membrane oxygenator, or prosthetic heart valve. Arteriolar stenosis causes abnormal shear stress in the circulation, resulting in the entrapment and fragmentation of red blood cells. It is often associated with thrombocytopenia because thrombosis is the most common cause of arteriolar stenosis. In some patients, thrombocytopenia may result from a different process such as decreased megakaryopoiesis in the bone marrow in patients with metastatic neoplasm. Arteriolar stenosis also leads to ischemic injury and dysfunction of the affected organs

Table 6.2 A mechanistic definition of TTP and a list of its various clinical presentations

A propensity to arteriolar thrombosis due mutations or autoimmune inhibitors of ADAMTS13
• Active thrombosis: ADAMTS13 is less than 10 % – Diad (thrombocytopenia, MAHA), triad (diad plus neurological deficits), or pentad (triad plus fever and renal abnormalities) (conventional syndrome of TTP) – Thrombocytopenia only (often mistaken to be idiopathic thrombocytopenic purpura) – Stroke or transient ischemic attack, with or without thrombocytopenia – Myocardial infarction, with or without thrombocytopenia – Thrombocytosis and MAHA – No or vague symptoms, normal platelet counts, and no MAHA (plasma therapy increases the platelet count)
• Subclinical platelet consumption with no active thrombosis (clinical remission): ADAMTS13 may be normal, decreased or less than 10 % – No symptoms, normal platelet counts, no MAHA

In this definition, the diagnosis of TTP is focused on whether a patient has genetic mutations or autoimmune inhibitors of ADAMTS13 rather than on whether the patient has thrombocytopenia or MAHA. Thus, the definition not only includes patients who present with the conventional diad, triad, or pentad but also includes the less common and less well-known groups of patients such as those presenting with thrombocytopenia only and are often mistaken to have ITP; those presenting

with strokes, transient ischemic attacks, or myocardial infarction, with or without thrombocytopenia but without MAHA; those presenting with thrombocytosis and MAHA; and those with subclinical thrombosis whose disease activity is revealed only when plasma or blood transfusion leads to an increase in the platelet count.

6.1.2 The Basis for the Mechanistic Definition of TTP

This mechanistic definition of TTP is based on two lines of evidence. Firstly, a subset of patients presenting with the acquired syndrome of thrombocytopenia and MAHA are found to have severe (<10 % of normal) deficiency of ADAMTS13 activity in plasma (Fig. 6.2). The percentage of patients with severe ADAMTS13 deficiency varies, depending on whether patients with renal failure or certain comorbid conditions are excluded [2–13]. It is 100 % when patients with renal failure (maximal creatinine greater than 2.5 mg/dL) or comorbid conditions are excluded [3, 7].

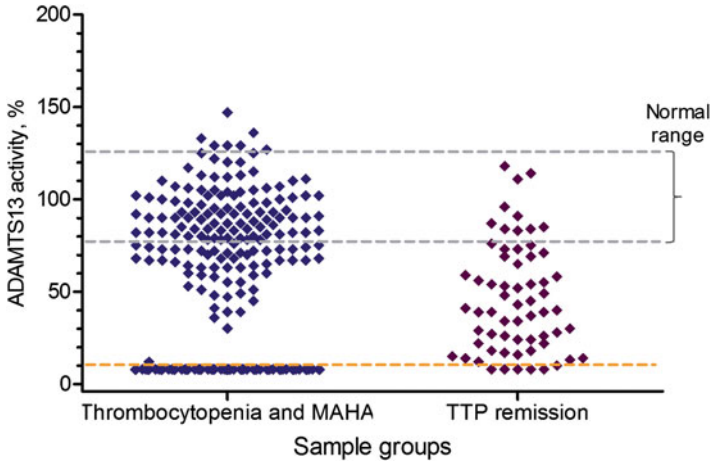

Fig. 6.2 Segregation of plasma ADAMTS13 activity among patients presenting with the syndrome of thrombocytopenia and microangiopathic hemolytic anemia (MAHA). Of the entire group of 384 patients with thrombocytopenia and MAHA, the plasma ADAMTS13 activity is below the detection limit of the assay (10 % of normal, except one of hereditary TTP at 12 %) in 230 cases (60 % of the entire group). For the group of patients without TTP, the plasma ADAMTS13 activity was 85 % (mean) ± 22 % (standard deviation), indicating that a patient with plasma ADAMTS13 activity level less than 20 % (mean – 3 standard deviations) after plasma or blood transfusion is also likely to have TTP. However, the patient would be in the process of recovery or in clinical remission. In the random group of 63 cases of TTP in remission (TTP remission), the plasma ADAMTS13 activity is normal, decreased, or below the detection limit of 10 %. In fact, serial analysis reveals that the plasma ADAMTS13 activity fluctuates in most patients during remission. The two *gray lines* encompass the normal range of plasma ADAMTS13 activity (78–126 %); the *lowest line* indicates the detection limit (10 %)

Secondly, genome-wide linkage analysis of patients of the hereditary form of TTP and their family members maps the defect to the long arm of chromosome 9 (q34) where the ADAMTS13 gene is identified and mutations in both alleles of ADAMTS13 are found in the patients and their relatives patients [14]. Together these two lines of evidence provide the basis for defining TTP as a disorder caused by autoimmune or genetic ADAMTS13 deficiency.

Serial investigation of TTP patients at acute presentation, during the course of plasma exchange therapy and during clinical remission, shows that in patients presenting with active thrombosis, the plasma ADAMTS13 activity is invariably less than 10 % of normal. During clinical remission (i.e., no active thrombosis), the ADAMTS13 activity may be normal or decreased. ADAMTS13 < 10 % of normal does not necessarily lead to thrombosis, as long as it is above the threshold level of thrombosis. However, such patients continue to have the disease and are at risk of thrombotic complications any time.

6.1.2.1 Is There TTP Without ADAMTS13 Deficiency?

It has been suggested that severe ADAMTS13 deficiency is not the only cause of TTP, as it is not detected in all patients of "TTP." However, those studies did not carefully exclude patients with other causes of the syndrome of thrombocytopenia and MAHA.

6.1.2.2 Does TTP Require a Second Hit in Addition to ADAMTS13 Deficiency?

Since some patients with severe ADAMTS13 deficiency are asymptomatic, it has been suggested that the disease of TTP requires a second hit in addition to ADAMTS13 deficiency. This is analogous to the argument that hemoglobin S is not sufficient to cause sickle-cell anemia because some patients with the β^s mutation do not have complications of sickle-cell anemia. In fact, the phenotypes of a molecular defect are often affected by genetic and environmental factors. These modifiers of disease presentation are different from "second" hits.

6.2 From ADAMTS13 Deficiency to Thrombosis

6.2.1 Pathology

The pathology of TTP is quite distinctive, with widespread hyaline thrombi in the terminal arterioles and capillaries of multiple organs, most extensively in the heart, pancreas, spleen, kidney, adrenal gland, and brain [15].

In chronic cases, the thrombi of TTP may be infiltrated by fibroblasts or converted by proliferating endothelial cells to become subendothelial deposits. Pseudoaneurysmal dilatation may form upstream of the stenosis or occlusion.

Immunochemical staining and electron microscopy show the thrombi of TTP are comprised primarily of von Willebrand factor (VWF) and platelets, with no or scanty presence of fibrin [15, 16].

6.2.1.1 TTP Does Not Cause TMA

Although TTP is often viewed as a disorder with TMA, this is a misconception. An essential feature of TMA is endothelial injury, as evidenced by the findings of endothelial swelling or disruption and subendothelial expansion. Such evidence of endothelial injury is absent in TTP. In the literature, prominent endothelial injury was reported in some studies of "TTP." However, close reviews of the cases suggest that the patients in those reports most likely had the atypical hemolytic-uremic syndrome (aHUS) rather than TTP.

6.2.1.2 Thrombosis in Large Arteries

Occasionally a patient with TTP may present with myocardial infarction or stroke due to thrombosis of a large vessel. It is believed that large-vessel thrombosis is due to injury inflicted by microvascular thrombosis in the vasa vasorum.

6.2.2 Shear Stress and Platelet Thrombosis in TTP

In vitro studies demonstrate that VWF in its compact conformation is not active in mediating platelet aggregation and is also not susceptible to cleavage by ADAMTS13. Exposure to shear stress renders VWF susceptible to cleavage by ADAMTS13; shear stress also activates VWF, leading to VWF-platelet aggregation.

6.2.2.1 The Hemostatic Activity of VWF Is Linked to Its Responsiveness to Shear Stress

The prominent presence of VWF in the thrombi of TTP indicates that it plays an important role in the development of thrombosis. VWF is a large multimeric adhesive glycoprotein whose primary function is to support the adhesion and aggregation of platelets at sites of vessel injury.

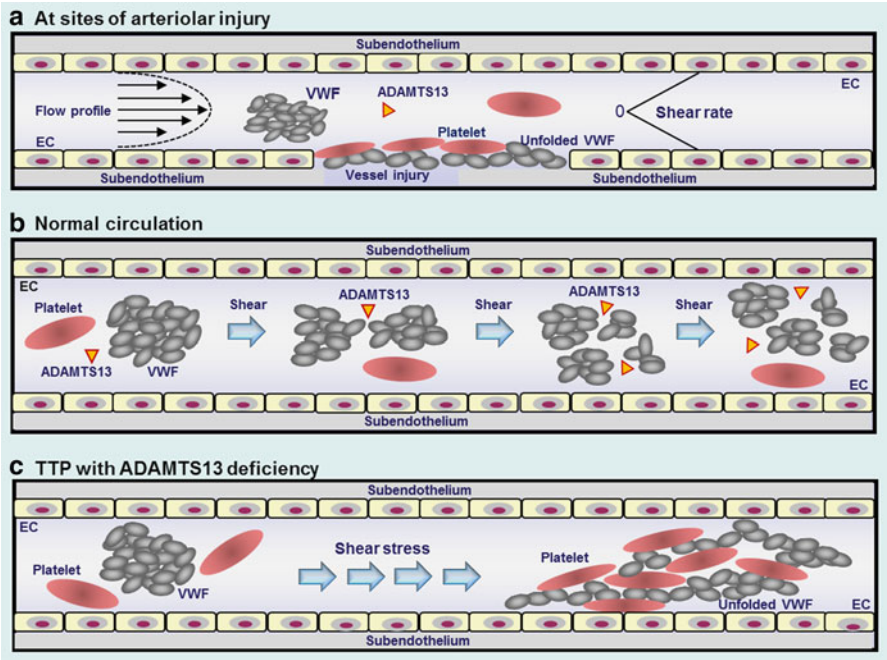

Fig. 6.3 A scheme depicting how ADAMTS13 deficiency may lead to VWF-platelet thrombosis. (**a**) At sites of arteriolar injury, VWF binds to the subendothelium. This binding exposes VWF to the high shear stress at the boundary, unfolding and activating it to support platelet adhesion and aggregation, a critical step in normal hemostasis process. (**b**) In normal circulation, VWF is cleaved by ADAMTS13 whenever it is beginning to be unfolded by intermittent exposure to high levels of shear stress in the arterioles. This proteolysis helps maintain VWF in its compact, inactive configuration, while its size becomes progressively smaller during repeated cycles of proteolysis, generating a series of multimers found in normal plasma. (**c**) When ADAMTS13 is not cleaved by ADAMTS13, it will become unfolded and activated after repeated cycles of exposure to high shear stress in the arterioles, resulting in VWF-platelet aggregation characteristic of TTP. In a flow chamber, VWF-platelet aggregation only occurs under high shear stress conditions (~80 dynes/cm^2), which is in the range seen in the human arteriolar circulation, although it typically varies widely in the circulation. Thrombosis does not occur if the shear stress does not exceed the threshold level

At sites of endothelial injury, VWF binds to vascular components such as type VI collagen, fibrillin, or sulfatides [17–19]. Bound VWF, exposed to wall shear stress, rapidly unfolds to secure its binding to the vessel wall at multiple points of attachment thereby providing the substrate to support platelet adhesion and aggregation (Fig. 6.3a).

Among adhesive proteins, VWF is unique in two aspects. Firstly, it is capable of supporting platelet adhesion under high shear stress conditions; in fact, shear stress increases rather than decreases VWF-supported platelet adhesion [20]. Secondly, VWF exists as a series of high molecular weight multimers (molecular weight ranging from 0.5×10^6 to more than 20×10^6 Da) in circulation. Indeed, the

large molecular size of VWF confers the responsiveness of VWF activity to shear stress; smaller multimers are less responsive to shear stress and therefore are also less effective than larger multimers for hemostasis.

6.2.2.2 ADAMTS13 Prevents Activation of VWF by Shear Stress

VWF can also become unfolded and activated by shear stress in the circulation, albeit much less efficiently than on endovascular surface. The process of conformational activation by shear stress, although slow, will eventually lead to VWF-platelet aggregation if it is left unchecked [21–23]. The function of ADAMTS13 is to prevent the activation of VWF in the circulation by shear stress: ADAMTS13 cleaves VWF whenever its conformation is beginning to become altered by shear stress.

By cleaving VWF before it becomes activated to cause platelet aggregation, ADAMTS13 helps maintain VWF in its compact, inactive configuration while progressively decreasing its size (Fig. 6.3b). When ADAMTS13 is severely deficient, VWF is not cleaved and becomes activated by shear stress, resulting in intravascular platelet thrombosis seen with TTP (Fig. 6.3c).

Shear stress causes physically detectable conformational changes of VWF [23–25]. The conformational change that makes VWF susceptible to cleavage by ADAMTS13 can also be induced by exposure to chaotropic agents such as guanidine hydrochloride or urea. Interestingly, small-angle neutron scattering studies show that the changes at the sub-domain level, without obvious elongation of the VWF molecules, is sufficient to make VWF cleavable by ADAMTS13 [26, 27].

A model of VWF remaining attached to endothelial surface after secretion has also been proposed. In this model, cleavage of VWF by ADAMTS13 and generation of VWF multimers occur on the endothelial surface. However, the phenomenon of endothelial adherence is observed only under profound endothelial perturbation, and the model is not supported by the immunochemical findings of VWF distribution in TTP: the endothelial surface is not decorated with VWF. Furthermore, the VWF released from the endothelial surface by ADAMTS13 is not different in molecular composition from the VWF directly released in culture media in its molecular composition [28]. Therefore, the physiological and pathological significance of VWF adherence remains undetermined.

6.2.2.3 Thresholds of Thrombosis in Patients with ADAMTS13 Deficiency

Clinically, no thrombosis is observed in patients with TTP when the plasma ADAMTS13 activity is greater than 10 % of normal. However, a plasma ADAMTS13 activity less than 10 % does not necessarily lead to VWF-platelet aggregation and intravascular thrombosis. This is because the occurrence of platelet thrombosis is not only determined by ADAMTS13 but also by the shear stress profile in the circulation, the responsiveness of VWF to shear stress, thrombospondin [29], and possibly other factors.

6.2.2.4 Factors Affecting the Plasma ADAMTS13 Level

In addition to autoimmune inhibitors, which can vary widely, the plasma ADAMTS13 level is also affected by conditions such as pregnancy, which progressively decreases ADAMTS13 activity [30, 31]; various inflammatory conditions such as infection, sepsis, disseminated intravascular coagulopathy, and active autoimmune diseases, presumably due to the downregulating effects of cytokines such as IFN-γ, TNF-α, and IL-4 on its biosynthesis [32]; the inactivating effect of thrombin or plasmin [33]; and other unknown mechanisms.

These physiological or pathological conditions per se do not decrease the plasma ADAMTS13 activity to cause platelet thrombosis; yet they may trigger the onset of thrombotic complications in patients with TTP by further suppressing already decreased ADAMTS13 activity levels.

6.2.2.5 Factors Affecting the Threshold of Thrombosis

Conceptually, one can envision a threshold level of ADAMTS13 below which platelet thrombosis occurs. This threshold is below 10 % of normal. However, its exact level varies, as it is constantly affected by factors such as the shear stress profile in the circulation, the secretion of VWF from endothelial cells, the reactivity of platelets, and the responsiveness of VWF to shear stress. In each individual patient, thrombosis occurs only when the ADAMTS13 activity is below the threshold level of thrombosis.

In vitro, the shear stress necessary to cause VWF activation is approximately 80 dynes/cm^2, which is in the range of physiological shear stress in the arterioles [3, 23, 34]. However, physiological shear stress levels vary widely among individual subjects. When the shear stress in the circulation is not sufficiently high to activate VWF, no platelet aggregation will occur at any level of ADAMTS13 deficiency. Low levels of shear stress may explain why the lung and liver are usually spared in TTP and why some patients with severe ADAMTS13 deficiency do not develop active thrombosis.

Conditions such as fever, infection, and surgery increase the shear stress profile in the circulation. These same pathological conditions and pregnancy also increase the secretion of VWF from endothelial cells but decrease the plasma ADAMTS13 activity. Together these changes may be sufficient to trigger platelet thrombosis and clinical presentation of TTP in patients with critically low levels of ADAMTS13 activity.

Evidence of activation of the classic complement system is detected in many patients presenting with TTP [35]. This is an expected consequence of circulating immune complexes of ADAMTS13 and its inhibitors. However, there is no definitive evidence that complement activation plays a role in triggering the onset of thrombotic complications in TTP.

6.2.3 Animal Models of ADAMTS13 Deficiency

Two types of animal models have been developed to examine the role of ADAMTS13 in preventing microvascular thrombosis: mice with inactivated ADAMTS13 gene and baboons given an inhibitory monoclonal antibody of ADAMTS13 [36–38]. Both models confirm that ADAMTS13 deficiency creates a propensity to arteriolar and capillary VWF-platelet thrombosis of TTP. The mouse model also highlights the complex genetic heterogeneity of the various murine strains.

Inactivation of the ADAMTS13 gene fails to induce microvascular thrombosis in some but not other strains of mice. In susceptible strains, infusion of Shiga toxins induces the release of VWF from endothelial cells and triggers thrombosis before spontaneous fatal thrombosis occurs. This supports the concept of VWF secretion as a modifier of thrombosis threshold.

In the resistant murine strains, infusion of large amounts of human VWF leads to the development of microvascular thrombosis, suggesting that the ADAMTS13-deficient mice are protected from thrombosis because endogenous murine VWF is ineffective in causing platelet thrombosis [39].

In the baboon model, intravenous infusion of an inhibitory monoclonal antibody of ADAMTS13 leads to the development of arteriolar and capillary thrombosis, recreating human autoimmune TTP in mice.

Overall, both the murine genetic model and the baboon antibody model not only confirm that ADAMTS13 deficiency results in a propensity to platelet thrombosis in the arterioles; they also reveal the diversity of factors that may modify the severity of the thrombosis phenotype.

6.2.4 Changes of VWF Multimers in TTP

Unusually large VWF multimers with molecular sizes larger than those found in normal plasma were first detected in patients with relapsing TTP during remission [40]. It was proposed that a VWF depolymerase is missing in those patients, and the unusually large multimers were intrinsically active in causing platelet thrombosis in TTP. ADAMTS13 corresponds to the putative “depolymerase.” Nevertheless, the hypothesis that unusually large VWF multimers cause thrombosis is not supported by the changes of VWF observed in TTP patients. Specifically, the depletion of VWF is not limited to unusually large forms during periods of active thrombosis [41].

VWF is secreted from endothelial cells as a disulfide-bonded large polymer. This polymer is converted via repetitive proteolysis by ADAMTS13 to a series of multimers (Fig. 6.4) [42]. The process of repeated proteolysis is shear stress dependent and is essential for preventing activation of VWF by shear stress.

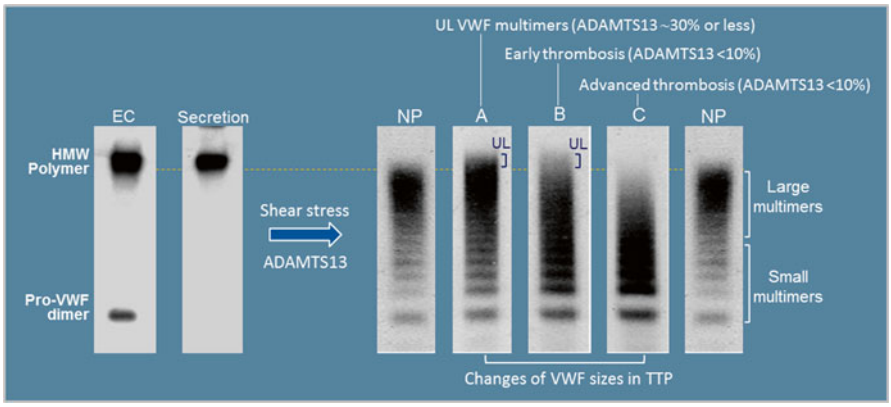

Fig. 6.4 Changes of VWF multimers in TTP. In endothelial cells (EC), VWF exists in two forms: a dimer of pro-VWF in the endoplasmic reticulum with a molecular weight of approximately 800 kDa and a high molecular weight (HMW) polymer in the storage granules of Weibel–Palade bodies from which the polymeric form is secreted. In normal plasma (NP), the HMW form is converted to a series of multimers by ADAMTS13 via a process of repeated proteolysis. Unusually large (UL)-VWF multimers are detectable by SDS-agarose gel electrophoresis when the plasma ADAMTS13 activity is 30 % or less. The precise level depends on the agarose content in gels used to separate the multimers. When the plasma ADAMTS13 activity is less than 10 %, platelet thrombosis and consumption of VWF may begin to occur. This causes a progressive depletion of VWF. At its early stage, this consumption creates a complex pattern with a gradient of decrease from the top; yet the UL multimers are still visible. Further consumption depletes both the UL and normal large multimers, a common pattern observed in TTP patients presenting with profound thrombocytopenia. The course of change in VWF is reversed when plasma exchange raises the ADAMTS13 activity in the circulation

In normal circulation, the size distribution of VWF multimers is determined by the balance of its secretion from endothelial cells and its proteolysis by ADAMTS13. When ADAMTS13 activity decreases to less than ~30 %, unusually large forms of VWF are detectable with SDS-agarose gel electrophoresis (Fig. 6.4, lane A). At this stage, the patient does not have thrombosis and the platelet count is not decreased. This contradicts the hypothesis that unusually large VWF multimers are intrinsically active in causing platelet thrombosis.

When ADAMTS13 level is further decreased to less than 10 %, VWF may begin to become unfolded and activated by shear stress in the circulation to cause platelet aggregation, resulting in progressive depletion of VWF (Fig. 6.4, lanes B and C). The depletion of VWF always begins from the largest multimers at the top of the gels because large size makes the VWF molecule more responsive to shear.

Thus, the appearance of unusually large multimers reflects defective proteolysis due to ADAMTS13 deficiency in TTP. These unusually large forms are detected before thrombosis occurs. When thrombosis begins to occur, progressive depletion of VWF occurs, starting from but not limited to the unusually large forms. In most de novo cases of TTP presenting with severe thrombocytopenia, both the unusually large and large multimers are decreased or depleted, a reflection of widespread platelet thrombosis.

6.3 Pathogenesis of ADAMTS13 Inhibitors

In most cases of acquired TTP, the causes of the autoimmunity are unknown (i.e., idiopathic). It is speculated that an otherwise innocuous infection or trigger may induce autoimmune reaction to ADAMTS13 in genetically susceptible individuals. A genetic predisposition is suggested by the finding that the HLA DRB1*11 allele is overrepresented among patients with acquired TTP [43].

An ADAMTS13 polymorphism (R1060W) is reportedly more prevalent in acquired TTP patients than in the population, raising the speculation that certain ADAMTS13 polymorphisms may predispose the affected individuals to develop ADAMTS13 inhibitors [44]. This association remains to be confirmed.

ADAMTS13 inhibitors may occur in patients with autoimmune disorders such as Still's disease, anti-glomerular basement membrane nephropathy, ulcerative colitis, and systemic lupus erythematosus. Indeed 10–40 % of TTP patients exhibit positive autoimmune reactions to various self-antigens such as DNA, suggesting regulation of the immune system is defective in many patients with TTP.

As will be further discussed later, HIV infection is associated with the development of TTP. This association is also likely related to defective regulation of the immune system, which is commonly observed among HIV-infected individuals before their effector immune system is severely decimated. Antiretroviral therapy appears to decrease the risk of TTP.

Acquired TTP occasionally develops in patients after hematopoietic stem cell therapy, especially among those who do not require immunosuppressive drugs for lack of graft-versus-host disease. It may also occur in women during the postpartum period, presumably. In both conditions, it is assumed that autoreactive B-cell clones emerge because of defective regulation of the immune system.

Ticlopidine is the only drug that has been shown to cause ADAMTS13 inhibitors [45–47]. Ticlopidine therapy increases the risk of developing ADAMTS13 inhibitors by 50–300-fold. The inhibitors occur between 2 and 8 weeks after institution of ticlopidine therapy, recede after discontinuation of the culprit drug, and generally do not recur. The binding of ADAMTS13 with inhibitors does not require the presence of ticlopidine. Therefore, it is assumed that ticlopidine-associated ADAMTS13 inhibitor may be analogous to the development of red blood cell antibodies induced by alpha-methyldopa.

An association between clopidogrel and ADAMTS13 inhibitors was suspected but has not been validated in drug surveillance studies [47]. Other drugs such as chemotherapeutic agents and calcineurin inhibitors cause MAHA due to TMA instead of ADAMTS13 inhibitors.

The factors that promote the development of ADAMTS13 inhibitors should be distinguished from factors such as infection, fever, surgery, pregnancy, and inflammation that trigger microvascular thrombosis by affecting the balance between the plasma ADAMTS13 activity level and the level of platelet thrombosis in patients with preexisting TTP.

6.4 Characteristics of ADAMTS13 Inhibitors

In most TTP patients, the levels of the ADAMTS13 inhibitors are low (<10 U/mL) [48] and often spontaneously decrease to undetectable levels after a few weeks to months. These characteristics of ADAMTS13 inhibitors are the main reason that plasma therapy is effective in raising the circulating ADAMTS13 level and preventing death for most patients.

The ADAMTS13 inhibitors of TTP are comprised primarily of IgG, with IgA and IgM antibodies detected infrequently. All four subclasses of IgG have been detected, although IgG4 appears to be the most prevalent (IgG_1, 52 %; IgG_2, 50 %; IgG_3, 33 %; and IgG_4, 90 %) [49]. The VH1-69 germline heavy chain gene appears to be used most frequently in producing the ADAMTS13 antibodies [50].

6.4.1 Cell Biology of ADAMTS13

ADAMTS13 is synthesized primarily in the stellate cells of the liver [51, 52]. ADAMTS13 may also be expressed in the spleen and other organs. However, compared to its expression in the liver, extrahepatic expression of ADAMTS13 is lower by at least one order of magnitude.

Stellate cells react to liver injury by activation and proliferation. The activated stellate cells continue to express ADAMTS13. The localization of its biosynthesis to the stellate cells instead of hepatocytes may explain why plasma ADAMTS13 activity level does not correlate with the severity of hepatic insufficiency. The expression of ADAMTS13 in stellate cells may be downregulated by cytokines such as IFN-γ, TNF-α, and IL-4 [32].

Expression of ADAMTS13 has been described in the renal glomerular podocytes and endothelial cells and vascular endothelial cells in culture, albeit at miniscule levels [53, 54]. The ADAMTS13 expressed in renal glomeruli may cleave VWF before its activity is neutralized by circulating inhibitors. This local protective effect may explain why renal injury is generally milder in acquired TTP than in hereditary TTP.

6.4.2 The Targets of ADAMTS13 Inhibitors

Nascent ADAMTS13, comprising 1427 amino acid residues, is a member of the ADAMTS metalloprotease family, which shares a common domain structure of metalloprotease (MP)-disintegrin (Dis)-thrombospondin type 1 repeat (TSR)-cysteine-rich region (Cys) and spacer (Spa) domains. ADAMTS13 contains 7 additional TSRs downstream of the Spa domain, followed by two unique CUB (complement C1r/C1s, Uegf, Bmp1) domains. The metalloprotease domain of ADAMTS13 contains a catalytic 224-HEIGHSFGLEHD-235 module characteristic of the ADAMTS proteases (conserved residues are underlined).

The Spa sequence, in which four of the ten *N*-glycosylation sites are located, is an essential component of the epitope recognized by TTP inhibitors [55, 56]. Nevertheless, while a recombinant peptide that comprises the sequence of distal part of MP and Dis-TSR-Cys-Spa exhibits binding with TTP inhibitors, peptides comprising the Spa or Cys-Spa sequence are not recognized by the inhibitors. The broad sequence suggests that the epitope of TTP inhibitors is likely to be B-cell-dependent three-dimensional surface of ADAMTS13 between the catalytic site and the spacer sequence.

ADAMTS13 variants truncated upstream of the spacer domain exhibit markedly decreased (<1 % of wild type) but detectable VWF cleaving activity [55]. This decreased activity is consistent with the existence of an exosite in the spacer domain that facilitates the engagement of catalytic site in the metalloprotease domain with its cleavage target in the VWF A2 domain. Spacer-truncated ADAMTS13 variants may potentially be developed to bypass the inhibitors of acquired TTP.

Further mapping studies show that residues Arg660, Tyr661, and Tyr665 in the spacer domain are critical for binding with the VWF A2 domain sequence [57]. These residues and Arg568 and Phe593 are also critical constituents of the target of TTP inhibitors [58]. Substitution of residues Arg660, Tyr661, or Tyr665 in with Ala abolishes the binding of ADAMTS13 with the inhibitors of TTP patients.

Some studies have suggested that the antibodies of TTP patients may also target other sequences of ADAMTS13 [59, 60]. However, the role of these antibodies in causing ADAMTS13 deficiency remains to be determined.

Peptides derived from the CUB2 domain sequence are preferentially presented on HLA-DRB1*11 of dendritic cells pulsed with rADAMTS13 in culture [61]. Since antibodies targeting the CUB domain are not found in many patients and the MHC class II antigens of dendritic cells only present short peptides with 9–16 residues, it remains to be determined whether the peptides presented on dendritic cells in culture have relevance to the genesis of ADAMTS13 inhibitors, which likely target the surface of ADAMTS13 formed by the sequences of the MP-Spa domains.

6.5 Clinical Features of TTP

6.5.1 Incidence Rates and Patient Characteristics

The clinical characteristics of TTP are illustrated in Table 6.3. The series of 39 consecutive non-referral cases encountered between 1999 and 2006 at an urban medical center in the Bronx, New York, where the prevalence of HIV infection (0.3 %) is among the highest in the USA.

Overall, similar to a recently reported series [62], acquired TTP occurs primarily in adolescents and adults, with the mean, median, and mode age around 40 years. Young children <10 years of age, who only account for 3 of the author's entire referral series of more than 200 cases, with the youngest being 5 years of age.

Table 6.3 Clinical features of acquired TTP

HIV infection	No	Yes
Number of cases	26	13
Age, years, median (range)	39.0 (14.2–62.2)	37.5 (9.4–65.3)
Sex, F, no. (%)	22 (85 %)	6 (46 %)
Black, no. (%)	22 (84.6)	12 (92.3)
Prior episodes, no. (%)	2 (7.7)	2 (15.4)
Hb, gm/L, median (range)	84 (36–121)	70 (51–84)
Platelet count, per μL, median (range)	11.0 (4–60)	12.5 (4–28)
LDH, U/L, median (range)	1231 (170–2920)	1075 (602–3271)
ADAMTS13 inhibitor, U/mL, mean (SD)	1.20 (0.86)	1.21 (1.14)
ADAMTS13 antibody, U/mL (range)	–	–
Serum creatinine, μmol/L, median (range)	84.0 (44.2–159.1)	97.2 (44.2–132.6)
Serum creatinine$_{max}$, μmol/dL, median (range)	97.2 (44.2–185.6)	106.1 (61.9–159.1)
CD4 cells, per mL, median (range)	–	187 (16–634)
Viral copies, per mL, median (range)	–	9.1×10^4 (<50–>7.5×10^5)
No. of plasma exchange, median (range)	13 (3–42)	16 (7–39)
Follow-up duration, months, median (range)	61 (0.1–120)	39 (0.4–117)
Number of death (%) (TTP, not TTP) During initial episode After remission	 2 (7.7%) (2, 0) 2 (8.3%) (1, 1)	 2 (15.4%) (0, 2) 1 (9.1%) (0, 2)

The female predominance (2–3:1) in the group of idiopathic cases, is not observed in the group of patients with HIV infection. Among the de novo cases, the platelet count is almost universally below 30×10^9/L.

The median number of plasma exchanges required is around 15. However, the range is quite broad. With prompt plasma exchange therapy, the risk of death is approximately 5–10 %. The risk of death may be higher in the HIV group. Nevertheless, the death in the HIV group was due to infectious complications or concurrent disorders (e.g., hepatic failure due to viral hepatitis) rather than TTP.

The series of non-referral cases gives rise to an estimated age- and sex-adjusted incidence rate of 14.5 cases per 10^6 person-years, which is much higher than the reported incidence rate of 1.74 cases per 10^6 person-years in the Oklahoma registry [12]. It is suspected that the higher incidence rate is due at least in part to the high prevalence of HIV infection (0.3%). None of the patients were being treated with antiretroviral therapy.

Indeed, among the four risk factors of TTP identified in univariate analysis (female gender, age between 30 and 50 years, African descent, and HIV infection), only HIV infection and female gender are found to be independent risk factors (Table 6.4). Furthermore, HIV infection is by far the stronger risk factor. It is speculated that the risk of TTP is related to defective regulation of the immune system in patients with untreated HIV infection. HIV infection has also been observed to be associated with various autoimmune disorders.

Table 6.4 The relative risk (95 % confidence interval) of TTP according to HIV status, gender, race, and age

Risk factor	HIV+ vs. HIV−	Female vs. male	Black vs. nonblack	Age 30–49 vs. others
Univariate analysis	36.2 (18.6, 70.5)	2.5 (1.23, 5.2)	3.4 (1.3, 8.8)	2.6 (1.4, 5.1)
Multivariate analysis[a]	38.5 (19.7 75.0)	2.7 (1.3, 5.7)	–	–

[a]Poisson regression

6.5.2 Presentation

6.5.2.1 Thrombosis, Thrombocytopenia, and MAHA

The symptoms and signs of TTP are primarily the complications of arteriolar and capillary thrombosis. When the plasma ADAMTS13 level is less than 10 % and below the threshold of platelet aggregation, platelet thrombosis begins to occur in the arterioles and capillaries of multiple organs. Thrombosis becomes symptomatic when it affects a vital function, most commonly of the brain. With progression of thrombosis, more complications such as paresis or paralysis, loss of cortical vision, altered mental status, and seizures ensue.

Less frequent complications include fever; abdominal pain, nausea, and vomiting with or without pancreatitis; myocardial infarction or heart failure; and, occasionally, sudden death.

Thrombocytopenia is detectable when platelet consumption in thrombosis exceeds compensatory thrombopoiesis. The period from decreasing ADAMTS13 activity to the onset of thrombocytopenia may be weeks to months. The period of thrombocytopenia may last for days to months before MAHA is apparent. Occasionally thrombocytopenia may spontaneously revert to normal platelet counts.

MAHA is noted when extensive arteriolar thrombosis causes fragmentation of red blood cells and hemolytic anemia. Before MAHA is detectable, the patients are often mistaken to have immune thrombocytopenic purpura (ITP).

Thrombocytopenia may cause petechiae, and MAHA may cause fatigue, weakness, pallor, and jaundice. More serious complications of thrombocytopenia such as intracranial hemorrhage or massive gastrointestinal hemorrhage occur infrequently.

6.5.2.2 Modes of Presentation

Most patients have no significant medical history and the early symptoms of headache and fatigue are often attributed to viral infections. By the time a de novo case presents for medical care, the disease has evolved in most cases to severe thrombocytopenia and MAHA (diad), often with neurological deficits (triad), renal abnormalities, and fever (pentad).

Occasionally, thrombosis affects the motor strength of an extremity or the function of speech or vision before it is widespread to cause overt thrombocytopenia and MAHA. This explains why a patient with TTP may present with ischemic stroke, paresis, dysarthria, or blurred vision without obvious thrombocytopenia and MAHA.

Some patients have chronic smoldering platelet thrombosis from TTP that causes thrombocytosis and MAHA. Thus, normal or high platelet counts do not exclude the process of active thrombosis in patients with TTP.

Overall, most patients present with the diad, triad, or pentad. Nevertheless, with heightened awareness of the disease and the aid of ADAMTS13 assays, less common modes of presentation as listed in Table 6.2 are increasingly recognized.

Occasionally a major stroke or myocardial infarction may occur due to thrombosis of a large vessel. For patients presenting with stroke or myocardial infarction, the diagnosis of TTP should be suspected if the patient has a history of TTP or there is unexplained thrombocytopenia.

6.5.2.3 Distinction Between Triggers of TTP and Inducers of ADAMTS13 Inhibitors

Conditions as infection, surgery, trauma, pregnancy, inflammation, or intravenous contrast agents, by raising the ADAMTS13 threshold of platelet thrombosis or further decreasing the plasma ADAMTS13 level, may trigger or worsen platelet thrombosis in patients with critical ADAMTS13 deficiency, giving rise to the clinical impression that these conditions "cause" TTP. These triggers should be distinguished from inducers of ADAMTS13 inhibitors such as ticlopidine and autoimmune dysregulation in association with HIV infection, postpartum period, hematopoietic stem cell therapy, lupus, or other systemic autoimmunity.

6.5.3 Renal Failure in Patients with TTP

Although hematuria and proteinuria are common in TTP, serum creatinine is normal or only minimally increased in acquired TTP (Table 6.3). Advanced renal failure with oliguria, anuria, fluid retention, electrolyte abnormalities, hypertension, or uremia is not a feature of acquired TTP.

The lack of advanced renal failure is consistent with findings at autopsy, which only show isolated areas of arteriolar and glomerular thrombosis, but no extensive glomerular or tubular destruction.

When overt renal failure (e.g., maximal serum creatinine greater than 221 μmol/L) occurs in a patient with acquired TTP, it should not be directly attributed to TTP. First, the validity of the diagnosis should be reassessed. After validity of the diagnosis of acquired TTP is confirmed, it is mandatory to search for other causes of renal failure, which, in the author's experience, includes one case of renal

transplantation, one case of concurrent anti-glomerular basement membrane nephropathy, and one case of atypical hemolytic-uremic syndrome. In the literature, two cases of TTP have been reported with renal failure due to concurrent Shiga toxin-associated HUS [63, 64].

Renal failure with serum creatinine levels >221 μmol/L is observed in up to 47 % of patients with ticlopidine-associated TTP [47]. However, those patients often had intravenous contrast agents immediately prior to the onset of TTP or comorbid conditions such as hypertension and diabetes that may amplify the severity of renal function impairment in association with TTP.

In contrast to its rarity in acquired TTP, renal failure is not uncommon in patients with hereditary TTP who are not being treated with maintenance plasma infusion. In the author's series of 27 cases, 20 % of the patients had at least one episode of serious renal failure (maximal serum creatinine greater than 221 μmol/L) and 12 % had chronic renal insufficiency. It is believed that the kidney function is compromised due to long-term cumulative injury of the kidney by chronic subclinical thrombosis and the lack of protection by locally expressed ADAMTS13. A case of hereditary TTP with renal failure due to concurrent atypical hemolytic-uremic syndrome of complement factor H mutations has also been described [65].

6.6 Diagnosis

A suspicion of TTP is most commonly raised in patients presenting with thrombocytopenia and MAHA. It should also be on the list of differential diagnosis for patients with idiopathic thrombocytopenia, especially if the thrombocytopenia is not severe, yet is accompanied by headache or other neurological symptoms or signs. Most patients presenting with acute ischemic stroke do not have TTP. Nevertheless, the diagnosis of TTP should be suspected if the patient has unexplained thrombocytopenia or a history of TTP.

6.6.1 Plasma ADAMTS13 Activity

Diagnosis of TTP requires the demonstration of severe ADAMTS13 deficiency. With ADAMTS13 assays, the diagnosis of TTP can be made in patients with atypical presentations. In the past, the correct diagnosis of TTP was not possible for such patients until the disease further progressed to cause both thrombocytopenia and MAHA.

For patients with decreasing platelet counts or thrombocytopenia that is not resolving, ADAMTS13 activity should be less than 10 %. On the other hand, the ADAMTS13 activity level may be normal, decreased, or less than 10 % during clinical remission of TTP.

An ADAMTS13 activity greater than 10 % should be sufficient to prevent VWF-platelet thrombosis. Therefore, other causes should be explored to account for persistent thrombocytopenia in a patient with TTP with ADAMTS13 activity greater than 10 %.

6.6.1.1 Other Causes of Decreased ADAMTS13 Activity

As previously discussed, the plasma ADAMTS13 activity may be decreased in patients with various pathological conditions such as sepsis, noninfectious inflammation, disseminated intravascular coagulopathy, active liver disease, and the HELLP syndrome of pregnancy. The mechanisms of ADAMTS13 decrease appear to be multifactorial and may include decreased synthesis, increased clearance and inactivation by other proteases such as plasmin or activated factor XI.

The decrease of ADAMTS13 activity in patients with various pathological conditions is not severe enough to cause platelet thrombosis. Yet, the decrease may be sufficient to trigger platelet thrombosis in patients with genetic mutations or autoimmune inhibitors of ADAMTS13.

ADAMTS13 is quite stable in normal plasma. However, its activity may be decreased in vitro in partially clotted plasma samples and plasma samples of patients with various pathological conditions such as DIC, sepsis, liver disease, or the HELLP syndrome. Some of the observed low ADAMTS13 levels in pathological conditions, especially when levels are less than 20–30 %, may be due to loss of ADAMTS13 activity during sample processing and storage.

6.6.2 Potential Pitfalls of ADAMTS13 Assays in Clinical Application

6.6.2.1 Substrates

Since ADAMTS13 does not cleave VWF isolated from normal plasma, all ADAMTS13 activity assays include a step to render the substrate cleavable. This is accomplished in four different ways: pretreatment of plasma-derived VWF with 1.5 mol/L guanidine hydrochloride or urea before it is incubated with a test sample, usually at a volume ratio of 1:10 or 1:20 to minimize the effect of chaotropic agents on the reaction [3], incubation of VWF with a test plasma in the presence of 1.5 mol/L urea [2], application of shear stress on the mixture of VWF and test sample [66–68], and use of a VWF sequence peptide that is constitutively cleavable by ADAMTS13 [13, 69, 70].

In design 2, urea at 1.5 mol/L decreases the cleavage of VWF by ADAMTS13. The decrease is approximately 50 % (mean) for wild-type ADAMTS13 and may be as much as 90 % for an ADAMTS13 variant p.P457S [71, 72]. This artifact led to the incorrect observation that P457S variation markedly decreases the activity of the

ADAMTS13 protease. The effect of urea on the activity of ADAMTS13 is also affected by plasma proteins and other components. Together these interferences contribute to the variability of ADAMTS13 results using urea at 1.5 mol/L in the reaction mixtures. The effect of urea on the activity of ADAMTS13 can be minimized by pretreating VWF with urea and incubating it with a test sample at a dilution of 1:10 to 1:20, as is the procedure in design 1.

Shear stress may be applied in a capillary tube [66], a parallel plate flow chamber [67], or a micro-centrifuge tube on vortex [68]. Shear stress is presumed to be more physiological than chaotrophic agents. However, compared to chaotropic agents, the advantage of shear stress has not been definitively demonstrated. Shear stress is more prone to operator differences and is not practical for concurrent testing of large numbers of samples.

The use of an abbreviated VWF peptide has the advantage of custom design to facilitate detection of cleavage. However, it can potentially overestimate the ADAMTS13 activity in patients with truncating or possibly other types of mutations.

6.6.2.2 Detection

Several designs have been developed to detect VWF cleavage: SDS-PAGE and immunoblotting to visualize VWF fragments generated during proteolysis [3], SDS-agarose gel electrophoresis to detect a decrease in the size of VWF multimers [2], ELISA to detect the loss of a tag signal or neo-epitopes created by cleavage of a VWF peptide or VWF multimers [7, 73, 74], detection of decrease in VWF activity in binding collagen or supporting platelet aggregation after proteolysis by ADAMTS13 [6, 74, 75], and fluorescence resonance energy transfer (FRET)-based detection, in which fluorescence is generated when VWF cleavage separates two fluorescent probes on the opposite sides of the cleavage bond [13, 69, 70].

SDS-PAGE detects specific VWF fragments; false ADAMTS13 activity due to abnormal cleavage of VWF by plasmin or other proteases is readily apparent. This assay clearly segregates TTP from other causes of MAHA (Fig. 6.2). It is also the only assay that not only clearly identifies patients with congenital TTP but also separates carriers (heterozygotes) from normal family members.

Multimer analysis with SDS-agarose gel electrophoresis does not distinguish proteolysis by ADAMTS13 from VWF size decrease due to plasmin or other proteases. Both SDS-PAGE and SDS-agarose gel electrophoresis involve laborious steps of gel electrophoresis and immunoblotting.

VWF sequence peptide, especially VWF73 (Asp1596 to Arg1668), is the most popular substrate in clinical laboratories because it is a small molecule that can be modified to simplify the detection of cleavage using FRET. Unfortunately, the FRET-based assay is prone to yield falsely high or low activity values in some samples. The causes of deviant results are not entirely understood and may include the low pH value in the reaction mixture, which may dissociate inhibitors

of low avidity from ADAMTS13, hydrolysis of the fluorescent probes or cleavage of the peptide at another site, giving rise to falsely high activity. On the other hand, high plasma bilirubin or hemoglobin concentrations, or other unknown factors, may quench fluorescence, yielding falsely low activity. Because the FRET-VWF73 assay produces falsely high or low ADAMTS13 activity in some samples, interpretation of the assay results requires caution and correlation with clinical features.

6.7 ADAMTS13 Inhibitors

Inhibitors of ADAMTS13 are detectable in 80–90 % of acquired TTP patients. Therefore, a negative test for ADAMTS13 inhibitors does not exclude the presence of ADAMTS13 inhibitors and the diagnosis of acquired TTP. In patients with acquired TTP but negative inhibitor assay results, mixing tests at higher patient to normal plasma volume ratios often yield positive results. Alternatively, the immunoglobulin molecules may be isolated from the patient's plasma for analysis of inhibitory activity at higher immunoglobulin concentrations. However, these extra steps are not performed in clinical laboratories.

In patients with negative inhibitor assay results, the presence of inhibitors may be inferred if the increase of the ADAMTS13 activity level is less than expected after plasma therapy or if the ADAMTS13 activity increases to greater than 10 % unrelated to plasma therapy.

Inhibitor assays are based on ADAMTS13 activity assays on mixing normal plasma with a patient's plasma preheated at 56 °C to inactivate its endogenous ADAMTS13 activity. Therefore, pitfalls in association with ADAMTS13 activity assays also affect the inhibitor assays.

6.7.1 ADAMTS13 Antibody and Antigen Assays

Assays have been developed to measure ADAMTS13 antigen and antibody levels. Both use the ELISA formats. The antigen assay, which detects free ADAMTS13 as well as ADAMTS13/inhibitor complex, yields low ADAMTS13 antigen levels in <50 % of TTP patients and has limited utility in clinical practice. The ADAMTS13 antibody assay is highly sensitive (>95 %) for acquired TTP but may yield false-positive results in 5–10 % of individuals without acquired TTP, presumably due to antibodies that are reactive with the component proteins used in the ELISA. A variant of ELISA for ADAMTS13 binding IgG uses ADAMTS13 expressed on cell surface via a glycosylphosphatidylinositol (GPI)-anchored linker [76]. Nevertheless, the performance of this assay remains uncertain in clinical practice.

6.7.2 Differential Diagnosis

TTP is merely one of the multiple causes of the syndrome of thrombocytopenia and MAHA. With the aid of a reliable ADAMTS13 assay, a diagnosis of TTP can be made in patients with typical or atypical presentations.

Most TTP patients do not have comorbid conditions. For the small fraction of patients with a comorbid condition, it is important to determine what roles each comorbid condition may play in the pathogenesis of the syndrome of MAHA and thrombocytopenia: a trigger of thrombotic complications of TTP; a cause of ADAMTS13 inhibitors; a cause of TMA; a cause of fibrin thrombosis, vasculitis/vasculopathy, or intravascular clusters of cancer cells; or merely an unrelated illness complicating the clinical features (Table 6.5).

Understanding the roles of comorbid conditions helps manage the complexity of disease processes in individual patients. Nevertheless, the existence of a comorbid disorder is not always clinically obvious. Therefore, a search for other causes is indicated when TTP is associated with unusual features such as renal failure, hypertension, fluid accumulation, or pulmonary infiltrates. Examples of comorbid conditions complicating the clinical features are anti-glomerular basement membrane nephropathy (personal unpublished data) or atypical hemolytic-uremic syndrome causing renal failure in a patient with TTP [77].

Some of the comorbid conditions such as pregnancy, hematopoietic stem cell therapy, autoimmune disorders, or kidney transplantation may potentially contribute to the development of MAHA and thrombocytopenia by more than one mechanism (Table 6.6).

6.7.3 Distinction Between Acquired and Hereditary TTP

Distinguishing acquired TTP from hereditary TTP is straightforward when inhibitors of ADAMTS13 are detected. However, the inhibitor assay is only positive in 80–90 % of acquired TTP patients. Measurement of ADAMTS13 antibodies with ELISA is more sensitive (>95 %) than inhibitor assays in detecting autoimmunity of ADAMTS13 but may yield false-positive results in ~5–10 % of patients without acquired TTP.

In patients with no detectable inhibitors to ADAMTS13, hereditary TTP is excluded when the plasma ADAMTS13 activity increased to greater than 10 % during remission. If the plasma ADAMTS13 activity is persistently less than 10 % during remission and no inhibitors or antibodies of ADAMTS13 are detected, a kinetic study with serial measurement of the plasma ADAMTS13 activity level after plasma infusion or exchange may help distinguish between acquired and hereditary TTP. Familial studies may help provide the answer if ADAMTS13 assay shows partial deficiency in the parents or offspring. Genetic sequence analysis is performed primarily for research and may yield negative results in some patients with hereditary TTP.

Table 6.5 Comorbid conditions and their potential roles in TTP patients

Comorbidity	Role	Mechanism	Management strategy
• Infection, surgery, trauma, pregnancy, etc.	Triggers of thrombosis in patients with TTP	• Decrease of ADAMTS13 activity; increase of thrombosis threshold due to increase in VWF or shear stress profile	• Avoid exposure to infection or other stresses; close monitoring of platelet count and ADAMTS13 activity; preemptive rituximab or plasma
• Ticlopidine • HIV infection • HSCT, autoimmune disorders	Inducers of ADAMTS13 inhibitors	• Unknown • Immune dysregulation • Immune dysregulation	• Discontinue the culprit drug • Antiretroviral therapy • ADAMTS13 assay for suspected patients
• Infection with STEC • Pneumococcal sepsis • Anti-VEGF • Other drugs • aHUS	Causes of TMA	• Shiga toxin-induced EC injury • Neuraminidase-induced EC injury • VEGF signaling deprivation in EC • EC injury via unknown mechanisms • EC injury due to incessant complement activation	• Supportive and dialysis as needed • Control of infection, plasma exchange, dialysis • Discontinue the culprit drug • Anticomplement C5 therapy
• Lupus vasculitis • Renal crisis of scleroderma • *R. rickettsii*, anthrax, viremia, fungemia	Causes of vasculitis/ vasculopathy	• Autoimmunity of vessel wall? • Activation of renin–angiotensin system? • Infection of vessel wall	• Immunosuppressive therapy • ACE inhibitors • Control of infection
• DIC, CAPS, HIT, PNH, HELLP syndrome	Causes of fibrin thrombosis	• Activation of coagulation system	• Management of the underlying cause; termination of pregnancy
• Metastatic neoplasm	Causes of intravascular cancer cells	• Intravascular invasion	• Management of the underlying neoplasm
• Miscellaneous	Unrelated to MAHA	• Various – e.g., anti-GBM nephropathy causing renal failure in a patient with TTP	• Management of underlying disorders

Abbreviations: *ACE* angiotensin-converting enzyme, *aHUS* atypical hemolytic-uremic syndrome, *CAPS* catastrophic antiphospholipid syndrome, *CFH* complement factor H, *DIC* disseminated intravascular coagulopathy, *GBM* glomerular basement membrane, *HELLP* hemolysis, elevated liver enzymes, and low platelets, *HIT* heparin-induced thrombocytopenia, *PNH* paroxysmal nocturnal hemoglobinuria, *TMA* thrombotic microangiopathy, *TTP* thrombotic thrombocytopenic purpura

Table 6.6 Some comorbid conditions that may cause MAHA and thrombocytopenia by more than one mechanism

Comorbidity	Mechanisms of MAHA and thrombocytopenia
Hematopoietic stem cell therapy	• TMA due to myeloablation or calcineurin inhibitors • Vasculopathy/vasculitis due to viremia or fungemia • TMA triggered by cell injury and complement activation in patients with preexisting aHUS • Development of ADAMTS13 inhibitors and TTP or anti-CFH and aHUS due to immune dysregulation, usually in patients not receiving drugs for GVHD
Pregnancy	• The HELLP syndrome • Trigger of thrombotic complications in patients with preexisting TTP • Trigger of TMA in patients with preexisting aHUS • Development of ADAMTS13 inhibitors and TTP or anti-CFH and aHUS due to immune dysregulation during the postpartum period
Autoimmune disorders	• Vasculopathy/vasculitis • TMA triggered by activation of the complement system in patients with preexisting aHUS • Development of ADAMTS13 inhibitors and TTP or anti-CFH and aHUS due to immune dysregulation
Kidney transplantation	• TMA due to calcineurin inhibitors • Vasculopathy/vasculitis due to viremia or fungemia • Trigger of TMA in patients with preexisting aHUS
Severe hypertension	• Previously thought to cause MAHA and TMA • Severe hypertension and TMA is more likely a consequence of TMA or scleroderma vasculopathy

Abbreviations: *aHUS* atypical hemolytic-uremic syndrome, *CFH* complement factor H, *GVHD* graft-versus-host disease, *HELLP* hemolysis, elevated liver enzymes, and low platelets, *HSCT* hematopoietic stem cell therapy, *MAHA* microangiopathic hemolytic anemia, *TMA* thrombotic microangiopathy, *TTP* thrombotic thrombocytopenic purpura

6.8 Management

There are three aims in the management of TTP: prevention of death, attainment of clinical remission, and prevention of relapse.

6.8.1 Causes of Death and Their Prevention

Without treatment, the risk of death due to TTP is greater than 90 % for patients presenting with both thrombocytopenia and MAHA, usually due to failure of the brain or heart functions. Plasma exchange and plasma infusion are the only therapies effective in preventing death of patients presenting with thrombosis. With immediate diagnosis and prompt plasma exchange therapy, the risk of death is decreased to less than 10 %.

Plasma infusion is less effective in preventing death (to 40 %) [78] and is used primarily as an emergent substitute when plasma exchange is not immediately available. Historically, a small fraction of patients not treated with plasma therapy experienced spontaneous remission. Spontaneous remission is more likely to occur in patients presenting with thrombocytopenia.

Before the era of plasma exchange therapy, antiplatelet drugs such as acetylsalicylate, dipyridamole, and dextran were used to treat TTP. With plasma exchange therapy, the additional benefit of antiplatelet drugs is miniscule, most likely only for patients with ADAMTS13 activity around the threshold level.

Plasma exchange is typically performed daily at one to 1 1/2 total plasma volumes until the platelet count is normal. It is believed that plasma exchange therapy removes the inhibitors and replenishes the missing ADAMTS13. After a period of one or a few days, a steady increase in the platelet count to the normal range is observed in most patients with plasma exchange therapy. Increasing platelet counts usually signify that the immediate risk of death from TTP is over.

In approximately 15–20 % of patients, platelet response may be delayed for days before rising to the normal range. Death may occur during the period of worsening thrombocytopenia. Death may also occur due to vital organ dysfunction immediately after admission or because of delay in the diagnosis of relapse after the patient achieves remission.

6.8.1.1 Advanced Dysfunction of Vital Organs at Presentation

A patient may present with advanced dysfunction of the brain or heart, leading to death before plasma therapy can be instituted and begin to exert its therapeutic effect (Fig. 6.5a). For a patient presenting with serious neurologic or cardiac dysfunction, immediate increase of the plasma ADAMTS13 activity is essential but may not always be achieved with plasma exchange therapy.

6.8.1.2 Rising ADAMTS13 Inhibitor Levels

A drastic increase in ADAMTS13 inhibitor levels can occur at the time of presentation or after a period of response to plasma exchange (Fig. 6.5c). Death can occur because plasma exchange therapy is inadequate to raise the ADAMTS13 activity.

There are presently no effective measures to prevent such death other than intensive plasma exchange therapy performed twice daily. Early rituximab therapy may decrease the risk of death in some patients, presumably by suppressing autoimmunity. However, rituximab does not decrease the risk of early death because its effect is often not evident for two or more weeks. In the future, blockers of VWF-platelet aggregation or recombinant ADAMTS13 variants that are not suppressible by ADAMTS13 inhibitors may be life saving for patients with high inhibitor levels or advanced organ dysfunction.

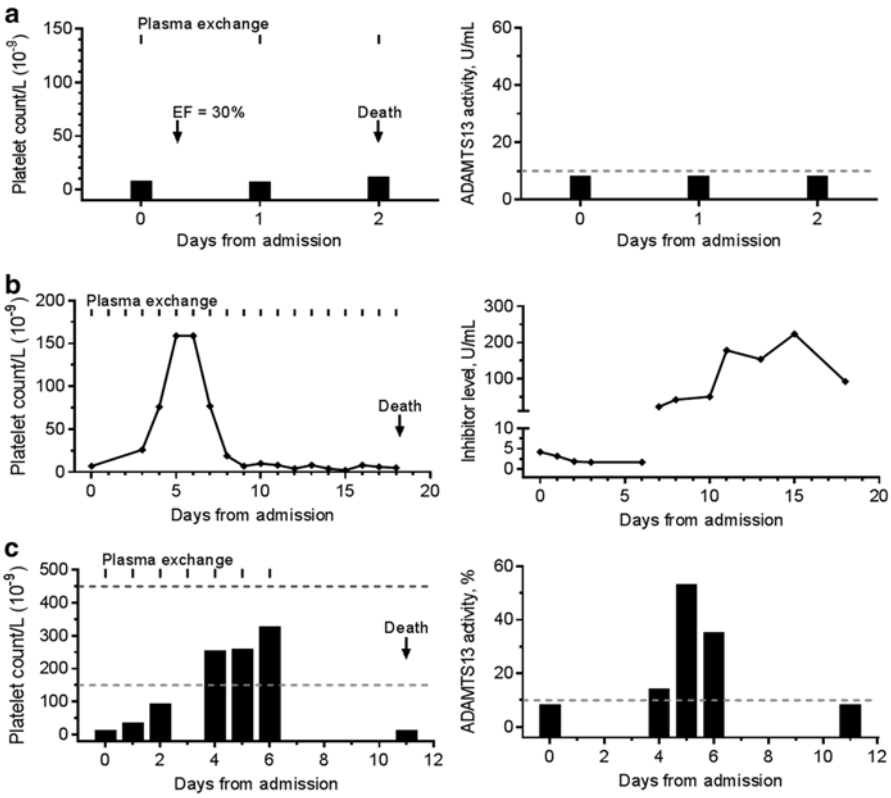

Fig. 6.5 Three types of death due to TTP. (**a**) Early death due to advanced organ dysfunction. Patient A had cardiac failure (ejection fraction 30 %) at admission and died on day 2 before the ADAMTS13 activity was increased by plasma exchange. (**b**) Death due to high inhibitor levels. Patient C had relapse of TTP on day 7 while still on plasma exchange therapy and died on day 18 because of rising ADAMTS13 inhibitors (>200 U/mL). (**c**) Death due to delay in the diagnosis of relapse. Patient B had a steady response to plasma exchange, achieving remission by day 5. However, he failed to have daily platelet counts performed after discharge from the hospital and became unconscious on day 5 when he died before plasma therapy could be reinstituted for relapse

6.8.1.3 Delay in Diagnosis or Management

Death may occur because there is delay in diagnosis (Fig. 6.5c) or no immediate access to plasma exchange therapy. If the diagnosis of TTP is suspected or established, but plasma exchange is not immediately available, the patient should be treated with plasma infusion until plasma exchange therapy can be initiated.

Although plasma exchange therapy is highly effective in preventing death, most of the patients will eventually have relapse of TTP complications in subsequent years (discussed below). It is very important that the patient is aware of this risk and continues to be closely monitored after achieving remission. The patient should refrain from traveling to locations where advanced medical care is not readily available.

Delay in the diagnosis of TTP may also result from lack of familiarity with the disease. Since TTP is uncommon, some physicians are not familiar with the disease.

6.8.1.4 Death due to Other Causes

Death may occur due to other causes such as catheter-associated sepsis or another comorbid condition (e.g., advanced hepatitis C disease) rather than TTP. HIV-infected patients are more prone to this type of death. For patients with HIV infection, special caution should be directed toward aggressive prevention and treatment of catheter-related infection. The catheter should be removed as soon as possible. The patients should also start antiretroviral therapy.

6.8.2 Attainment of Clinical Remission

Most patients achieve clinical remission if death is prevented with plasma exchange therapy. Plasma exchange does not alter the natural course of the ADAMTS13 inhibitors. Clinical remission, which is achieved after 15 (median, range 3–40) sessions of plasma exchange (Table 6.3), is a consequence of spontaneous abatement of the autoimmunity to ADAMTS13.

Serial analysis shows that the plasma ADAMTS13 activity and inhibitor levels are often quite unstable for a few days to weeks before they gradually settle at a steady-state range that may be normal, decreased, or less than 10 %. This fluctuation explains why clinical remission may take weeks. Therefore, it is important to closely monitor the platelet count while plasma exchange therapy is being tapered. Close monitoring should continue for at least a few more weeks until the platelet counts and ADAMTS13 activity are stable.

Monitoring of plasma ADAMTS13 activity helps identify patients who have falling ADAMTS13 activity or rising inhibitor levels and are at high risk of early relapse. However, the long turnaround time of the test relegates the ADAMTS13 assay results to the help dissect clinical events a posteriori.

6.8.2.1 Promoting Remission

Occasionally, a patient cannot be weaned off plasma therapy because the ADAMTS13 level does not remain steadily above the threshold level of platelet thrombosis. Rituximab is quite effective (70–90 %) in helping the patients to achieve clinical remission [79–82]. With rituximab now commonly used in TTP, protracted cases are less commonly encountered.

Early rituximab therapy may prevent protracted courses of plasma exchange therapy. However, there is no easy way to identify the small group of patients a priori who will have a protracted course. In one approach, rituximab therapy is

instituted for all patients once the diagnosis of TTP is established [62, 83]. However, early rituximab therapy has not been found to decrease the average number of plasma exchange sessions. This is because many patients achieve clinical remission in less than 2 weeks, before the rituximab effect occurs, often 2–5 weeks after the first dose.

Before the era of rituximab, other drugs such as antiplatelet agents, intravenous immunoglobulins, protein-A adsorption columns, corticosteroids, vincristine, cyclophosphamide, azathioprine, and splenectomy were used for protracted cases, often with equivocal results. More recently, *N*-acetylcysteine and calcineurin inhibitors such as cyclosporine A have been advocated. However, the efficacy of *N*-acetylcysteine in promoting remission of TTP remains hypothetical. Calcineurin inhibitors are slow acting, target T cells rather than B cells, and are unlikely to be a practical and effective measure of promoting remission.

6.8.3 Prevention of Relapse

With plasma therapy, the median duration of relapse-free survival is only 3.2 months after achieving clinical remission, defined as two consecutive normal platelet counts. Furthermore, nearly all patients will have at least one relapse by 7 years after achieving remission (Fig. 6.6a). The risk of relapse is at its highest during the first month, gradually decreasing thereafter. Relapse can occur anytime without warning or after exposure to trigger conditions. Many patients have more than one relapse during this period. Patients should be made aware of this risk and have immediate access to medical care for any early symptoms or signs of TTP.

The risk of relapse is very low in patients with ticlopidine-associated TTP after the drug is discontinued and in the HIV groups after 1 year of retroviral therapy, suggesting the risk of TTP is related to the drug or active HIV infection. Other studies also find antiretroviral therapy is effective in preventing relapses [84–86].

For patients without an obvious cause of ADAMTS13 autoimmunity, the conventional approach is to follow the blood cell counts for a few weeks to months after the patients achieve clinical remission. Monitoring of blood cell counts may detect relapse earlier and prevent death but does not obviate the need for plasma exchange therapy.

Preemptive rituximab therapy soon after the diagnosis is established may decrease the risk of early relapse [62]. Further analysis shows that rituximab increases the duration of relapse-free survival to 31.3 months from 9.4 months (Fig. 6.6b). However, rituximab therapy does not eliminate the problem of relapses.

Anecdotal experience suggests that splenectomy may be effective in promoting relapse in patients who are unable to wean off plasma exchange therapy or who have frequent relapses [87]. However, the procedure is invasive; its role has been largely replaced by rituximab therapy.

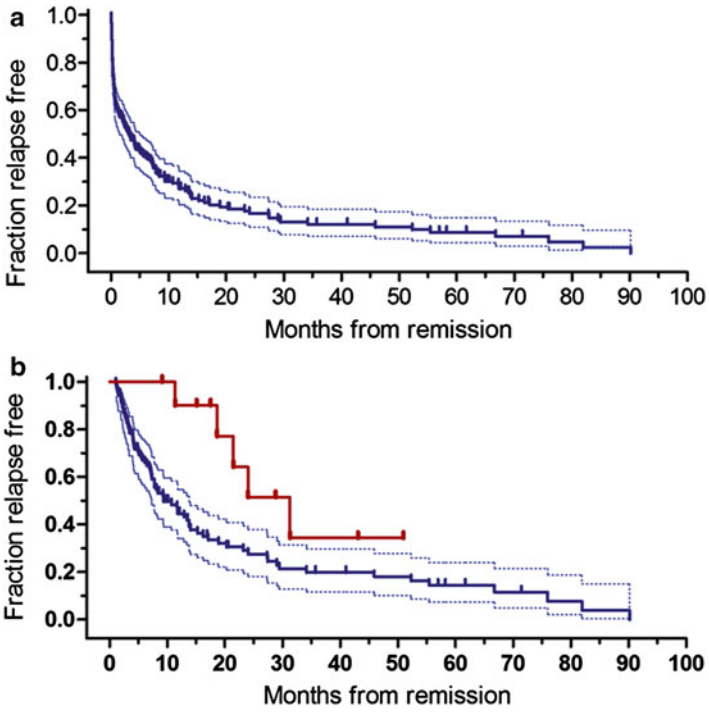

Fig. 6.6 Kaplan–Meier analysis of relapse-free survival after an acute episode of TTP requiring plasma exchange therapy. (**a**) Relapse-free survival of 182 episodes of acquired TTP complications due to ADAMTS13 inhibitors. Remission is defined as two consecutive normal platelet counts. HIV-infected patients accounted for nine of the episodes. Only three of the censored events were death. Relapses occurring after rituximab therapy are excluded for this analysis. The median duration of relapse-free survival is only 3.2 months. (**b**) Comparison of relapse-free survivals of 11 cases who were treated with rituximab with those of 105 cases who were not treated with rituximab. To account for the delay in the effect of rituximab, only relapses occurring at least 4 weeks after remission are included in this analysis. Rituximab increases the median duration of relapse-free survival to 31.3 months from 9.4 months, yet it does not eliminate the risk of relapse

6.8.3.1 ADAMTS13-Guided Rituximab for Prevention of Relapse

Serial monitoring of plasma ADAMTS13 activity during remission reveals that ADAMTS13 levels gradually decrease in a zigzag manner over the course of several weeks to months before a clinical relapse occurs. This period of gradual decrease in plasma ADAMTS13 activity provides a window for intervention with preemptive rituximab therapy.

For preemptive rituximab therapy to be effective, the ADAMTS13 activity should be checked at remission and at least weekly when plasma therapy is being tapered. A course of rituximab is indicated if the ADAMTS13 activity is less than 10 % of normal after plasma therapy is discontinued [88]. Since ADAMTS13 assay results are often not available for 1–2 weeks, in practice the threshold level for ritux-

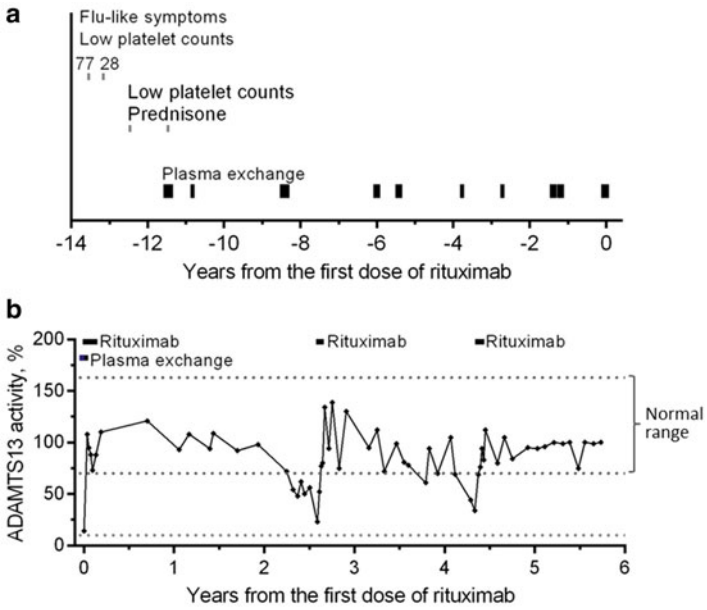

Fig. 6.7 ADAMTS13-guided prophylaxis of TTP relapses. (**a**) Over a course of 14 years, the patient had 10 episodes of TTP relapses that required plasma exchange therapy. She also had three episodes of thrombocytopenia which in retrospect are believed to be formes frustes of TTP. (**b**) With ADAMTS13-guided rituximab therapy, the patient has been free of relapse for nearly 6 years and ongoing. The ADAMTS13 curve shows that the strategy likely prevented two episodes of relapse during this period. The two *upper dashed lines* encompass the normal range of ADAMTS13 activity. The *lowest dashed line* indicates the ADAMTS13 activity level below which clinical relapses are likely to occur

imab therapy is set at 30–40 %. This provides a buffer for decline before rituximab therapy takes its course.

Since rituximab therapy does not eliminate the risk of relapse, it is necessary to continuously monitor plasma ADAMTS13 activity after each course of rituximab therapy. Plasma ADAMTS13 activity often begins to decrease after 2 ± 1 years. A preemptive course of rituximab is repeated when the ADAMTS13 again decreases to less than 30–40 % of normal.

This strategy of ADAMTS13-guided rituximab therapy is effective in preventing relapse in patients whose plasma ADAMTS13 activity is increased with rituximab therapy (Fig. 6.7). The strategy carries minimal adverse effects and is clearly preferable to the alternative of CBC monitoring or a blindly fixed schedule of rituximab therapy. The overall efficacy of this strategy remains to be determined in a larger series of cases.

Long-term immunosuppressive therapy with corticosteroids, cyclophosphamide, or azathioprine is of questionable efficacy in preventing relapse and has unacceptable adverse effects.

6.8.4 Special Consideration of Pregnancy

TTP occasionally occurs in women during pregnancy. With the aid of ADAMTS13 analysis, a better understanding of the relation between pregnancy and TTP has ensued. Normal pregnancy progressively decreases the ADAMTS13 level by 30 % at term and approximately 60 % if the pregnancy is complicated by preeclampsia or the HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome. Pregnancy also increases plasma VWF levels. These changes increase the risk of TTP relapse or exacerbation in women with acquired or hereditary TTP. On the other hand, autoimmunity often abates during pregnancy. Overall, unlike the clear risk of exacerbation with hereditary TTP, it is difficult to predict whether pregnancy may lead to exacerbation of acquired TTP in individual patients known to have the disease. In the postpartum period, autoantibodies may occasionally develop against ADAMTS13 causing TTP or against complement factor H causing aHUS.

The management of pregnancy in women with a history of TTP should not only aim to prevent relapse of TTP but also to minimize adverse fetal outcomes.

In general, when a woman with a diagnosis of hereditary TTP becomes pregnant, she should go on periodic plasma infusion therapy if the patient is not already receiving the treatment. For optimal fetal outcome, the interval of plasma infusion should be adjusted to minimize subclinical thrombosis. This is assessed by the magnitude in the increase of the platelet count following plasma infusion.

A more difficult challenge is counseling and management of women with a history of acquired TTP who want to become pregnant. Before pregnancy is to proceed, blood cell counts and plasma ADAMTS13 activity should be evaluated. If there is thrombocytopenia indicative of subclinical thrombosis or the ADAMTS13 activity is less than 40 % of normal, the patient should be treated with a course of rituximab to raise the ADAMTS13 activity. During pregnancy, serial monitoring of the platelet count and ADAMTS13 activity is critical. If plasma ADAMTS13 activity level exhibits a trend of decrease toward 10 %, rituximab may be used to preemptively increase ADAMTS13 levels before clinical relapse occurs. With meticulous measures to prevent relapses, a good outcome of pregnancy is expected [89].

Rituximab is assigned class C for pregnancy. This is based on the adverse effect of lymphocytopenia observed in animal reproductive studies. In theory, its deleterious effect may last for 12 months. Rituximab use during pregnancy has been reported in women without causing adverse consequences. There are no well-controlled studies in humans. Thus, potential benefits and risk should be fully discussed with patients in advance.

Patients presenting with thrombotic complications of TTP during pregnancy should be treated like other patients with plasma exchange, with tapering of the treatment to be guided by serial platelet counts and ADAMTS13 levels.

Pregnancy is also associated with other causes of MAHA and thrombocytopenia such as the HELLP syndrome and aHUS. In most cases, clinical features and ADAMTS13 analysis provide clear distinction of TTP from other causes of MAHA and thrombocytopenia.

6.8.5 *Future Perspectives*

Further improvement in the performance and availability of ADAMTS13 assays should facilitate the translation of advances in bench research to better diagnosis and management of TTP in clinical practice.

Recombinant ADAMTS13 (rADAMTS13) is under development for replenishing ADAMTS13 in patients with TTP. While the advantage of rADAMTS13 over plasma is obvious for hereditary TTP, its use for acquired TTP is likely to be complicated by the frequent variation of ADAMTS13 inhibitor levels in many patients. The amount of rADAMTS13 would need to be constantly adjusted to meet these varying requirements.

Variants of ADAMTS13 truncated upstream of the spacer domain are not suppressible by the inhibitors of TTP patients [55]. Such truncated variants may have advantages over full-length ADAMTS13, as they are not affected by variation in the inhibitor levels. With such non-suppressible ADAMTS13 variants, it may be possible to raise plasma ADAMTS13 activity immediately and consistently, preventing death from TTP and eliminating the need for plasma exchange. Similarly, ADAMTS13 variants with amino acid substitutions at the residues Arg660, Tyr661, or Tyr665 of the spacer domain are also active but not suppressible by TTP inhibitors [57]. However, immunogenicity is a concern with rADAMTS13 variants containing substituted amino acids.

Transplantation of hematopoietic cells with copies of functional ADAMTS13 gene may potentially provide a long-term solution for hereditary TTP [90]; yet it is unlikely to be practical for acquired TTP.

N-acetylcysteine, presently used for acetaminophen liver toxicity and chronic lung diseases, decreases the size of VWF in vitro by reducing the disulfide bonds of VWF multimers [91]. It may be an attractive therapy for TTP if its VWF-reducing activity is confirmed in human subjects.

Blockers of VWF-platelet aggregation such as anti-VWF aptamer ARC1779 or nanobody ALX-0081 may inhibit platelet thrombosis in TTP [92, 93]. Such blockers may serve as bridge therapy to suppress life-threatening thrombosis until ADAMTS13 is increased to prevent death.

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Chapter 7
Thrombotic Microangiopathies and Their Distinction from TTP

Melissa J. Bentley

Thrombotic microangiopathies (TMAs) comprise a number of distinct disorders with shared findings of platelet thrombi within small arterioles with resultant thrombocytopenia and mechanical destruction of red blood cells within small vessels. As this is often a result of increased shear stress, a key common feature is that of schistocytes on peripheral blood smear. With this in common, however, the pathology underlying these findings is variable for different forms of TMA. Because the morbidity and mortality of these diseases, in the absence of appropriate treatment, is significant, accurate and timely diagnosis is of critical importance. For many forms of TMA, including thrombotic thrombocytopenic purpura (TTP), effective treatment exists but must be instituted without undue delay, and the treatment itself may be costly and not without risk.

Over the past decade, research has clarified the pathology underlying many TMAs, and this had led to the development of specific diagnostic tests for these diseases, as well as to an understanding of clinical features which differ among the disorders. An understanding of these can facilitate diagnosis and treatment and thereby improve patient outcomes.

7.1 Thrombotic Thrombocytopenic Purpura

TTP is a TMA caused by a severe deficiency of ADAMTS13, a serine protease responsible for cleavage of ultra-large von Willebrand factor multimers [1–3]. Insufficient enzyme activity (<5–10 % of normal depending upon the laboratory)

M.J. Bentley, M.D., F.A.C.P. (✉)
Ogden Internal Medicine, 5405 South 500 East, Suite 203, Ogden, UT 84405, USA
e-mail: melissa.bentley@hsc.utah.edu

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leads to lack of multimer processing. In the absence of appropriate degradation, widespread platelet aggregation, thrombotic occlusion, and subsequent end-organ damage occurs [4]. Untreated mortality is approximately 90 % [5]. With appropriate treatment, plasma exchange, the great majority of patients survive [6].

7.1.1 Laboratory Assessment

As in other forms of TMA, initial laboratory data include a complete blood count (CBC) showing anemia and thrombocytopenia. Peripheral blood smear characteristically has decreased numbers of platelets, polychromasia, and schistocytes, a hallmark of microangiopathic hemolysis, as noted above. Platelet counts are usually very low (<30,000/μl). Comprehensive metabolic panel (CMP) may show an elevated bilirubin; if fractionated, indirect bilirubin is responsible for the preponderance of the elevation. Contrary to early descriptions of the disease, TTP patients typically have relatively preserved renal function. Indeed, significant elevation of serum creatinine should lead to reconsideration of a TTP diagnosis. Other useful laboratory data for diagnosis include D-dimer assay, which should not be significantly elevated, and an increased reticulocyte count, typically seen in TTP [7–10].

Of paramount diagnostic importance is ADAMTS13 analysis. This laboratory test should be sent prior to beginning plasma exchange, though in cases with a high level of suspicion, plasma exchange may be initiated while awaiting the test result. TTP is characterized by ADAMTS13 activity levels that are significantly below normal; various studies have used <15, <10, or <5 % as cutoff levels for diagnosis, and to some extent the laboratory used may influence the definition of severe deficiency. Many laboratories perform reflex testing for ADAMTS13 inhibitors; their presence in the setting of severe ADAMTS13 deficiency further suggests acquired TTP, as opposed to a rare congenital deficiency (Upshaw–Schulman syndrome) [11].

7.1.2 Clinical Features

Patients with TTP, as with many other forms of TMA, are often critically ill at presentation. No constellation of symptoms is pathognomonic for the diagnosis of TTP, and of the classically described pentad, hemolytic anemia, thrombocytopenia, neurologic abnormalities, fever, and renal disease [12, 13], only the first two are accurate descriptors. Neurologic and gastrointestinal symptoms may be present, but are not invariable, and may also be present in other forms of TMA. A history of prior episodes of TTP increases chances of a recurrent episode and is therefore useful in diagnosis. A history of bloody diarrhea may be suggestive of toxin-mediated hemolytic-uremic syndrome (see below) but is not specific and does not rule out TTP or other TMAs [14]. Rheumatologic disease, pregnancy, valvular heart disease, known malignancy, known infection or infectious symptoms, medications, and other historical information may be useful as suggestive of other forms of TMA

Table 7.1 Point-based thrombotic thrombocytopenic purpura (*TTP*) clinical prediction score

Variable	Assigned points
Creatinine >2.0 mg/dl	−11.5
Platelets >35/mc/l	−30
D-dimer >4.0 μg/ml	−10
Reticulocytes >3 %	+21
Indirect bilirubin >1.5 μg/ml	+20.5

Total score by model	Severe ADAMTS13 deficiency and response to plasma exchange (%)
<20 points	0 (low likelihood)
20–30 points	40 (moderate likelihood)
>30 points	100 (high likelihood)

The clinical prediction score uses data obtained at patient presentation. Points in the upper panel are obtained and added together. The point total is interpreted in the lower panel as shown. Patients with a moderate or high likelihood of TTP should receive urgent plasma exchange. Patients with a low likelihood of having TTP should be reevaluated for an alternative TMA diagnosis

(see below). In a retrospective review of the history, symptoms, and laboratory features of 110 patients presenting with clinically suspected TTP, only five variables, all laboratory values, were found to be of significance in predicting or excluding severe ADAMTS13 deficiency and thus TTP [7]. These variables were used to create a clinical prediction score, which was subsequently assessed in an independent cohort [8] and may be used to rapidly assess the likelihood of TTP as a cause of TMA while awaiting ADAMTS13 and other test results (see Table 7.1).

The mainstay of treatment for TTP is plasma exchange and in general, patients with TTP should show symptomatic and laboratory improvement within several days of initiation, though the recovery course is somewhat variable [15]. It is important to note that plasma exchange may lead to improvement in thrombocytopenia and other laboratory abnormalities in patients with HUS and other forms of TMA as well; however, if the underlying disorder is not corrected, this improvement will not be sustained with cessation of plasma exchange [16]. Indeed, the initial laboratory improvement may give treating clinicians false reassurance. In the case of sepsis, for example, a patient may die of overwhelming infection, while receiving plasma exchange without appropriate antimicrobial therapy. In addition, the process of plasma exchange carries expense, not insignificant risks, and should be reserved for patients for whom it is truly indicated [17, 18]. See Chap. 6 for additional information on TTP.

7.2 Hemolytic-Uremic Syndrome

HUS is currently thought to be primarily a complement-mediated TMA. The underlying pathology is unregulated complement hyperactivation, which leads to endothelial cell damage, platelet aggregation, microangiopathic hemolysis, and subsequent

Table 7.2 Features useful in distinguishing thrombotic thrombocytopenic purpura (*TTP*) from hemolytic-uremic syndrome (*HUS*)

Favors TTP	Favors HUS
Platelets <30,000 mc/l	Less severe thrombocytopenia
Normal or near-normal renal function	Significant renal impairment
Response to plasma exchange	Lack of complete response to plasma exchange
ADAMTS13 activity less than 5–10 % normal[a]	Positive Shiga toxin[a]

[a]Confirms diagnosis

end-organ damage, notably with renal involvement [19, 20]. Its distinction from TTP may be a clinical challenge. This is clearly evidenced by the fact that in the past the term TTP–HUS has been used to describe patients who presented with thrombocytopenia, microangiopathic hemolysis, and generally some form of neurologic, renal, and/or gastrointestinal symptoms (see Table 7.2). However, despite similar features, current knowledge of the distinct pathology triggering the TMA in TTP, the availability of ADAMTS13 testing, and the recognition of key characteristics of patients with severe deficiency make it possible to distinguish clinically between these two clearly different diseases [21]. And indeed, as treatment for each is different and inappropriate treatment carries high morbidity and mortality, this distinction is crucial.

In approximately 90 % of cases, HUS may be linked to an infection, typically a gastrointestinal infection with *Escherichia coli*, serotype 0157:H7 [22]. Less commonly, other *E. coli* serotypes, *Shigella dysenteriae*, *Citrobacter freundii*, or other bacteria may be implicated [5, 23, 24]. These organisms produce powerful exotoxins which lead to unregulated complement activation. This in turn causes unregulated platelet activation, aggregation, and consumption, endothelial cell activation, microvascular occlusion with subsequent microangiopathic hemolysis, and organ damage [25].

In approximately 5 % of cases, a genetic defect in a factor needed for complement inhibition underlies the uncontrolled activation of the complement system that results in HUS [20]. These cases have been termed atypical HUS (aHUS). Multiple genetic mutations have been identified, the most common affecting the complement regulatory protein factor H [26]. Table 7.3 lists the currently identified mutations thought to cause aHUS.

7.2.1 Laboratory Assessment

The CBC of a patient with HUS, as expected, reveals anemia and thrombocytopenia. The latter is usually less severe than in cases of TTP and may even be within the normal range, though a decrease from baseline platelet values may be seen for a given

Table 7.3 Genetic mutations identified in patients with atypical hemolytic-uremic syndrome (*aHUS*)

Gene mutation	Approximate frequency (%)
Complement factor H	30
Membrane cofactor protein	10–15
Complement C3	5–10
Complement factor I	4–10
Thrombomodulin	5
Complement factor B	1–2

It should be noted that 30–50 % of aHUS patients have no identified mutation
Data from Ref. [26]

patient [27]. Evidence of hemolysis such as schistocytes on peripheral blood smear, elevated LDH, and decreased haptoglobin are to be expected. A key distinguishing feature from TTP is the presence of significant renal dysfunction, which may often be easily noted as elevation in BUN and creatinine on initial laboratory assessment. Hematuria, proteinuria, and urinary casts are commonly seen as well. Liver transaminases and both direct and indirect bilirubins may be elevated.

More specific testing includes assays for Shiga-toxin exposure. This may be done through identification of Shiga toxin in stool or through serum detection of IgG or IgM antibodies to enterohemorrhagic *E. coli* (EHEC). Laboratory detection of EHEC toxin by EIA and PCR is increasingly available. Complement system testing is generally not clinically useful in the diagnosis of HUS or in distinguishing it from TTP [28]. ADAMTS13 levels are normal or slightly decreased, a key in distinguishing HUS from TTP, as noted above [28]. Testing for the most common genetic mutations associated with aHUS is available, and it may be useful for counseling patients and for research purposes; however, it is expensive, 30–50 % of patients with aHUS have no mutation identified, and results are usually not available for weeks to months, making it unhelpful in initial diagnosis and treatment of a critically ill patient [28, 29].

7.2.2 Clinical Features

Patients with HUS may present with a history of gastrointestinal illness with diarrhea, often bloody, several days or up to 2 weeks prior to the development of HUS. It is more common in children, but affects adults as well [5]. Patients may note decrease in urine output, hematuria, or very dark urine. Neurologic symptoms such as confusion, a change in level of consciousness, seizures, and stroke may occur as a result of thrombotic, hemorrhagic, or direct-toxin-mediated damage and are not, therefore, suggestive of a diagnosis of TTP or useful in distinguishing between the two [14].

Supportive care is indicated for typical (infectious) cases of HUS; no specific treatment exists. Plasma exchange may be initiated in cases where distinction from TTP has not yet been made, but its usefulness in treatment of HUS has not been demonstrated [16, 23]. Dialysis may be required, in some cases with eventual recovery of renal function; in other cases, a degree of renal impairment may persist, with some patients requiring permanent renal replacement therapy or transplant. Acute mortality from Shiga-toxin-associated HUS is 3–5 % [20].

Though accounting for a small percentage of HUS cases, several features of aHUS are worth mentioning. The first is its comparatively poor prognosis, with 30–40 % of patients dying or suffering end-stage renal disease at the time of the first clinical illness. Within the first year of diagnosis, approximately 65 % of patients die, require dialysis, or have permanent renal damage. There is, however, a monoclonal antibody, eculizumab, which has been approved for use in patients with aHUS. It blocks the complement cascade through inhibition of C-5 and may significantly improve outcomes for these patients [16].

7.3 Other Forms of Thrombotic Microangiopathy

A complete review of all forms of TMA is beyond the scope of this chapter. Several categories of disease and features useful for distinguishing them from TTP will be briefly discussed.

7.3.1 Infection Associated

Specific types of viral infection, notably human immunodeficiency virus, Epstein–Barr virus, and cytomegalovirus, as well as infection caused by *R. rickettsii*, *B. anthracis*, *C. difficile*, and *S. pneumonia* may cause TMA [5, 23, 30]. Sepsis or the systemic inflammatory response syndrome (SIRS) may lead to TMA or may result in disseminated intravascular coagulation (DIC) which shares features of TMA [31]. An elevated D-dimer is suggestive of DIC and is not characteristic of TTP. An elevated white blood cell count, CRP, suggestive history, and microbiological data are all helpful in distinguishing infection from TTP.

7.3.2 Pregnancy Associated

TMA in the setting of pregnancy occurs as part of the HELLP syndrome—hemolysis, elevated liver enzymes, and low platelets. Antiphospholipid syndrome and DIC may also occur as complications of pregnancy and lead to TMA. These conditions should

be suspected in pregnant women presenting with TMA, and treatment may involve supportive care, treatment of hypertension, delivery as soon as possible, or assessment for retained products of conception as a trigger for DIC [13, 30, 31]. However, it is important to note that TTP can also occur in pregnancy, and ADAMTS13 testing is a critical part of the evaluation of such patients, with a severe ADAMTS13 deficiency indicative of TTP and the need for plasma exchange.

7.3.3 Transplant Associated

Transplantation of solid organs including kidney and lung transplants, as well as stem cell and bone marrow transplant, has been associated with TMA. The disorder may occur at any time, from weeks to years after transplantation. The cause is not always known but may be related to drugs used for immunosuppression and prevention of transplant rejection (calcineurin inhibitors) or in other cases may be a form of immunologic activation, transplant rejection, or graft-versus-host disease [5, 30].

7.3.4 Other Hematologic/Malignancy Associated

Various forms of leukemia, bone marrow carcinosis, tumor cell embolism, and other malignant processes may exhibit features of TMA. Ovarian and other cancers are associated with DIC. Patients with paroxysmal nocturnal hemoglobinuria (PNH) exhibit features of TMA. In addition, chemotherapeutic agents and the process of bone marrow transplantation used to treat malignancies may themselves cause a form of TMA [5, 30–32]. Each of these should be suspected based on supportive history and other diagnostic laboratory and radiologic assessment.

7.3.5 Immune Associated

A very broad range of immunologic disorders may cause thrombocytopenia, and some forms of systemic autoimmune disease may cause TMA. These include severe forms of systemic lupus erythematous (SLE), vasculitis, antiphospholipid antibody syndrome, and others [30, 33]. Again it is important to note that patients with autoimmune disease may also develop classical TTP or HUS, and assessment of ADAMTS13 activity, in addition to other specific diagnostic tests, is crucial in evaluation.

7.3.6 Drug Associated

Pharmacologic agents which have been associated with TMA include cyclosporine, tacrolimus, mitomycin C, clopidogrel, ticlopidine, protamine, quinine, and cocaine [5, 13, 30]. For some agents, it appears autoantibodies are triggered by the causative drug, but in many cases the pathophysiology is unclear. A high level of suspicion and careful medication history is required for diagnosis. Withdrawal of the offending agent and avoidance of its use in the future is crucial for resolution of the TMA, and the said resolution also confirms the agent as the underlying cause.

7.3.7 Others

Additional cases of TMA may occur in the setting of mechanical damage to platelets in the setting of prosthetic heart valves, vascular grafts, indwelling catheters, and intra-aortic balloon pumps or with extracorporeal life support. Significant atherosclerosis with endothelial changes, stenotic native heart valves, and vascular changes in the setting of advanced chronic kidney disease or metastatic cancer has also been associated with TMA [30, 31, 34, 35]. Even after exhaustive evaluation, the etiology of some cases of TMA remains idiopathic.

7.4 Suggested Approach to Diagnosis and Treatment

Though certain features of individual cases of TMA may be suggestive of specific diagnoses, a commonality of most patients at presentation is the severity of their illness and high morbidity and mortality without efficient diagnosis and institution of appropriate treatment. Timely and appropriate intervention is therefore of utmost importance. Use of a diagnostic algorithm may be helpful in guiding evaluation (Fig. 7.1).

A willingness to continually reassess a diagnosis and treatment plan, avoiding the heuristic trap of anchoring, is essential. Obtaining further clinical history from a patient, family, or other providers may be helpful, as will additional diagnostic studies as they become available. While it may be prudent to begin empiric therapy, antibiotics, for example, in a patient in whom sepsis is a legitimate concern or plasma exchange in a patient in whom it is felt, TTP cannot be excluded, failure to respond to treatment as expected, or pieces of contradictory information obtained during ongoing assessment must not be ignored. Instead, this should prompt a clinician's reconsideration of the initial diagnosis and increased diligence in determining the correct diagnosis.

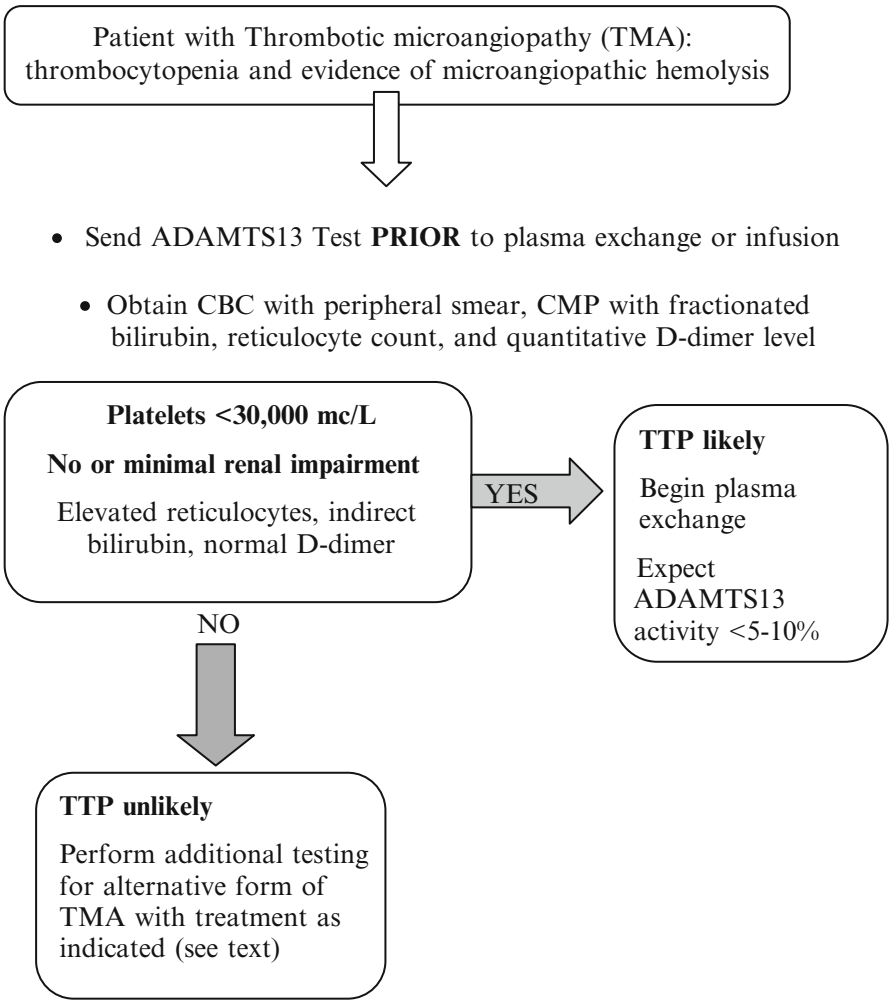

Fig. 7.1 Algorithm for evaluation of TMA etiology; *CBC* complete blood count, *CMP* comprehensive metabolic panel, *TTP* thrombotic thrombocytopenic purpura

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Chapter 8
Laboratory Assays for ADAMTS13

Kristi J. Smock and Nahla M. Heikal

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

K.J. Smock, M.D. (✉) • N.M. Heikal, M.D.
Department of Pathology, University of Utah Health Sciences Center and ARUP Laboratories, 500 Chipeta Way, Mail Stop 115-G04, Salt Lake City, UT 84108, USA
e-mail: Kristi.smock@aruplab.com; Nahla.heikal@aruplab.com

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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

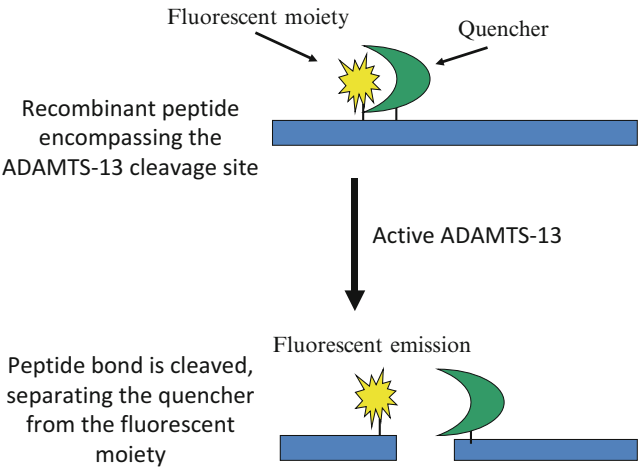

Fig. 8.1 Depiction of a FRET assay utilizing a fluorochrome and quencher that are separated by ADAMTS13 cleavage, resulting in fluorescent emission

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

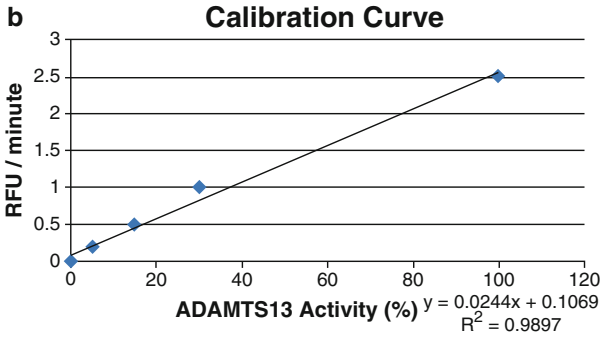

Fig. 8.2 (**a**) Representative data from duplicate wells (A5 and A6) in a kinetic FRET assay. A baseline fluorescence reading is taken (time 0), followed by multiple additional readings as the reaction progresses. The raw data is used to determine the slope of the kinetic curve (RFU/minute), which is 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.

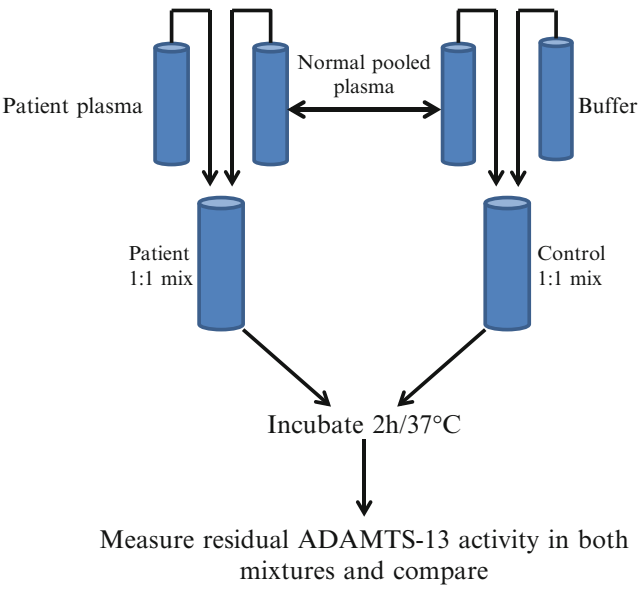

Fig. 8.3 Bethesda assay for identifying and quantifying inhibitory ADAMTS13 autoantibodies

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.

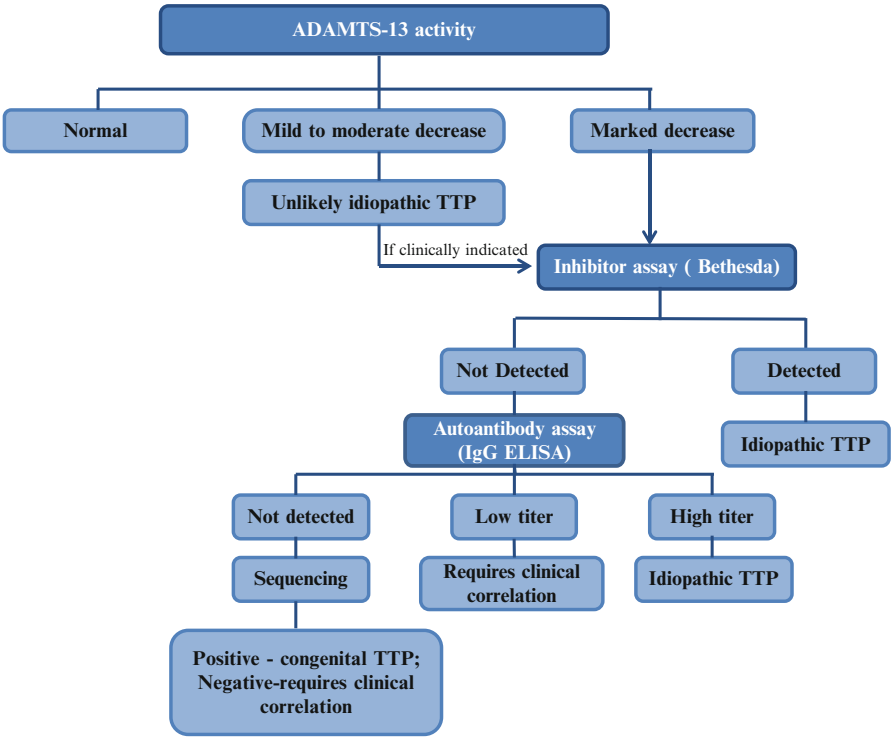

Fig. 8.4 Suggested testing algorithm for evaluating patients with suspected TTP

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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Chapter 9
Potential Clinical Use of Recombinant Human ADAMTS13

Silvia Ferrari, Hanspeter Rottensteiner, and Friedrich Scheiflinger

9.1 Introduction

ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type-1 repeats), also known as von Willebrand factor (VWF)-cleaving protease, is a plasma enzyme responsible for cleaving unusually ultra-large von Willebrand factor (ULVWF) multimers that circulate in the blood. Such multimers are hyperactive, and their conversion into smaller and less active forms is crucial for balanced hemostasis. Lack of functional *ADAMTS13* due to mutations in the *ADAMTS13* gene (congenital) or autoantibodies against *ADAMTS13* (acquired) causes accumulation of ULVWF multimers, which can lead to platelet clumping and thrombotic occlusions within the microvasculature. Accompanying microangiopathic hemolytic anemia and thrombocytopenia are the hallmarks for thrombotic thrombocytopenic purpura (TTP), a life-threatening disease caused by a severe (<5 %) functional deficiency of *ADAMTS13*. In recent years, mild to moderate *ADAMTS13* deficiency has been also associated with thrombotic conditions of different origins. The reduced levels of *ADAMTS13* are usually accompanied by increased VWF antigen and/or activity levels, and an elevated VWF to *ADAMTS13* ratio seems to play a causal role in disease pathogenesis [1, 2].

Herein, we review current best practice in treating patients with severe *ADAMTS13* deficiency as manifested in congenital and acquired TTP. We also explore the literature for other thrombotic complications associated with *ADAMTS13* deficiency and, where available, present insights from animal models mimicking these diseases. Finally, we discuss the potential use of recombinant human *ADAMTS13* (rhADAMTS13) in the treatment of clinical conditions associated with

S. Ferrari • H. Rottensteiner • F. Scheiflinger (✉)
Baxalta Innovations GmbH, Vienna DC Tower, Donau-City Straße 7, 1220, Vienna, Austria
e-mail: friedrich.scheiflinger@baxalta.com

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reduced levels of *ADAMTS13* and/or elevated VWF to *ADAMTS13* ratios, from possible replacement therapy in congenital TTP to intervention in stroke with supraphysiological doses of the VWF-cleaving protease.

9.2 Thrombotic Thrombocytopenic Purpura

9.2.1 Congenital Thrombotic Thrombocytopenic Purpura

Congenital TTP, also known as Upshaw–Schulman syndrome, is a very rare genetic disorder characterized by a severe *ADAMTS13* deficiency due to homozygous or double heterozygous mutations in the *ADAMTS13* gene and resulting in a constitutional plasma deficiency or nonfunctional protein [3, 4]. The clinical presentation of congenital TTP is highly heterogeneous, with a variable age of onset, severity, and frequency of TTP episodes. Half of patients experience an early onset, with their first acute TTP episode occurring between the first days of life and 5 years of age. The other half experiences their first bout of TTP later in life, often triggered by stress factors such as infection, pregnancy, or drug use. After an acute episode, patients tend to develop chronic recurrent TTP, often with unpredictable relapses with intervals of several years [5].

9.2.1.1 Current Therapies for Congenital TTP

The mainstay of treatment of acute episodes in patients with congenital TTP is infusion of fresh frozen plasma (FFP) containing normal functional levels of *ADAMTS13*. Acute events are treated with 10–15 mL FFP/kg/day until clinical conditions have improved. Some patients, mainly those with adult onset, require treatment only in situations of increased risk such as pregnancy or infection. Approximately 50 % of patients, however, have a recurrent form of the disease and require regular prophylaxis with FFP every 2–4 weeks to prevent relapse or complications [6–8].

Although only one case of alloantibody development against *ADAMTS13* in a patient receiving frequent plasma transfusions has been reported to date [9], continuous exposure to large volumes of FFP increases the risk of transmission of pathogens (virus and/or prions), transfusion-related side effects, and immunogenic responses.

As an alternative to FFP, off-label use of plasma-derived FVIII–VWF concentrates in treating congenital TTP has been described [10, 11].

The potential upsides of such treatment are the smaller volumes required and the enhanced safety of such products which have undergone extended virus inactivation procedures. Their *ADAMTS13* content, however, is usually low and varies substantially between products [12, 13], with the highest *ADAMTS13* activity (9 U/mL) for Koate®-DVI [12]. Furthermore, since *ADAMTS13* activity is not a release parameter for any FVIII–VWF concentrate, the lot-to-lot variability within a product may be significant.

9.2.1.2 Potential for Treatment of Congenital TTP with Recombinant Human *ADAMTS13*

Replacement therapy with a human *ADAMTS13* concentrate may reduce or even prevent some of the above-listed detrimental complications associated with FFP treatment in patients with congenital TTP and also circumvent the shortcomings observed with FVIII–VWF concentrates. To our knowledge, however, a plasma-derived *ADAMTS13* concentrate for clinical use is currently not under development, perhaps partly due to the reported difficulties in isolating fully functional protein and the low yields experienced [14].

A recombinant version of human *ADAMTS13* (rhADAMTS13) has been produced by Baxter in a genetically engineered Chinese hamster ovary (CHO) cell line on industrial scale under serum- and protein-free conditions [15]. Purification of rhADAMTS13 is achieved through a multistep chromatography procedure utilizing commercially available resins, including two independent virus inactivation steps. The purified enzyme has high specific activity and has been shown through thorough biochemical characterization to be very similar to *ADAMTS13* purified from plasma [16]. It is expected that administration of the purified recombinant protein concentrate to patients would result in higher *ADAMTS13* activities with a much smaller administered volume when compared to the use of FFP or FVIII–VWF concentrates. Moreover and in analogy to common practice in the hemophilia area, administration of rhADAMTS13 might be adapted to home administration, avoiding the current need for reaching a specialized center and thereby improving the quality of life of patients.

The ability of rhADAMTS13 to restore in vitro the defective VWF-cleaving protease activity was demonstrated a decade ago. When rhADAMTS13 was spiked into plasma samples of two brothers with congenital TTP, a normal VWF-processing pattern was restored [17], highlighting for the first time the potential of the protein as a substitution therapy in congenital *ADAMTS13* deficiency.

More recently, nonclinical in vivo studies specifically addressed the prophylactic and therapeutic effect of rhADAMTS13 in an animal model mimicking congenital TTP [18]. Mouse strains lacking a functional *ADAMTS13* protease (*ADAMTS13* gene knockout strain) were challenged with recombinant human VWF (rhVWF) containing a high portion of ULVWF multimers, thereby simulating an acute event of TTP. Intravenous injection of a very high dose of rhVWF (2000 U/kg body weight) reproducibly triggered a rapid onset of TTP-like symptoms including thrombocytopenia, schistocytosis, and extensive platelet aggregation. In these studies, the heart was shown to be the most sensitive target organ. Prophylactic administration of rhADAMTS13, i.e., prior to rhVWF challenge, completely prevented the development of TTP-like symptoms. In the therapeutic setting, where rhADAMTS13 was injected at various time points up to 3 h after rhVWF administration, symptoms could not be prevented, but were mitigated in a treatment interval-dependent manner, with the most beneficial effect of rhADAMTS13 observed at the first measurement time point. These results not only confirmed that intravenous injection of rhADAMTS13 can regulate the size of VWF in vivo, but also suggested

that a prophylactic rhADAMTS13 treatment regimen for TTP patients may suffice to eliminate the occurrences associated with a deficiency of VWF-cleaving protease. Notably, the pharmacokinetics of rhADAMTS13, as established in *ADAMTS13*-deficient mice, seems to support such a regimen. In these experiments, an in vivo recovery of 65 % and a terminal half-life of about 24 h were measured [19]. In the case of a therapeutic use, the mouse data suggest that rhADAMTS13 may have significant potential also in this clinical setting, but that it will be important to intervene in an acute episode as early as possible to achieve significant benefit [18].

A clinical phase I trial studying the safety and pharmacokinetic properties of rhADAMTS13 in patients with congenital TTP has been initiated. The in vivo recovery and terminal half-life of the recombinant product in humans are not currently known. Such pharmacokinetic parameters may inform the design of future treatment regimens and will particularly affect the dosing intervals in prophylaxis.

9.2.2 Acquired Thrombotic Thrombocytopenic Purpura

Acquired idiopathic TTP is an autoimmune disorder characterized by severe *ADAMTS13* deficiency due to anti-*ADAMTS13* autoantibodies [20, 21]. Neutralizing antibodies, which inhibit *ADAMTS13* activity or enhance its clearance, have been identified in 60–90 % of patients with acquired TTP [22–24].

9.2.2.1 Current Therapies for Acquired TTP

Current standard therapy for acquired TTP involves plasma exchange (PEX) [25]. The efficacy of this procedure has been attributed to the combination of infusion with plasma which supplies *ADAMTS13* and apheresis which removes autoantibodies and ULVWF multimers which are directly associated with the pathogenesis of TTP. Despite having increased patient survival from 10 to 80 %, PEX is not entirely harmless, with adverse events reported to complicate about 20 % of procedures [26].

In addition to first-line PEX therapy, supportive therapy with corticosteroids is often used, although clinical trials that support their efficacy or the rationale for their use are not available [25, 26]. In relapsing or refractory cases, patients are treated with other immunosuppressive drugs such as cyclosporine, cyclophosphamide, and vincristine. More recently, the anti-CD20 monoclonal antibody, rituximab, has been shown to be an effective treatment option for patients with relapsing or refractory forms of TTP [27, 28] and safe and effective in those with acute TTP as confirmed by a phase II non-randomized clinical trial [29]. Patients receiving rituximab during an acute event required less PEX to achieve remission and a shorter inpatient stay (apart from those in intensive care units) and had a lower risk of relapse [30]. These results encourage widespread rational use of rituximab in acquired TTP.

9.2.2.2 Potential for Treatment of Acquired TTP with Recombinant Human *ADAMTS13*

In contrast to congenital TTP, where administration of rhADAMTS13 is expected to dose-dependently restore *ADAMTS13* activity, the situation in acquired TTP is complex. The therapeutic efficacy of plasma-derived or recombinant *ADAMTS13* is negatively affected by neutralizing anti-*ADAMTS13* antibodies, which vary not only in concentration but also in inhibitory properties. Formation of immune complexes between *ADAMTS13* and cognate antibodies, as well as the clearance of these complexes, would clearly influence the pharmacodynamics and pharmacokinetics of the *ADAMTS13* protein administered. Furthermore, repeated administration of *ADAMTS13* might boost the immune response, as observed in some acquired TTP patients undergoing repeated PEX during an acute episode and described as exacerbation [31, 32]. These concerns notwithstanding, whether or not a recombinant and therefore pure protein concentrate with high specific activity is able to overcome the inhibitory antibody is currently unknown, but warrants clinical investigation. Dependent on the outcome, the rhADAMTS13 may be useful in acquired TTP as an adjunctive therapy or even as a stand-alone therapy.

The first line of evidence for a beneficial effect of rhADAMTS13 in acquired TTP comes from an in vitro study where spiking of rhADAMTS13 into plasma samples from a cohort of TTP patients reversed the antibody-inhibiting capacity and restored *ADAMTS13* activity [33]. The amount of rhADAMTS13 needed to restore a certain *ADAMTS13* activity level was shown to be mainly determined by the inhibitor titer (BU/mL) of the samples. No relevant difference was seen between samples from patients experiencing an acute phase and those in remission; neither did the anti-*ADAMTS13* immunoglobulin class and subclass distribution affect the amounts of rhADAMTS13 required. Data processing generated a formula that allowed estimation of the amount of rhADAMTS13 required under in vitro (static) conditions to achieve a measurable *ADAMTS13* activity of 0.5 U/mL at a given inhibitor titer [33].

This equation may already support the rationale for a dosing scheme in acquired TTP patients; however, for the reasons outlined above, in vivo use of rhADAMTS13 is likely to be more challenging. For instance, the feasibility of a stand-alone therapy where rhADAMTS13 is administered at a concentration high enough to override the inhibitory activity of the anti-*ADAMTS13* antibodies may be limited to patients with low inhibitor titers (<2 BU/mL), who account for approximately 30 % of those with TTP. Not only were low doses of rhADAMTS13 able to restore plasma *ADAMTS13* activity, but the risk of side effects due to formation of immune complexes is considered low in this subgroup. A preclinical proof-of-concept study in rats appears to support the use of rhADAMTS13 in an acute TTP setting [34]. Rats were treated to a defined inhibitor titer by injection of goat antihuman *ADAMTS13* IgG antibodies and then treated with increasing doses of rhADAMTS13. In this setup, dose-dependent inactivation of the neutralizing activity of the inhibitor with eventual restoration of *ADAMTS13* activity was observed. Importantly, formation of *ADAMTS13*-specific immune complexes and their clearance over time was also

demonstrated, and no pathological findings were revealed by histological examination. Another unknown factor potentially affecting the efficacy of rhADAMTS13 in the acquired TTP setting is the recently shown variance in inhibitor capacity of anti-*ADAMTS13* antibodies in plasma purified from different patients. Antibody samples with the same inhibitory capacity as measured by Bethesda assay showed vastly different inhibitory kinetics under flow conditions [35].

In the case of an adjunct therapy, modifying the PEX procedure by administering rhADAMTS13 in addition to human plasma exchange could be an option worth pursuing. The hope would be that such an additional treatment might allow decreasing the number of PEX required to bring the patient into remission. A more remote possibility would be the combination of anti-*ADAMTS13* antibody removal through immune-adsorption and rhADAMTS13 therapy, which would circumvent the infusion of exogenous plasma. Nonetheless, the potential clinical use of rhADAMTS13 in the treatment of acquired TTP requires further debate among experts and needs to be accompanied by the establishment and thorough testing of the compound in animal models of acquired TTP.

9.2.2.3 Other Treatment Options Under Development

A novel potential therapy for patients with acquired TTP has recently been proposed by Jian et al. [36] and employs a rhADAMTS13 variant engineered by substituting critical amino acids within exosite 3 of the spacer domain. This exosite is part of the predominant immune epitope recognized by anti-*ADAMTS13* antibodies from a majority of patients with acquired TTP. Interestingly, the mutated rhADAMTS13 enzyme was not only resistant to binding and inhibition by anti-*ADAMTS13* antibodies but also exhibited significantly enhanced specific proteolytic activity [36]. On the other hand, antibodies to *ADAMTS13* are not restricted to exosite 3 of the spacer domain [37]; antibodies against other parts of *ADAMTS13* might also be reactive against the mutant, thereby possibly compromising the mutant's pharmacokinetic and pharmacodynamic properties. Therefore, extensive analysis of this gain-of-function rhADAMTS13 variant in animal models of acquired TTP is required to prove its efficacy in vivo before proceeding to clinical testing. It is also essential to determine whether introducing point mutations in the wild-type molecule may create neo-epitopes that trigger antibody formation and thus inactivation of the therapeutic drug upon repeated administration.

Other approaches under development focus on intervention at the level of VWF activity, either targeting the multimeric size of VWF or its interaction with the platelet receptor GPIbα [38]. The former strategy employs *N*-acetylcysteine (NAC), an FDA-approved drug to reduce the size of polymeric mucin multimers in the treatment of chronic obstructive lung disease and acetaminophen toxicity. In vitro studies showed that NAC reduced the multimeric size of VWF through limited disulfide bond reduction, causing a decrease in VWF-dependent platelet aggregation and collagen binding. Administration of NAC to *ADAMTS13*-deficient mice consistently reduced the size of VWF multimers in plasma and led to decreased platelet thrombus formation in calcium ionophore-treated vessels [39]. A recent case report describes the recovery of a

patient with refractory TTP after supplementation of the standard treatment (PEX, corticosteroids, and rituximab) with NAC [40]. However, it would be important to determine whether such a drug exerts a pleiotropic effect on other proteins with disulfide bonds amenable to reduction under mild conditions.

Another therapeutic option for targeting the interaction of VWF with platelets is the ARC1779 aptamer, which has already been evaluated in patients with TTP. ARC1779 is a modified DNA/RNA aptamer that binds to the A1 domain of VWF and thus prevents interaction with platelet GPIbα, which blocks VWF-mediated platelet function [41]. Administration of ARC1779 in patients with congenital or acquired TTP was shown to inhibit VWF activity and platelet function in a dose- and concentration-dependent manner, increase platelet count, and improve clinical symptoms [42–46]. The aptamer was also well tolerated, with no safety issues, including bleeding complications [44, 46]. ARC1779 is intended for use in patients with acquired TTP as adjunctive therapy to PEX and/or immune suppression.

Other compounds that interfere with the VWF–GPIb α axis include the anti-VWF nanobody ALX-0681 and monoclonal anti-VWF antibody GBR600, both of which were shown to preclude the onset of TTP symptoms in a baboon model for acquired TTP [47, 48]. A striking aspect of this model is that inhibition of endogenous *ADAMTS13* suffices to trigger thrombocytopenia and other typical symptoms of TTP [47], a phenomenon not observed in mice or humans. Tersteeg and colleagues [49] recently discovered that the fibrinolytic enzyme plasmin acts as a physiological backup enzyme for *ADAMTS13* to process unusually large VWF multimers, which may explain this interspecies difference; they also discuss a potential therapeutic value for plasmin in acquired TTP.

9.3 Cardiovascular Diseases

In theory, almost any thrombotic complication can be influenced by the activity levels of *ADAMTS13*, given that its substrate, VWF, is directly involved in the fundamental events of thrombus formation, i.e., platelet adhesion and platelet aggregation, which ultimately lead to vessel occlusion. Other functions include involvement in endothelial leukocyte adhesion and extravasation as part of the inflammation process accompanying many cardiovascular diseases. Nonetheless, experimental (preclinical) evidence for a beneficial role of rhADAMTS13 is available only for a few indications, and the doses required to improve the course of the disease have not yet been determined.

9.3.1 Stroke

Stroke is an acute clinical syndrome that develops after a vascular insult to the brain and is the second most common cause of death and permanent disability worldwide [50]. Strokes are either ischemic or hemorrhagic, and clinical distinction between

the subtypes is critical for their management. Acute ischemic stroke is the most common (about 80 %) type of stroke; brain ischemia results from thromboembolism or, less frequently, from in situ thrombosis. Hemorrhagic strokes represent about 20 % of all strokes and are mainly caused by hypertension or vessel wall pathology [50]. Most subarachnoid hemorrhages (SAHs) are due to rupture of saccular aneurysms within the subarachnoid space. Although less common than other forms of stroke, SAH is a life-threatening disease associated with significant morbidity and mortality and affects 10 in 100,000 people a year [51].

9.3.1.1 Ischemic Stroke

Acute ischemic stroke is caused by sudden occlusion of a blood vessel by a thrombus or through embolism. Our understanding of stroke pathogenesis has significantly improved during the last years, and it is presumed that the *ADAMTS13*–VWF axis plays a crucial role in its development. Clinical studies have shown that patients affected by stroke have higher VWF antigen and VWF ristocetin cofactor levels than controls [52–56] and that VWF levels are an independent predictor of stroke [55, 57]. By contrast, low levels of *ADAMTS13* are also associated with an increased risk of adult [53, 54] and pediatric [58] stroke. This association was confirmed by a meta-analysis of previously gathered data [53, 54, 58, 59], which concluded that there is a higher risk for ischemic stroke at low *ADAMTS13* levels [60]. Moreover, single nucleotide polymorphisms in both VWF [61] and *ADAMTS13* [62, 63] were found to be associated with an increased risk of ischemic stroke. However, the significance of these studies is limited due to their case–control design and small sample size.

Although these patient-derived data provide evidence for the potential role of VWF and *ADAMTS13* in the development of ischemic stroke, animal models offer important insight into the pathophysiological mechanism involved. Particularly relevant is the so-called transient middle cerebral artery occlusion (MCAO) model of ischemia/reperfusion injury in which some aspects of human ischemic acute stroke can be reproduced experimentally by temporary occlusion of this artery in animals [64].

Experiments using a MCAO model of ischemia/reperfusion injury in mice showed that the interaction of VWF with collagen and platelet glycoprotein GPIbα, but not GPIIb/IIIa, plays a central role in the development of stroke [65]. Inhibition of GPIbα or absence of VWF protected mice from ischemia/reperfusion injury without increasing cerebral hemorrhage, thus confirming the pathophysiologic role of VWF and its platelet receptor GPIbα during experimental ischemic stroke injury [65–67]. Furthermore, studies performed with *ADAMTS13* knockout mice demonstrated that mice lacking *ADAMTS13* developed larger infarction areas and more severe neurological tissue damage than wild-type mice [66, 68].

In animals, *ADAMTS13* deficiency also exacerbates ischemia/reperfusion brain injury by inducing an acute inflammatory response characterized by neutrophil recruitment in the infarcted and peri-infarcted regions, increased myeloperoxidase

activity, and elevation of pro-inflammatory cytokines [69, 70]. These inflammatory processes contribute to an increase in infarct size and tissue damage after ischemic stroke. By contrast, VWF deficiency eliminates the inflammatory response, suggesting that VWF promotes brain tissue inflammation after injury and that *ADAMTS13* modulates this effect [69, 70]. Another noteworthy aspect of *ADAMTS13* biology is its reported upregulation in astrocytes and microglia after spinal cord injury in wild-type rats, suggesting that local production of *ADAMTS13* in activated glial cells may help to increase VWF degradation and thereby suppress thrombosis and inflammation after brain injury and reduce the blood–brain barrier permeability [71].

9.3.1.2 Hemorrhagic Stroke

Aneurysmal SAH is frequently complicated by cerebral vasospasm leading in about 25–30 % of patients to delayed cerebral ischemia (DCI) with onset from 4 to 10 days after the hemorrhage [72]. The DCI may be reversible, but can also progress to cerebral infarction and brain injury, thereby increasing the likelihood of disability or death. Calcium channel blockers, which have been shown to reduce the incidence of DCI and the risk of poor outcome, are now the mainstay treatment for hemorrhagic stroke. However, their efficacy and true impact on outcome after a SAH event are still controversial [72, 73]. The cause of DCI onset is poorly understood, but current evidence suggests that in addition to vasospasm, inflammation and microthrombosis play an important role. Supporting this hypothesis are patient data showing that those who experienced SAH with a DCI complication had high levels of both VWF propeptide and VWF antigen but reduced *ADAMTS13* activity [74].

9.3.1.3 Current Treatment of Stroke and the Potential for Using rhADAMTS13

The only medication currently available for acute ischemic stroke is recombinant tissue plasminogen activator (tPA) which facilitates rapid reperfusion of brain tissue by promoting thrombolysis of the occluding thrombus. To be beneficial for patients, administration of tPA needs to be initiated within the first 4.5 h of stroke onset as only then the affected ischemic area can be partially rescued [75]. This narrow therapeutic time window allows only a small number of patients to be treated, and 6–7 % of these patients develop symptomatic intracranial hemorrhage, which is a devastating major adverse effect associated with high mortality [50, 76]. Other limitations of tPA therapy include the low rate of arterial recanalization, only moderate efficacy in non-eligible patients, and a low incidence of patients eligible for treatment due to various exclusion criteria or contraindications [77]. Moreover, a significant number of patients who receive reperfusion therapy remain at risk for developing progressive infarction and secondary neuronal injury, a process referred to as “reperfusion injury.”

Experimental evidence for a beneficial effect of rhADAMTS13 in acute ischemic stroke comes from studies using the MCAO mouse model. In a seminal study by Zhao et al., infusion of a high dose of rhADAMTS13 into wild-type mice immediately before reperfusion reduced infarct volume and improved functional outcome without producing cerebral hemorrhage [66], suggesting that *ADAMTS13* protects the brain from ischemia–reperfusion injury. This hypothesis is supported by the observed antithrombotic and thrombo-destabilizing effect of rhADAMTS13 upon administration to *ADAMTS13* knockout mice [78]. Nonetheless, the high therapeutic dose (~3500 U/kg) of rhADAMTS13 used in the study might overestimate its effect, and further studies with lower doses are required to better assess the clinical efficacy of rhADAMTS13 in stroke.

Compared with tPA treatment, the pharmacologic profile of rhADAMTS13 appears to be safe. Generally, single intravenous administration of rhADAMTS13 at doses up to 32,000 U/kg to *ADAMTS13* knockout mice caused no complications or adverse effects [19]. In the MCAO mouse model, animals treated with rhADAMTS13 showed a reduced rate of symptomatic intracranial hemorrhage [66, 68]. Furthermore, when applied topically, rhADAMTS13 reduced occlusive thrombus size and increased recanalization in the venous circulation of mice in a similar manner to tPA without causing hemorrhage in the treated area [79].

A further aspect to consider in the treatment of stroke with tPA is that the blood–brain barrier may become compromised. Disruption of this barrier is triggered by activation of matrix metalloproteinases and results in increased ischemic endothelial injury [80]. According to Wang et al. [81], rhADAMTS13 may exert the opposite effect. Injection of tPA or VWF into the cerebrospinal fluid of nonischemic mice induced blood–brain barrier permeability, whereas coadministration of rhADAMTS13 with tPA left the barrier intact. Furthermore, tPA-associated cerebral hemorrhage was prevented, and the neurological outcome in a MCAO model of ischemic stroke improved in the concomitant presence of rhADAMTS13 [81].

Based on these preliminary results, rhADAMTS13 also appears to exert a neuroprotective effect in stroke. Future studies should therefore not only aim to substantiate the therapeutic potential of rhADAMTS13 in the treatment and possibly prevention of stroke but also explore a combination therapy of tPA and rhADAMTS13, which may reduce bleeding complications and extend the time window for tPA administration. An important yet currently unresolved question is the plasma level of *ADAMTS13* or ratio between plasmatic *ADAMTS13* and VWF activity, required to prevent or treat ischemic stroke.

As for hemorrhagic stroke, two independent groups have recently demonstrated the efficacy of rhADAMTS13 in different experimental SAH mouse models [82, 83]. Systemic administration of rhADAMTS13 shortly after induction of SAH reduced the extent of microthrombosis and the amount of apoptotic and degenerative neurons. In addition, rhADAMTS13 reduced neuronal inflammation and brain injury and improved neurological performance [82, 83]. These results suggest that rhADAMTS13 might be a new option to prophylactically treat patients who experienced SAH in order to prevent DCI and progression to brain injury.

9.3.2 *Myocardial Infarction*

Acute myocardial infarction is caused by thrombotic occlusion of a coronary artery resulting in damage or dysfunction of the cardiac tissue. Although early and rapid restoration of coronary blood flow is critical to reduce the size of a myocardial infarct and improve clinical outcome, reperfusion itself can induce further cardiomyocyte injury, a phenomenon known as myocardial reperfusion injury. The most effective myocardial reperfusion strategies involve thrombolytic therapy or primary percutaneous coronary intervention [84].

There is increasing clinical evidence that the *ADAMTS13*–VWF axis is involved in the pathogenesis of myocardial infarction. Case-control studies showed that patients with acute coronary heart disease such as acute myocardial infarction and unstable angina pectoris have increased VWF antigen levels, reduced *ADAMTS13* activity, and high VWF antigen/*ADAMTS13* antigen ratios [85–89]. Moreover, individuals with high VWF antigen and reduced *ADAMTS13* antigen levels are at higher risk of developing myocardial infarction [54, 59, 90, 91]. Although these studies were observational and did not determine whether the *ADAMTS13*–VWF axis directly contributes to the pathogenesis of acute myocardial infarction, the results suggest that VWF plays a role in myocardial infarction by contributing to a proinflammatory state [92]. However, a recently published meta-analysis of all studies that provided odd ratios for a first myocardial infarction or coronary heart disease [54, 59, 89–91] found no significant association between *ADAMTS13* levels and these conditions [60].

In vivo studies using a mouse model of myocardial infarction ischemia/reperfusion injury showed that complete *ADAMTS13* deficiency exacerbates myocardial ischemia/reperfusion injury by promoting thrombosis and inflammation, whereas a complete deficiency of VWF exerts a protective role [19, 93, 94]. Interestingly, heterozygous deficiency of *ADAMTS13* had no effect, suggesting that moderate levels of *ADAMTS13* (~50 %) are sufficient to prevent myocardial ischemia/reperfusion injury [93].

9.3.2.1 Potential Use of rhADAMTS13 in Acute Myocardial Infarction

Data from experimental myocardial infarction animal models have shown that administering rhADAMTS13 before reperfusion has a cardioprotective effect, reducing infarct size and inflammation and improving overall cardiac function [19, 94, 95]. Notably, not only full-length but also a C-terminally truncated variant of rhADAMTS13 comprising roughly half the protein including the spacer domain was also efficacious in this model [94].

Most recently, Savchenko et al. [95] showed that myocardial ischemia/reperfusion injury in mice also caused an increase in nucleosomes in plasma and in neutrophil infiltration and histone H3 at the site of injury. Mice receiving a coadministration of rhADAMTS13 and the DNA-degrading enzyme deoxyribonuclease I (DNase I)

had smaller infarct areas and improved cardiac function than animals treated with DNase I or rhADAMTS13 alone [95]. In this model, rhADAMTS13 showed a stronger anti-inflammatory effect than DNase I [95], suggesting that the protective role of rhADAMTS13 in myocardial ischemia/reperfusion injury is due to reduced inflammation via modulation of VWF-mediated leukocyte recruitment.

9.3.3 Atherosclerosis and Inflammation

Atherosclerosis is a progressive chronic inflammatory disease of the arterial wall and is characterized by the development of atheromatous plaques in the inner lining of the large and medium arteries [96]. These plaques are formed through accumulation of lipids and inflammatory cells. The pathological sequence of events in atherosclerosis development includes endothelial dysfunction as an early sign of lesion. Atherosclerotic plaque rupture promotes atherothrombosis, vessel occlusion, and subsequent ischemic organ damage, which leads to acute cardiovascular events such as myocardial infarction or stroke [96].

Previous epidemiological studies have described elevated VWF antigen and activity levels and reduced *ADAMTS13* activity in plasma from patients with various acute and chronic inflammatory conditions including sepsis [97–102]. Some of these studies showed that levels of *ADAMTS13* and VWF correlate with disease severity, organ dysfunction, and/or outcome. Moreover, *ADAMTS13* levels decreased in murine [103] and porcine [104] sepsis models after induction of sepsis. These findings suggest a potential role of the *ADAMTS13*–VWF axis in chronic inflammatory processes such as atherosclerosis.

Intravital microscopy in a murine model of acute inflammation showed that *ADAMTS13* contributes to downregulating inflammation by cleaving hyperactive ULVWF multimers, thereby preventing leukocyte rolling in nonactivated veins and adhesion in activated endothelium [92]. Since these effects were VWF dependent, it is tempting to suggest that ULVWF multimers promote or accelerate vascular inflammation by slowing down leukocytes and facilitating their extravasation, a preliminary step required for the initiation of vascular lesion in atherosclerosis [92].

Studies performed in atherosclerotic-prone apolipoprotein E knockout mice fed with a high-fat Western diet showed that complete *ADAMTS13* deficiency increases vascular inflammation and early atherosclerotic plaque formation [105, 106], whereas a deficiency of VWF reduces atheromatous plaque development [107]. Furthermore, Gandhi et al. [108] demonstrated that *ADAMTS13* curbs early atherosclerotic lesion progression in the aortic sinus by reducing neutrophil and macrophage infiltration into the lesions via a VWF-dependent mechanism. Together, these findings suggest that *ADAMTS13* protects against early atherosclerosis by cleaving ULVWF multimers and thereby reducing leukocyte infiltration in areas of atherosclerotic lesions.

One limitation of these studies is that the experimental atherosclerosis performed in a monogenic apolipoprotein E-null mouse model to investigate the role of the *ADAMTS13*–VWF axis does not entirely mimic the human condition.

Human atherosclerosis is a multifactorial and complex disease; moreover, patients with acute and chronic inflammatory diseases usually have only partially reduced *ADAMTS13* levels rather than complete *ADAMTS13* deficiency. It is therefore essential to determine whether regularly administrated pharmacological doses of rhADAMTS13 curb the development of atherosclerosis in apoE mice. If this is the case, the use of rhADAMTS13 in patients at a certain stage of the disease may warrant further discussion. However, also here, as in other indications, rhADAMTS13 dose finding, as well as definition of an analytically accessible and reliable biomarker such as the *ADAMTS13*–VWF activity ratio, is of utmost importance.

9.3.4 Liver Disease

Chronic liver disease is characterized by complex changes in the hemostatic system such as decreased levels of most procoagulant and anticoagulant factors, alterations in the fibrinolytic system, thrombocytopenia, and platelet function defects. Despite these major changes, the hemostatic system remains in balance due to a comparable decline in both pro- and anticoagulant pathways. Depending on prevailing circumstantial risk factors, this precarious equilibrium can be shifted toward bleeding or thrombosis [109].

Patients with chronic liver disease, particularly in the end stage, have elevated plasma levels of VWF which gradually increase as functional liver capacity decreases. Various mechanisms have been proposed to be responsible for this increase, including increased VWF synthesis by expansion of the endothelial cellular surface due to collateralization and angiogenesis, enhanced secretion by bacterially damaged endothelial cells, and reduced VWF clearance [110]. However, elevated antigen levels do not necessarily correlate with the functional capacity of VWF as evidenced by occasional low VWF ristocetin cofactor/VWF antigen ratios [111, 112].

Several studies have shown that patients with liver cirrhosis have markedly reduced *ADAMTS13* activity and antigen levels [97, 111, 113, 114], with only one study reporting the presence of highly variable levels of both *ADAMTS13* activity and antigen [112]. This discrepancy is likely due to the inclusion of patients with different etiologies of liver disease, as only the last study reported cirrhosis to be caused mainly by alcohol abuse. Plasma levels of *ADAMTS13* decrease according to the severity of the disease [111, 114], and the VWF ristocetin cofactor/*ADAMTS13* activity ratio increases progressively as the functional capacity of the liver deteriorates [112, 115]. This development is reflected by a shift of the VWF multimer pattern from normal in early to ultra-large in end-stage disease [112, 115].

Due to these results, levels of VWF and *ADAMTS13* have been proposed as new predictor markers for clinical outcome in patients with advanced cirrhosis [114, 116, 117]. Moderately reduced plasma levels of *ADAMTS13* were also found in patients with acute liver disease [118], hepatic veno-occlusive disease [119, 120], alcoholic hepatitis [121–123], idiopathic noncirrhotic intrahepatic portal hyperten-

sion [124], and in those undergoing liver transplantation [125–128] or partial hepatectomy [129].

Various factors are likely involved in the observed decrease in *ADAMTS13* activity in patients with advanced liver disease. Rat models of acute liver injury [130] and liver fibrosis [131, 132] provide evidence that damage of hepatic stellate cells, the main source of *ADAMTS13* synthesis [133], indeed affects plasma *ADAMTS13* levels [130]. Enhanced consumption due to the degradation of large quantities of VWF antigen [97], inflammatory cytokines [123], and/or *ADAMTS13* plasma inhibitors [111] may also contribute to a lower *ADAMTS13* activity in plasma.

9.3.4.1 Current Treatment of Chronic Liver Diseases and the Potential for Using rhADAMTS13

Liver cirrhosis has long been considered to be a prototype of hemorrhagic coagulopathy as routine tests show coagulation abnormalities in most patients. When undergoing invasive procedures with an increased risk of bleeding such as liver biopsy or main surgery, patients have commonly been treated with blood products, mainly FFP and platelet concentrates [134]. However, since such regimens pose a high burden on patients with liver cirrhosis due to fluid overload that may complicate preexisting conditions such as portal hypertension, administration of low-volume coagulation factor concentrates, antifibrinolytics, or recombinant factor VIIa is most often used to reduce bleeding during surgery [134]. On the other hand, it has long been assumed that patients with chronic liver disease are protected against thrombotic disease due to their abnormal hemostatic values. Thus, antithrombotic therapy to prevent or treat thrombotic disease in this patient population has been used restrictedly, and published experience is limited.

Nonetheless, microthrombi within the hepatic microvasculature of patients with liver cirrhosis are frequently observed. Their occurrence may be explained by a distorted balance of the *ADAMTS13*–VWF ratio, in that ULVWF released by injured liver cells cannot be sufficiently cleaved in patients with low *ADAMTS13* levels, thereby inducing platelet thrombus formation locally [118]. It is therefore suggested that in patients with advanced liver cirrhosis with moderately to severely reduced (~25–<3 % of normal) *ADAMTS13* levels, administration of FFP might be of benefit as it allows replenishment of *ADAMTS13* [135]. Although clinical data that support this proposal are not yet available, this subgroup appears potentially eligible for rhADAMTS13 replacement therapy. The recombinant protein has the advantage over FFP that much lower volumes are required to reach a similar therapeutic efficacy, which would prevent fluid overload, a main complication of FFP therapy. Prophylactic administration of rhADAMTS13 is expected to suppress buildup of ULVWF multimers and reduce plasma VWF antigen levels, thereby lowering the overall thrombogenic potential, which could result in an increase in platelet counts and improvement of liver function.

Another possible field of application of *ADAMTS13* therapy is allogeneic stem cell transplantation. A multicenter, prospective, randomized, controlled study showed

prophylactic FFP administration to help prevent development of hepatic veno-occlusive disease after transplantation of stem cells [120]. Here, the *ADAMTS13* supplemented with FFP is considered to modulate the elevated levels of VWF released from damaged sinusoidal endothelial liver cells induced by chemotherapy and/or radiation accompanying the transplantation procedure. These preliminary data suggest prophylaxis with rhADAMTS13 is beneficial in allogeneic stem cell transplantation, and further studies appear justified.

A third group of patients that might benefit from rhADAMTS13 administration are those undergoing living-donor-related liver transplantation in whom a "local TTP" mechanism within the liver graft has been observed [125]. Immediately after reperfusion of the transplanted liver, a deposition of platelets on the sinusoidal endothelium, release of ULVWF multimers, and increase in plasma VWF levels, as well as a reduction in plasma *ADAMTS13* activity, have been observed in some patients, leading to thrombus formation in the hepatic sinusoid, organ dysfunction, and rejection of the transplant [110]. Administration of rhADAMTS13 throughout the transplantation procedure may therefore help to decrease the likelihood of rejection of the donor liver.

9.3.5 Malaria

Malaria is a parasitic disease associated with high morbidity and mortality. In humans, five species of the genus *Plasmodium*, mainly *Plasmodium falciparum* and *Plasmodium vivax*, cause malarial infections [136]. The pathogenesis of malaria is not fully defined, but it is considered to involve adherence of parasitized erythrocytes to the vessel wall. Sequestration of the infected erythrocytes in the microvasculature causes activation of endothelial cells and release of pro-inflammatory cytokines. Endothelial cell activation also induces release of ULVWF which is considered to mediate cytoadherence of infected erythrocytes to endothelial cells via platelets [137]. Interestingly, malaria shares many clinical features with TTP, including fever, thrombocytopenia, anemia, renal failure, and coma, symptoms that in malaria are still not fully understood. A potential contribution of VWF levels in the pathophysiological mechanism of malaria has been postulated.

A prospective study in patients from Ghana with different malaria presentations has shown that levels of VWF and its propeptide were two- to fourfold higher in malaria-infected patients than in controls and that they correlated with disease severity [138]. Moreover, VWF propeptide levels were higher in patients with cerebral malaria than in those with non-cerebral malaria and decreased more rapidly than VWF levels with antimalarial treatment.

Similar findings were obtained in a study of experimental human *P. falciparum* malaria infection where levels of VWF, propeptide, and "active" VWF were found to be increased and inversely correlated with platelet count in the early stage of blood infection [139]. Prospective studies in patients from Ghana, Malawi, and Uganda presenting with cerebral and non-cerebral malaria due to *P. falciparum*

infection further supported these results [140–142]. Moreover, a study in Indonesian patients with symptomatic *P. falciparum* or *P. vivax* malaria infection showed that infected patients had a significant increase in levels of both VWF and "active" VWF and a decrease in *ADAMTS13* activity and antigen levels, which resulted in high levels of circulating ULVWF [143].

Decreased *ADAMTS13* activity and antigen levels were also determined in patients with severe malaria and correlated with overall disease severity [140, 144]. Recently, an SNP (rs4962153) located in intron 28 of *ADAMTS13* gene was found to be associated with protection against cerebral malaria, with a higher frequency of rs4962153 observed in patients with mild malaria than in those with cerebral malaria [145]. Notably, the same SNP was correlated with an increased risk of ischemic stroke [62], suggesting that this SNP regulates the plasma level of *ADAMTS13*.

Taken together, acute endothelial cell activation appears to constitute an early feature of malaria infection, and decreased *ADAMTS13* activity combined with increased VWF concentrations may contribute to the development of TTP-like symptoms as seen in severe malaria. It is therefore tempting to suggest that administering rhADAMTS13 to patients with severe malaria might prevent or attenuate thrombotic complications during the acute infection phase.

9.4 Other Clinical Conditions Associated with Reduced Levels of *ADAMTS13*

Table 9.1 lists additional clinical conditions where *ADAMTS13* levels were reported to be lower than in the healthy population, but generally within the normal range.

9.5 Conclusions

Since its discovery in 2001, numerous studies have confirmed the crucial role of *ADAMTS13* in regulating the activity of VWF. Evidence is accumulating that qualitative or quantitative deficiencies of *ADAMTS13* may be involved in the development of several diseases, most likely through an increase in the concentration of ULVWF multimers and their pro-thrombotic and pro-inflammatory properties.

The most obvious indication for the therapeutic use of rhADAMTS13 is congenital TTP, where the recombinant protein would replace the missing or non-functional factor. The efficacy and safety of rhADAMTS13 has already been demonstrated in a mouse model mimicking this disorder. The ongoing clinical studies will have to provide insight into the safety, dosage, and clinical benefit of rhADAMTS13 in preventing disease recurrence in patients with congenital TTP.

The potential use of rhADAMTS13 in acquired TTP, either as adjunctive or stand-alone therapy, also requires further investigation, particularly in an animal model suitable for preclinical assessment. Studies first need to address whether the high

Table 9.1 Clinical conditions associated with reduced levels of ADAMTS13

Clinical conditions	ADAMTS13 activity	Reference
HELLP syndrome	31 % (12–43 %)	Lattuada et al. [146]
	74 % (53–95 %)	Hulstein et al. [147]
	28 % (20–50 %)	Pourrat et al. [148]
Preeclampsia	64 % (53–79 %)	Stepanian et al. [149]
	567 ng/ml (486–688 ng/ml)[a]	Alpoim et al. [150]
Disseminated intravascular coagulation	60 % (40–80 %)	Hyun et al. [151]
Antiphospholipid syndrome	79 % (<5–181 %)	Austin et al. [152]
Malignant hypertension	80 % (53–130 %)	Van den Born et al. [153]
Acute pancreatitis	37 % (20–54 %)[#]	Morioka et al. [154]

ADAMTS13 activity levels are expressed as median with the interquartile range between brackets, except for (#) where activity is expressed as mean ± standard deviation
[a]Only ADAMTS13 antigen levels were tested

doses of rhADAMTS13 required to overcome the circulating inhibitory antibodies do not cause undesirable side effects due to immune complex formation and whether such a treatment regimen can indeed improve clinical outcomes.

Several other diseases with thrombotic complications appear to be associated with moderately reduced *ADAMTS13* activity and/or antigen levels. Although the significance of some of these data needs to be confirmed, administering rhADAMTS13 to increase the plasma concentration of the protease to supraphysiological levels could be a strategy worth exploring. Nonclinical data are available mainly for stroke. The results obtained for rhADAMTS13 in experimental animal models mimicking ischemic or hemorrhagic stroke are encouraging; nonetheless, the limitations of these studies need to be adequately addressed before moving into the clinical setting. One shortcoming is that extremely high doses of rhADAMTS13 are required to obtain a therapeutic effect in mice, probably due to a different sensitivity/susceptibility for cleavage of mouse VWF. As a consequence, it is currently difficult to predict the appropriate rhADAMTS13 doses to treat or prevent stroke in humans.

Further indications in which rhADAMTS13 therapy may be clinically beneficial include myocardial infarction, liver disease, and malaria. The challenge now lies in defining the effect of rhADAMTS13 in suitable animal models, as such models usually do not closely emulate human pathophysiology or are simply not available and because human *ADAMTS13* does not efficiently cleave the respective animal VWF substrate in all species.

A remaining question is the selection of specific and reliable biomarkers to assess efficacy in the context of stroke and other diseases associated with reduced *ADAMTS13* levels. The most obvious one would be the VWF antigen/activity to *ADAMTS13* antigen/activity ratio mentioned throughout this review; however, prospective studies would have to provide validation for each indication.

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Index

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