Recent Advances in Thrombotic Thrombocytopenic Purpura

J. Evan Sadler, Joel L. Moake, Toshiyuki Miyata, and James N. George

Thrombotic thrombocytopenic purpura (TTP) is characterized by microangiopathic hemolytic anemia and thrombocytopenia, accompanied by microvascular thrombosis that causes variable degrees of tissue ischemia and infarction. Intravascular coagulation is not a prominent feature of the disorder. Plasma exchange can induce remissions in approximately 80% of patients with idiopathic TTP, but patients have a much worse prognosis when thrombotic microangiopathy is associated with cancer, certain drugs, infections, or tissue transplantation. Recently, acquired autoimmune deficiency of a plasma metalloprotease named ADAMTS13 was shown to cause many cases of idiopathic TTP. This review describes our current understanding of how to use this knowledge clinically.

In Section I, Dr. Joel Moake describes the presentation of thrombotic microangiopathy, emphasizing the pathophysiology of idiopathic TTP. Platelets adhere to ultra-large (or "unusually large") von Willebrand factor (ULVWF) multimers that are immobilized in exposed subendothelial connective tissue and secreted into the circulation in long "strings" from stimulated endothelial cells. ADAMTS13 cleaves ULVWF multimers within growing platelet aggregates under flowing conditions, and this normally limits platelet thrombus formation. If ADAMTS13 is absent, either congenitally or due to acquired autoantibodies, platelet-rich microvascular thrombosis proceeds unchecked and TTP ensues. Plasma exchange is effective therapy for idiopathic TTP, probably because it replenishes the deficient ADAMTS13 and removes some of the pathogenic autoantibodies and endothelial-stimulating cytokines. Some patients have a type of thrombotic microangiopathy after transplantation/chemotherapy but do not have severe ADAMTS13 deficiency. The pathogenesis of their disease must differ but remains poorly understood.

In Section II, Dr. Toshiyuki Miyata describes recent advances in assay methods that should facilitate routine laboratory testing of ADAMTS13 for patients with thrombotic microangiopathy. ADAMTS13 cleaves a single Tyr-Met bond in domain A2 of the VWF subunit. ADAMTS13 assays based on the cleavage of plasma VWF multimers have been used extensively but require considerable time and expertise to perform. A recombinant substrate containing 73 amino acid residues of VWF domain A2 has been devised that allows short incubation times and rapid product detection by gel electrophoresis or immunoassay. These results should encourage the development of even simpler assays that can be performed in most clinical laboratories.

In Section III, Dr. James George provides an update on the long-term prospective study of thrombotic microangiopathy in the Oklahoma TTP-HUS Registry. At presentation, the clinical distinction between idiopathic TTP, various forms of secondary thrombotic microangiopathy, and even Shiga toxin–associated hemolytic uremic syndrome (HUS) can be problematic because the symptoms and laboratory findings often overlap. Consequently, plasma exchange usually is administered to any patient with thrombotic microangiopathy if there is doubt about the cause. The role of ADAMTS13 testing in choosing therapy remains uncertain, but the results do appear to have prognostic significance. Severe ADAMTS13 deficiency is specific for idiopathic TTP and identifies a subgroup with a high likelihood of response to plasma exchange, and high-titer ADAMTS13 inhibitors correlate strongly with a high risk of relapsing disease. Patients with normal ADAMTS13 activity have a much worse prognosis, although many factors probably contribute to this difference. Longitudinal study of these patients will continue to clarify the relationship of ADAMTS13 deficiency to the clinical course of thrombotic microangiopathy.

I. Idiopathic Thrombotic Thrombocytopenia Purpura

*Joel L. Moake, MD**

Clinical Presentations of TTP

Thrombotic thrombocytopenia purpura (TTP) is a severe microvascular occlusive "thrombotic microangiopathy" characterized by systemic platelet aggregation, organ ischemia, profound thrombocytopenia (with increased marrow megakaryocytes), and fragmentation of erythrocytes.[1] The RBC fragmentation occurs, presumably, as blood flows through turbulent areas of the microcirculation partially occluded by platelet aggregates. Schistocytes, or "split" red cells, appear on the peripheral blood smear (> 1% of total RBCs) as an indication of "microangiopathic hemolytic anemia." Serum levels of lactate dehydrogenase (LDH) are extremely elevated as a consequence of hemolysis and the leakage of LDH from ischemic or necrotic tissue cells.

The systemic platelet clumping in TTP is often associated with platelets below 20,000/μL. Occlusive ischemia of the brain or the gastrointestinal tract is common, and renal dysfunction may occur. A "pentad" of signs and symptoms was long associated with TTP: thrombocytopenia; microangiopathic hemolytic anemia; neurologic abnormalities; renal failure; and fever. In current clinical practice, thrombocytopenia, schistocytosis, and an impressively elevated serum LDH value are sufficient to suggest the diagnosis.[1] Clotting studies are usually normal.

A clinically similar thrombotic microangiopathy can occur weeks to months after exposure to mitomycin C; inhibitors of the Ca^{2+}-activated phosphatase, calcineurin (cyclosporine or tacrolimus [FK 506]); quinine; combinations of chemotherapeutic agents; total-body irradiation; or allogeneic bone marrow, kidney, liver, heart, or lung transplant.[1] The microvascular thrombi may be either predominantly renal or systemic in these heterogenous entities. Other thrombotic microangiopathies include the hemolytic-uremic syndrome (HUS), which can be acquired by ingestion of enterohemorrhagic bacteria that produce Shiga toxin (e.g., *Escherichia coli* of serotype 0157:H7).[1] Familial types of HUS are caused by a regulatory protein defect in the alternative complement pathway (plasma factor H or membrane-cofactor protein [CD46]) or defective intracellular cobalamin reduction/cofactor function. The discussion to follow is limited to TTP associated with deficient plasma von Willebrand factor (VWF)–cleaving metalloprotease (ADAMTS13) **(Table 1)**.

Familial TTP is rare, usually (but not always) appears initially in infancy or childhood, and may recur as "chronic relapsing TTP" episodes at about 3-week intervals.[1-3] Acquired idiopathic ("out-of-the-blue") TTP has become a commonly recognized disorder that occurs in adults and older children[1,2] and, following successful treatment, recurs at irregular intervals in 11%–36% of patients. A small fraction of patients treated for arterial thrombosis with the platelet adenosine diphosphate receptor-inhibiting thienopyridine drugs, ticlopidine (Ticlid) or clopidogrel (Plavix) develop TTP within a few weeks after the initiation or therapy.[1] TTP occurs occasionally late in pregnancy or immediately after delivery.

* Baylor College of Medicine, Methodist Hospital, 6565 Fannin St., MS 902 Main, Houston TX 77030

Table 1. ADAMTS13 deficiency and thrombotic thrombocytopenia purpura (TTP).

ADAMTS13 Plasma Activity Absent*	Clinical Presentation
ADAMTS13 mutations	Familial TTP; chronic relapsing TTP
Disease presentation in infancy/childhood	
Disease presentation delayed	
Autoantibodies against ADAMTS13	Acquired idiopathic TTP
Transient	Single episode TTP
Recurrent	Recurrent (intermittent) TTP
Thienopyridine-associated	Ticlopidine/clopidogrel-TTP
ADAMTS13 transient production or survival (?) defect	Acquired idiopathic TTP (?)
Pregnancy+	Pregnancy-associated TTP

* < 5% of normal
+ Autoantibodies may also be present

The ADAMTS13-Deficient Types of TTP

In 1982, "unusually large" (UL) VWF multimers found in plasma samples taken repeatedly from 4 patients with chronic relapsing TTP were proposed as the systemic agglutinating agents.[2] The patients described were believed to have defective ULVWF multimeric "processing."[2] Convincing evidence for ULVWF multimers

as the cause of platelet clumping in TTP has accumulated in the subsequent 22 years.[1]

Brief comments on VWF biochemistry and physiology are included here for orientation. Monomers of VWF (280,000 daltons) are linked by disulfide bonds into multimers with varying molecular masses that range into the millions of daltons. Multimers of VWF are constructed within megakaryocytes and endothelial cells, and stored within platelet α-granules and endothelial cell Weibel-Palade bodies. Most plasma VWF multimers are derived from endothelial cells. Both endothelial cells and platelets produce VWF multimers larger than the multimers in normal plasma. These ULVWF multimers bind more efficiently than the largest plasma VWF multimers to the glycoprotein (GP) Ibα components of platelet GPIb-IX-V receptors.[4,5] The initial attachment of ULVWF multimers to GPIbα receptors, and subsequently to activated platelet GPIIb-IIIa complexes, induces platelet adhesion and aggregation in vitro in the presence of elevated levels of fluid shear stress.[4] After retrograde secretion by endothelial cells, ULVWF multimers become entangled in subendothelial collagen, thereby maximizing the VWF-mediated adhesion of blood platelets to any subendothelium exposed by vascular damage and endothelial cell desquamation. An efficient "processing activity"[2,6] in normal plasma prevents the highly adhesive ULVWF multimers that are also secreted antegrade into the vessel lumen from persisting in the bloodstream.

The ULVWF "processing activity" is now known to be a specific VWF-cleaving metalloprotease present in normal plasma.[3,7,8] The enzyme degrades ULVWF multimers by cleaving 842Tyr-843Met peptide bonds in susceptible A2 domains of VWF monomeric subunits. The VWF-cleaving metalloprotease is number 13 in a family of 19 distinct ADAMTS-type enzymes identified to date. The ADAMTS enzymes are numbered 1–20, but ADAMTS5 and ADAMTS11 have been found to be identical. ADAMTS13 is *a* *d*isintegrin *a*nd *m*etalloprotease with eight *t*hrombo*s*pondin-1-like domains composed **(Figure 1)** of an amino-terminal reprolysin-type metalloprotease domain followed by a disintegrin domain; a thrombospondin-1-like domain; a cysteine-rich domain containing an arginine-glycine-aspartate (RGD) sequence and an adjacent spacer portion; 7 additional thrombospondin-1-like domains; and 2 similar CUB domains at the carboxyl-terminal end of the molecule. CUB domains, found only in ADAMTS13 among the ADAMTS enzymes, contain peptide sequences present in *C*omplement subcomponents C1r/C1s; embryonic sea *U*rchin protein egf; and *B*one morphogenic protein-1. ADAMTS13 is a Zn^{2+}- and Ca^{2+}-requiring 190,000 dalton glycosylated protein that is encoded on chromosome 9q34 and produced predominantly in the liver. ADAMTS13 activity is inhibited in vitro by ethylenediaminetetraacetic acid (EDTA) and, therefore, functional assays of the enzyme are usually performed using plasma anticoagulated with citrate.[3,7-11] Plasma anticoagulated with heparin, D-phenylalanylprolylarginyl chloromethyl ketone (PPACK), hirudin and other direct thrombin inhibitors, or even serum, may also be satisfactory for testing.

ULVWF multimers are cleaved by ADAMTS13 as they are secreted in long "strings" from stimulated endothelial cells[12,13] **(Figure 2A)**. The ULVWF multimeric strings may be anchored in the endothelial cell membrane to P-selectin molecules that are secreted concurrently with the ULVWF multimers from Weibel-Palade bodies.[14] Endothelial cells are stimulated to secrete ULVWF multimers by histamine, Shiga toxin, tumor necrosis factor-alpha (TNFα), interleukin (IL)-8 and IL-6 in complex with IL-6 receptor.[15] CUB domains, as well as one or more of the thrombospondin-1-like domains, may be involved in binding the ADAMTS13 to ULVWF multimers as they are secreted by the endothelial cells. Specifically, ADAMTS13 enzymes may attach under flowing conditions to accessible A3 domains in the monomeric subunits of ULVWF multimers,[13] and then cleave Tyr 842-843 Met peptide bonds in adjacent A2 domains (**Figure 2B**). Partial unfolding of emerging ULVWF multimers by fluid shear stress may increase the efficiency of ADAMTS13 attachment to, or cleavage of, ULVWF multimers.[12]

Failure to degrade ULVWF multimers has long been suspected to cause the familial and acquired idiopathic types of

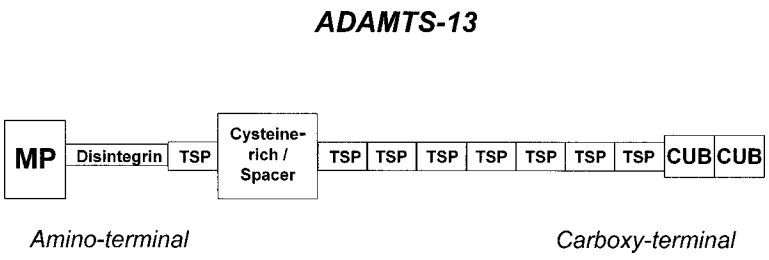
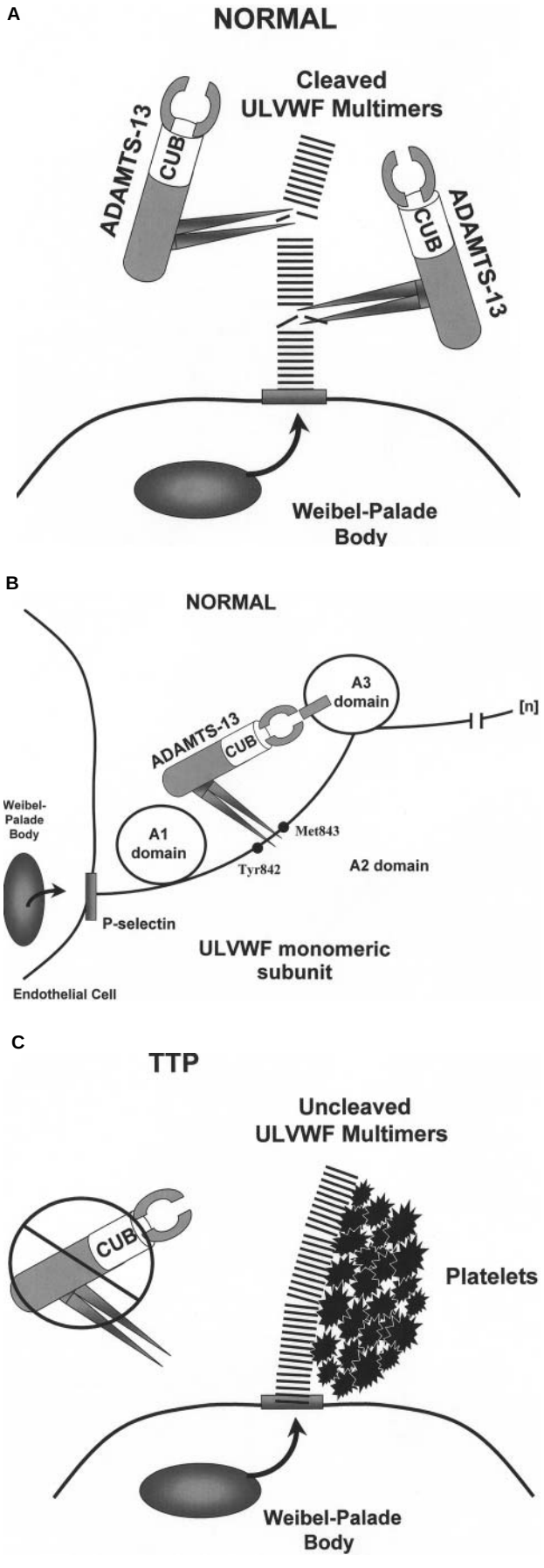


Figure 1. Domain structure of the plasma VWF-cleaving metalloprotease, ADAMTS13.

Abbreviations: MP, metalloprotease (proteolytic) domain; TSP, thrombospondin-1-like domain (a total of 8 are present); CUB, two similar domains containing peptide sequences found in the *c*omplement components, C1r/C1s, a sea *u*rchin protein, and a *b*one morphogenic protein.



TTP or predispose an individual to these disorders[1,2] (**Figure 2C**). Critical experiments verifying this concept were reported in 1997–1998. In 1997, Furlan et al[3] described 4 patients with chronic relapsing TTP who had a deficiency of VWF-cleaving protease activity in plasma. Because no inhibitor of the enzyme was detected, the deficiency was ascribed to an abnormality in the production, survival or function of the protease. The following year, elegant papers by Furlan et al[7] and Tsai and Lian[8] elucidated the pathogenesis of the more common acquired idiopathic type of TTP. The acquired idiopathic patients had little if any plasma VWF-cleaving protease activity during acute episodes; however, the activity returned to normal upon recovery. An IgG autoantibody produced transiently against the enzyme accounted for the lack of protease activity during the acquired idiopathic TTP episodes.[8]

Patients with familial, chronic relapsing TTP almost always have ULVWF multimers in their plasma[1,2] because they chronically have less than about 5% of normal plasma ADAMTS13—unless they have recently received plasma infusions. ULVWF multimers are also detectable in some patient plasma samples during acute episodes of acquired idiopathic TTP, but not after recovery,[1] due to transient inhibition of ADAMTS13 to less than about 5% of normal only during acute TTP episodes.[1,3,7,8,16] Severe deficiency of ADAMTS13 activity in TTP patient plasma often correlates with a failure to cleave ULVWF multimers as they emerge in

Figure 2. ADAMTS13 activity in normal and thrombotic thrombocytopenia purpura (TTP) plasma.

(A) In normal individuals, ADAMTS13 enzyme molecules from the plasma attach to, and then cleave, unusually large von Willebrand factor (ULVWF) multimers that are secreted in long "strings" from stimulated endothelial cells.

(B) The ULVWF multimeric strings may be anchored in the endothelial cell membrane to P-selectin molecules that are secreted concurrently with the ULVWF multimers from Weibel-Palade bodies. P-selectin molecules are retained in the endothelial cell membrane by a transmembrane portion. Each ADAMTS13 molecule may dock via one or both of its C-terminal CUB domains, possibly along with one or more thrombospondin-1-like domains, to exposed A3 domains in ULVWF monomeric subunits. The attached ADAMTS13 molecules then cleave Tyr 842-843 Met peptide bonds in the adjacent A2 domains of ULVWF monomeric subunits. The smaller VWF forms that circulate after cleavage do not induce the adhesion and aggregation of platelets during normal blood flow.

(C) Absent or severely reduced activity of ADAMTS13 in patients with TTP prevents the timely cleavage of ULVWF multimers secreted by endothelial cells. Uncleaved ULVWF multimers induce the adhesion and subsequent aggregation of platelets in flowing blood. Congenital deficiencies of ADAMTS13 activity caused by gene mutations or acquired defects of ADAMTS13 caused by autoantibodies result in TTP.

long strings from the surface of stimulated endothelial cells[12] **(Figure 2C)**.

ULVWF multimeric strings are anchored to the endothelial cells[12,13] via P-selectin molecules, which have transmembrane domains and are secreted along with ULVWF multimers from Weibel-Palade bodies.[14] P-selectin molecules are predominantly retained in the endothelial cell membrane. Passing platelets adhere via their GPIbα receptors to the long ULVWF multimeric strings anchored to P-selectin.[12] (Platelets do not adhere to the smaller VWF forms that circulate after cleavage of ULVWF multimers, perhaps because the binding site for platelet GPIbα in VWF A1 domains is not exposed on smaller VWF forms.[5]) Many additional platelets subsequently aggregate under flowing conditions, probably via their activated IIb-IIIa complexes, onto the ULVWF multimeric strings to form large, potentially occlusive, platelet thrombi.

ULVWF multimeric strings are capable of detaching from endothelial cells in the absence of ADAMTS-13 activity, the presence of fluid shear stress, and the increasing torque generated by ULVWF-platelet adherence.[12,13] The detached ULVWF-platelet strings may "embolize" to microvessels downstream and contribute to organ ischemia. The formation of ULVWF-platelet thrombi and emboli may account for the detection of ULVWF multimers in the plasma of familial TTP patients chronically and of acquired idiopathic TTP patients during acute episodes.[1,2,6] Increased VWF antigen has been found by flow cytometry on platelets during episodes of familial or acquired TTP, and abundant VWF antigen (but not fibrinogen) has been observed by immunohistochemistry on platelet occlusive lesions in TTP.[1]

The absent or severely reduced plasma ADAMTS13 activity in familial TTP[1,3,7] is usually a consequence of homozygous (or double heterozygous) mutations in both of the ADAMTS13 alleles located at chromosome 9q34.[10] Mutations in familial TTP have been detected all along the gene, in regions encoding different domains.[10] In severe familial deficiency of ADAMTS13 activity, episodes of TTP usually commence in infancy or childhood **(Table 1)**. In others, however, overt TTP episodes do not develop for years (e.g., during a first pregnancy), if ever. These latter observations suggest that (1) in vivo ADAMTS13 activity on ULVWF multimers emerging from stimulated endothelial cells may exceed the estimates of plasma enzyme activity using in vitro non-physiologic assays, and/or (2) that accentuated secretion of ULVWF multimers by endothelial cells induced by estrogen or pro-inflammatory cytokines[15] is required to provoke TTP episodes in some patients with severe plasma ADAMTS13 deficiency.

Many patients with acquired idiopathic TTP also usually have absent or severely reduced plasma ADAMTS13 activity only during an initial episode or later recurrence[1,7,8] **(Table 1)**. IgG autoantibodies that inhibit plasma ADAMTS13 activity during acquired episodes can be detected using the nonphysiologic techniques currently available in 44%–94% of patients,[1,7,8] suggesting the presence of a transient, or intermittently recurrent, defect of immune regulation. Antibodies that inhibit plasma ADAMTS13 have also been demonstrated in a few patients with ticlopidine- or clopidogrel-associated TTP.[1] It is not known if there is a transient, severe defect of metalloprotease production or survival in patients with acquired idiopathic TTP who do not have detectable autoantibodies against ADAMTS13, or if the results are explained by the limited test sensitivity.

In one recent study[17] of ADAMTS-13 autoantibodies in 25 acquired TTP patients, the epitope targets *always included the cysteine-rich/spacer domain* (100% of autoantibodies), and were *exclusively* directed against the cysteine-rich/spacer domain in 3 of the 25 autoantibodies. The other 22 autoantibodies reacted with the cysteine-rich/spacer domain plus either the CUB domains (64%), the metalloprotease/disintegrin-like/1st thrombospondin-1-like domain combination (56%), or the 2nd–8th thrombospondin-1-like domain combination (28%). The propeptide region was also identified by 20% of autoantibodies,[17] indicating that propeptide removal is not required for secretion of active ADAMTS13. The recent demonstration of acquired idiopathic TTP caused by IgG autoantibodies against ADAMTS13 in identical twin sisters emphasizes that production of these autoantibodies is genetically determined.[18]

Plasma ADAMTS13 activity in healthy adults ranges from about 50% to 178%. Activity is often reduced below normal in liver disease, disseminated malignancies, chronic metabolic and inflammatory conditions, pregnancy, and newborns.[19] With the exception of those peri-partum women who develop overt TTP, the ADAMTS13 activity in these conditions is not reduced to the extremely low values (< 5% of normal) found in most patients with familial or acquired idiopathic TTP.

Neither bone marrow transplantation-associated thrombotic microangiopathy nor "classical" HUS is usually associated with absent or severely reduced plasma ADAMTS13 activity.[1,7] The explanation for VWF abnormalities in the plasma of a few chemotherapy/transplant-associated thrombotic microangiopathy patients is not known.

Therapy

In 1977, Byrnes and Khurana[20] reported that relapses of TTP could be prevented or reversed by the infusion

of only a few units of fresh-frozen plasma or its cryoprecipitate-poor fraction (cryosupernatant), without concurrent plasmapheresis. It was shown in 1985 that the processing of ULVWF multimers was restored in patients with familial, chronic relapsing TTP by transfusing fresh-frozen plasma or cryosupernatant.[6] Tsai and Lian[8] and Furlan et al[7] demonstrated in 1998 that these plasma products, as well as solvent/detergent-treated plasma,[1] contain active ADAMTS13.

The infusion about every 3 weeks of normal ADAMTS13 into familial TTP patients producing defective ADAMTS13 molecules[3,10] is sufficient to prevent TTP episodes. The plasma $t_{1/2}$ of infused ADAMTS13 activity is about 2 days, so perhaps ADAMTS13 molecules dock and cleave one secreted ULVWF multimeric string after another over a longer time period.[1,12,13]

The sequence of ADAMTS13 has been determined, and it has been partially purified[1,9] and produced in recombinant active form[21] for ultimate therapeutic use. Because plasma ADAMTS13 of only about 5% is often sufficient to prevent or truncate TTP episodes,[1,16] gene therapy may eventually extend remissions in familial, chronic relapsing TTP.

Adults and older children with acquired idiopathic TTP episodes associated with ADAMTS13 deficiency require daily plasma exchange. This combines plasmapheresis (which may remove circulating ULVWF multimer-platelet strings, agents that stimulate endothelial cells to secrete ULVWF multimers, and autoantibodies against ADAMTS13) and the infusion of fresh-frozen plasma or cryosupernatant (containing uninhibited ADAMTS13). Both solvent-detergent–treated plasma[1,8] and methylene blue/light-treated plasma (for inactivation of lipid envelope viruses) also contain active ADAMTS13; however, protein S activity is below normal in solvent-detergent plasma.

Plasma exchange allows about 80%–90% of acquired TTP patients to survive an episode, usually without persistent overt organ damage. Lower titers of ADAMTS13 autoantibodies are associated with better responses to plasma exchange procedures. Production of ADAMTS13 autoantibodies may be suppressed by high-dose glucocorticoids, 4–8 weekly doses of rituximab (monoclonal antibody against CD20 on B-lymphocytes) or removal of autoantibody-producing cells by splenectomy.

II. New ADAMTS13 Assays and Clinical Applications

Toshiyuki Miyata, PhD, and Koichi Kokame, PhD*

Thrombotic thrombocytopenic purpura is typically characterized by thrombocytopenia and microangiopathic hemolytic anemia, with variable degrees of renal failure, neurological dysfunction, and fever. In most cases the underlying mechanism of idiopathic TTP is explained by the accumulation of ULVWF multimers in the plasma, leading to the formation of platelet thrombi in microvessels. In turn, the accumulation of ULVWF multimers is caused by congenital or acquired deficiency of a metalloprotease, ADAMTS13. By 2001, this enzyme had been purified and cloned by several groups.[1] Since then, our understanding of TTP and ADAMTS13 has greatly increased.

von Willebrand factor and ADAMTS13

VWF is synthesized primarily by vascular endothelial cells. In the endoplasmic reticulum, VWF dimer formation occurs through disulfide bond formation near the C-termini. The pro-VWF dimers transit to the Golgi apparatus, where the pro-sequence is cleaved and multimers form by N-terminal disulfide bond formation. ULVWF multimers, disulfide-bonded at both N- and C-terminal domains, are formed in their mature form and stored in Weibel-Palade bodies or secreted into the plasma. ULVWF multimers are highly active in collagen binding and platelet aggregation. Under normal physiological conditions, ULVWF multimers depolymerize into smaller multimers ranging in size from 500 to 20,000 kDa. Depolymerization is catalyzed by a plasma metalloprotease, ADAMTS13, which specifically cleaves the peptide bond between Y1605 and M1606 in the A2 domain of VWF. Functional deficiency of ADAMTS13, caused by genetic mutation, inhibitory autoantibodies, or other etiologies, leads to the accumulation of ULVWF multimers in plasma. These multimers promote microvascular thrombi of platelets that result in platelet consumption and hemolysis. Once microvascular thrombi are formed in the brain or kidney, patients may suffer neurological dysfunction or renal failure.

* National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita 565-8565, Japan

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ADAMTS13 has a multidomain structure like other ADAMTS proteases and its characteristics have been described in Section I. The ADAMTS13 precursor consists of 1427 amino acids and contains a signal peptide, a short propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type I repeat (TSP1), a Cys-rich domain, a spacer domain, 7 additional TSP1 repeats, and 2 CUB domains.[1] The metalloprotease domain of ADAMTS13 is necessary but not sufficient for VWF cleavage, which appears to require other domains as well. A mutant with a common single nucleotide polymorphism leading to the P475S substitution in the Cys-rich domain, found in Japanese populations with an allelic frequency of about 5%, showed reduced VWF cleavage, indicating a significant role for the Cys-rich domain.[2] The analysis of sequentially truncated C-terminal mutants suggests that both the Cys-rich and spacer domains are indispensable for VWF-cleaving activity.[3,4] In addition, epitope analysis of ADAMTS13 autoantibodies in acquired TTP has shown that the Cys-rich and spacer domains usually are involved in antibody reactivity.[4,5] Taken together, the Cys-rich and spacer domains seem to be essential for the VWF-cleaving activity of ADAMTS13. Although removal of the propeptide sequence in ADAM family members is usually essential for their enzymatic activity, pro-ADAMTS13 exhibited normal proteolytic activity toward VWF, indicating that propeptide removal is not required for the activity of ADAMTS13.[6]

Assay Methods for ADAMTS13

As of 2002, five main assays for ADAMTS13 activity were in use to study ADAMTS13 activity in thrombotic microangiopathies and other pathophysiological conditions. However, more accurate and simpler methods were needed. This year, we developed a more convenient ADAMTS13 substrate based on the amino acid sequence of the A2 domain of VWF. In this section, I will describe conventional assay methods using multimeric VWF and then introduce our novel method, which uses a recombinant monomeric protein as the substrate. The assay methods for ADAMTS13 are summarized in **Table 2**.

The first assay method was developed by Furlan et al.[7] The substrate for this method was protease-free plasma VWF. The plasma samples are first diluted, then activated by barium chloride, mixed with substrate and dialyzed overnight against low ionic strength buffer, pH 8.0, containing 1.5 M urea. The reaction products were separated by sodium dodecyl sulfate (SDS)–1.4% agarose gel electrophoresis followed by immunoblotting. The resolution of the ladders of the degraded VWF multimers was excellent and reproducible, but the method required several days to complete.

Table 2. A comparison of ADAMTS13 assay methods.

Authors	Substrate	Denaturant	Incubation Time	Detection Method	Indication of VWF Proteolysis	Ref.
Furlan et al	purified VWF	1.5 mol/L urea	overnight	SDS-agarose gel electrophoresis, immunoblotting	decreased multimer size	7
Tsai	purified VWF	0.15 mol/L guanidine HCl	1 hour	SDS-polyacrylamide gel electrophoresis, immunoblotting	generation of dimer of 176 kDa fragment	8
Obert et al	recombinant VWF	1.5 mol/L urea	overnight	two-site immuno-radiometric assay	decreased VWF antigen	9
Gerritsen et al	VWF in EDTA-treated and dialyzed plasma	1.5 mol/L urea	2 hour	residual collagen binding	decreased collagen binding	10
Böhm et al	purified VWF	1.5 mol/L urea	overnight	residual ristocetin cofactor activity	decrease of ristocetin induced platelet aggregation	11
Kokame et al	recombinant VWF fragment, VWF73	none	20–60 min	SDS-polyacrylamide gel electrophoresis, immunoblotting	generation of proteolytic fragment	13
Whitelock et al	VWF A2 domain	none	2 hour	enzyme linked immunosorbent assay	decrease of intact A2 domain	14
Zhou and Tsai	recombinant VWF fragment, VWF73	none	3 hour	enzyme immunoassay	decrease of VWF73	16

Abbreviations: VWF, von Willebrand factor

The second method was reported by Tsai.[8] Plasma samples were incubated with guanidine HCl-treated protease-free VWF for one hour. Next, the products were separated by SDS–polyacrylamide gel electrophoresis, followed by immunoblotting for dimers of C-terminal 176-kDa fragments of VWF. An advantage of this method is that dimer formation directly reflects the cleavage of the scissile bonds.

Obert et al reported an elegant and high-throughput method,[9] in which the plasma samples were incubated with recombinant VWF overnight and the degraded VWF fragments were detected by two-site immunoradiometric assay in microtiter plates. This test can be performed in the setting of a hospital laboratory and the cumbersome immunoblot technique is not required. In 102 healthy volunteers, the range (± 2 SD) of ADAMTS13 activity was 50%–178%, where the mean was 114%.

Gerritsen et al reported a functional assay based on the preferential binding of high molecular weight forms of VWF to collagen.[10] Plasma that was treated with EDTA and dialyzed against the buffer was used as substrate. Since EDTA is a chelator of divalent cations, EDTA treatment of plasma abolishes the VWF-cleaving activity. Depolymerized forms of VWF showed impaired binding to microtiter plates coated with collagen type III, and collagen-bound VWF was quantified using a specific anti-VWF antibody. In 177 healthy volunteers, ADAMTS13 activity was widely distributed, ranging from 40% to 170%.

Böhm et al used the ristocetin-induced platelet aggregation activity to quantitate the residual VWF multimers for their ADAMTS13 assay.[11] This assay is based on the positive correlation between VWF multimer size and ristocetin cofactor activity of platelet aggregation. The method requires no special laboratory equipment or expertise. In 80 healthy volunteers, the ADAMTS13 activity ranged from 52% to 134%.

To evaluate the various assays for ADAMTS13 activity, a pilot multicenter comparison of the four different assays—the quantitative immunoblotting of VWF (Furlan's method), the two-site immunoradiometric assay (Obert's method), the residual collagen binding assay (Gerritsen's method), and the residual ristocetin cofactor assay (Böhm's method)—has been performed.[12] The test consisted of 30 plasma samples from patients with hereditary and acquired TTP and other conditions associated with ADAMTS13 levels ranging from < 3% to > 100%. All assays identified plasma with severe ADAMTS13 deficiency (< 5%), notwithstanding some exceptions observed using the collagen-binding assay, suggesting that all were useful to screen for suspected TTP. For samples with normal to moderately reduced activity, results were less concordant.

Specific Minimum Recombinant Substrate for ADAMTS13 Activity

Coagulation proteases such as thrombin or factor Xa that are classified as "serine proteases" have a restricted substrate specificity recognizing short amino acid sequences on the N-terminal side of the scissile bonds. Based on these sequences, peptidyl substrates specific for many of these proteases were developed and utilized for activity assays. ADAMTS13 is a metalloprotease rather than a serine protease, and it cleaves a peptide bond between Y1605 and M1606 in the A2 domain of VWF. The development of peptidyl substrates for ADAMTS13 has been attempted but not achieved, suggesting that cleavage depends not only on specific residues in the close vicinity of the scissile bond, but also on more remote sequences in VWF.

We utilized a recombinant protein approach to develop an ADAMTS13 substrate, using the amino acid sequence of the A2 domain of VWF that contains the scissile bond.[13] To identify the minimal region of VWF required for cleavage by ADAMTS13, we prepared 5 recombinant proteins, each containing a region of the A2 domain of VWF (**Figure 3A**). Two criteria were set: (1) the region must contain the cleavage site by ADAMTS13, and (2) it must not contain Cys residues that often interfere with the proper folding of recombinant proteins. The longest region that meets the criteria was D1459 to R1668 of VWF. For purification and detection, the protein was flanked with N-terminal glutathione-*S*-transferase (GST) and C-terminal 6xHis (H) tags. This was designated GST-D1459R1668-H (substrate A). We also prepared 4 shorter recombinant proteins (substrates B-E), as shown in **Figure 3A**.

Five recombinant proteins were expressed in *E coli* and purified by affinity chromatography. The purified proteins were incubated with plasma for 1 hour at 37°C and the reaction mixture was electrophoresed on an SDS-polyacrylamide gel. The cleaved products were then detected by Western blot using anti-GST antibodies. Four out of 5 recombinant proteins were successfully cleaved with plasma ADAMTS13; however, substrate E (the shortest) was not (**Figure 3B**). Therefore, the minimum substrate for ADAMTS13 was substrate D, which contains the region from D1596 to R1668 (73 amino acid residues). We designated substrates D and E as GST-VWF73-H and GST-VWF64-H, respectively. The amino acid sequence of substrate D is shown in **Figure 3C**. As described, substrate E was resistant to cleavage. Therefore, the region of nine amino acid residues (EAPDLVLQR) underlined in **Figure 3C** was likely essential for cleavage by ADAMTS13. Mass spec-

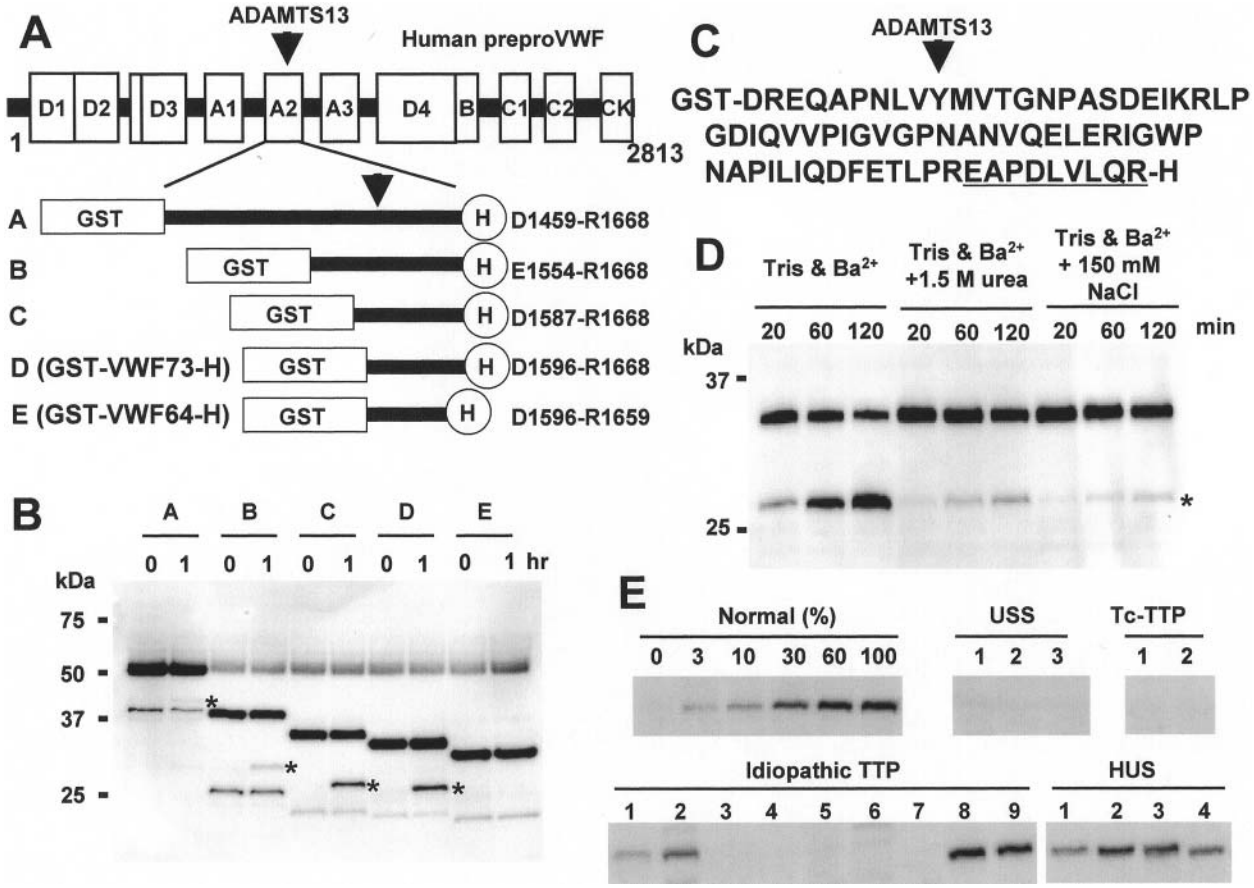

Figure 3. A novel assay for ADAMTS13.

(A) Structures of substrates A–E. The domain structure of human preproVWF is depicted. Five recombinant proteins flanked with GST- and H-tags were expressed for the ADAMTS13 assay. Substrates D and E were designated as GST-VWF73-H and GST-VWF64-H, respectively.

(B) Cleavage of recombinant proteins. The recombinant proteins were incubated with normal plasma at 37°C for 1 hour. The reaction products were run by SDS-polyacrylamide gel electrophoresis and the products were detected by anti-GST antibodies. The product bands are indicated by asterisks. The nonspecific bands of approximately 50 kDa are plasma albumin.

(C) Amino acid sequence of VWF73. Nine amino acid residues indispensable for cleavage are underlined.

(D) Effect of urea and ion concentration on cleavage. GST-VWF73-H was incubated with plasma for the indicated time in reaction buffer (5 mM Tris-HCl, 10 mM BaCl2, pH 8.0) or in the same buffer supplemented with 1.5 M urea or 150 mM NaCl. The products are marked by an asterisk.

(E) Cleavage of GST-VWF73-H by patients' plasma. GST-VWF73-H was incubated with serially diluted normal plasma (0%–100%) or with plasma from patients with Upshaw-Schulman syndrome (USS), ticlopidine-associated thrombotic thrombocytopenic purpura (Tc-TTP), idiopathic TTP, or hemolytic uremic syndrome (HUS), and the products were detected by anti-GST antibodies.

A part of these figures were originally published in *Blood*. Kokame K, Matsumoto M, Fujimura Y, Miyata T. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. Blood. 2004:103:607-612.

trometry analysis of both substrates, VWF73-H and VWF64-H, showed that their estimated molecular weights were exact, indicating no modification. To understand the substrate specificity of ADAMTS13, we determined the solution structure of ^{1}H and ^{15}N double-labeled substrates VWF73-H and VWF64-H by nuclear magnetic resonance (NMR). The results indicated an extended structure for both peptides, suggesting an induced-fit substrate recognition mechanism (unpublished observations).

It was previously reported that ADAMTS13 cleaves VWF in vitro in the presence of urea and low ionic strength conditions. In hypotonic buffer containing 5 mM Tris-HCl and 10 mM $BaCl_2$, GST-VWF73-H was cleaved by plasma in a time-dependent manner (**Figure 3D**). The supplementation of 1.5 M urea or 150

mM NaCl reduced the cleavage, indicating low ADAMTS13 activity under these conditions. GST-VWF73-H was cleaved in a dose-dependent manner with high sensitivity. This assay was able to detect as little as 3% activity (**Figure 3E**).[13] GST-VWF73-H was not cleaved by either plasma from patients with congenital deficiency of ADAMTS13 activity due to *ADAMTS13* mutations (designated as Upshaw-Schulman syndrome [USS]) or plasma from patients with acquired TTP associated with ticlopidine (Tc-TTP). ADAMTS13 activity was observed in 4 patients with idiopathic TTP, but no activity was found in 5 other patients. All patients with hemolytic uremic syndrome (HUS) exhibited activity. Most patients with acquired TTP have autoantibodies that inhibit ADAMTS13 activity in their plasma. GST-VWF73-H was also useful for the detection of autoantibody when normal plasma was incubated with plasma from the patients with acquired TTP.[13]

Another attempt at developing recombinant substrates has been reported in which the *E coli*–expressed A2 domain was used and proved to be a substrate for ADAMTS13 (**Table 2**).[14]

There are several advantages to our method. First, ours is a direct assay for measuring ADAMTS13 product generation, which should provide a more accurate analysis than indirect methods that measure substrate depletion. Second, the bacteria-expressed protein avoids the need to make protease-free VWF. The recombinant protein is simple to prepare: we can produce 1 mg of pure GST-VWF73-H, sufficient for more than 2000 assays, from 100 mL of *E coli* culture in 2 days. Third, the bacterial expression system is easily modified. For example, we have expressed a mouse version of GST-VWF73-H to measure the mouse ADAMTS13 activity.[15] Fourth, having the substrate tagged with two different molecules makes it easy to modify the detection of product. For example, Zhou and Tsai used GST-VWF73-H to develop an ELISA system to measure ADAMTS13 activity in a 96-well format (**Table 2**).[16] Finally, VWF73 will be used as the lead compound to develop more convenient and rational substrates for ADAMTS13 activity assays.

One potential disadvantage of this method is that GST-VWF73-H is not a natural substrate. A recent report showed that ADAMTS13 may recognize the A3 domain of VWF.[17] Therefore, if the defects of the enzyme in patients with TTP affect the ADAMTS13 binding site for the A3 domain, cleavage of GST-VWF73-H will not reflect these defects.

Clinical Applications

TTP is a life-threatening disease. If untreated, mortality may exceed 90%, but plasma exchange therapy significantly reduces mortality. Therefore, measurement of ADAMTS13 activity could be useful for early diagnosis, which is essential for successful treatment of an acute episode. For example, in July 2003, a Japanese pharmaceutical company began ADAMTS13 monitoring, using GST-VWF73-H, for the early diagnosis of patients with suspected TTP associated with ticlopidine. This approach may be effective in preventing the morbidity caused by drug-associated TTP. In addition, periodical ADAMTS13 measurements may be important for monitoring the therapeutic efficacy of plasma exchange or plasma infusion.

Low levels of ADAMTS13 activity have been observed occasionally in several conditions, including heparin-induced thrombocytopenia, severe sepsis, and HUS.[18] But ADAMTS13 activity in these conditions is consistently detectable, and severe ADAMTS13 deficiency appears to be specific for TTP.[19,20] Consequently, ADAMTS13 assays may help in the differential diagnosis of thrombocytopenia, especially when accompanied by microangiopathic changes. In particular, the discrimination of TTP from HUS can be an urgent issue for treatment. Therefore, an assay for ADAMTS13 activity with a narrow normal range is needed. We are currently developing more rapid and quantitative methods for determining ADAMTS13 activity. One of our goals is to develop a bed-side assay for ADAMTS13 activity, for the diagnosis of congenital or autoimmune TTP.

III. Clinical Course and Long-Term Outcomes of Thrombotic Thombocytopenic Purpura

*James N. George, MD**

Diagnosis of Thrombotic Thrombocytopenic Purpura

The evaluation of patients with suspected thrombotic thrombocytopenic purpura is difficult because the diagnostic criteria—(1) thrombocytopenia, (2) micro-

* University of Oklahoma Health Sciences Center, Hematology-Oncology Section, PO Box 26901, Oklahoma City OK 73190-0001

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angiopathic hemolytic anemia, and (3) no other clinically apparent etiology—are not specific.[1] The value of ADAMTS13 measurements for establishing the diagnosis of TTP and determining the indication for plasma exchange treatment remains uncertain. Although a severe deficiency of ADAMTS13 activity (< 5%) may be an abnormality that is specific for TTP,[2] the presenting symptoms of patients with severe ADAMTS13 deficiency are often not severe and not distinguishable from many other common illnesses. Patients can have the characteristic presenting features and clinical course of TTP without severe ADAMTS13 deficiency or even with normal ADAMTS13 activity (> 50%),[3] and also patients can have severe ADAMTS deficiency for many years with no illness.[4]

Patients with TTP and severe ADAMTS13 deficiency are heterogeneous, with remarkably variable presenting features (**Table 3**).[3,5] Nine of the 22 patients with severe ADAMTS13 deficiency (< 5%) in the Oklahoma TTP-HUS Registry (**Table 3**) had no neurologic abnormalities, not even mild symptoms such as headache and confusion. Nonspecific symptoms, such as weakness, abdominal pain, nausea, vomiting, and diarrhea, were common presenting complaints. Some patients had had symptoms for 3 weeks before their diagnosis; the median duration of symptoms prior to diagnosis was 6 days. The presenting symptoms of these patients were similar to patients diagnosed with idiopathic TTP-HUS but who did not have severe ADAMTS13 deficiency.[3]

Patients with severe illnesses that can mimic the presenting clinical features of TTP, such as sepsis and preeclampsia, may have low but detectable levels of ADAMTS13.[3,5,6] The clinical importance of less severe ADAMTS13 deficiencies is unknown. Furthermore, because of the long half-life of ADAMTS13 in plasma, estimated to be 2.6 days,[7] transfusion of red cells and platelets before the diagnosis of TTP is considered can increase plasma ADAMTS13 activity.

Initial studies reported that a severe deficiency of ADAMTS13 distinguished syndromes described as TTP from the related syndromes described as HUS.[8] Although this distinction is valid in many patients, overlap of these syndromes can occur. Acute renal failure, the characteristic clinical feature that is often used to designate syndromes as HUS, may rarely occur in adults with acquired severe ADAMTS13 deficiency.[3] Children with congenital TTP caused by mutations of the *ADAMTS13* gene may have severe renal failure.[9,10] Also children with typical HUS caused by *E coli* 0157:H7 infection may rarely have severe ADAMTS13 deficiency.[11]

Clinical Course of Thrombotic Thrombocytopenic Purpura

Although the value of ADAMTS13 measurements for establishing the diagnosis of TTP is uncertain, demonstration of severe ADAMTS13 deficiency and documentation of the inhibitor titer may help to anticipate the clinical course of patients with acquired TTP. Four

Table 3. Presenting features of 22 consecutive patients with severe deficiency (< 5%) of ADAMTS13 activity.*

Age	39 years (range, 19–71)	
Gender	18 (82%) female	
Race	10 (45%) African-American	
Obesity	12 (55%) BMI ≥ 30 kg/m^2	
Presenting symptoms:		
neurologic abnormalities	13	(59%)
nausea, vomiting, diarrhea	6	(27%)
abdominal pain	6	(27%)
weakness	4	(18%)
chest pain	3	(14%)
hematuria	2	(9%)
menorrhagia	1	(5%)
purpura	1	(5%)
flank pain	1	(5%)
Duration of symptoms	6 days (1–21)	
Neurologic abnormalities:		
severe	10	(45%)
mild	3	(14%)
none	9	(41%)
Renal function:		
acute renal failure	1	(5%)
renal insufficiency	8	(36%)
normal	13	(59%)
Platelet count	9000/μL (range, 4000–27,000/μL)	
Hematocrit	21% (range, 13%–30%)	
LDH	1431 U/L (range, 436–3909 U/L)	

* Data are presented on 22 consecutive patients from the Oklahoma TTP-HUS Registry, November 13, 1995-December 31, 2003, who had a severe deficiency of ADAMTS13 activity at the time of their initial presentation. This experience represents an extension of our previously published data.[3] The number of major presenting symptoms exceeds 22 because some patients had multiple major symptoms. The duration of symptoms describes the patient's history of the initial onset of symptoms until diagnosis of TTP-HUS. Severe neurologic abnormalities included coma, stroke, seizure, or fluctuating focal signs; mild neurologic abnormalities included headache, blurred vision, ataxia, or mental status changes with transient confusion. Acute renal failure and renal insufficiency have been previously defined.[3] Lactate dehydrogenase (LDH) values were adjusted to an upper limit of normal value of 200 U/L. Age, duration of symptoms, and laboratory data are median values. Laboratory data are the most abnormal values at the time of diagnosis of TTP-HUS ± 7 days, to account for transient effects of transfusion and potential worsening after diagnosis.

Table 4. Risks for death and relapse in thrombotic thrombocytopenic purpura (TTP) related to ADAMTS13 deficiency.*

	ADAMTS13 Deficient			ADAMTS13 Not Deficient		
Case Series (no. of patients)	**Total No. Patients**	**Death (no.)**	**Relapse (no.)**	**Total No. Patients**	**Death (no.)**	**Relapse (no.)**
Veyradier et al[15] (63)	44	–	10	19	–	0
Mori et al[12] (18)	12	2	–	6	4	–
Raife et al[14] (107)	50	4	–	57	10	–
Zheng et al[13]						
idiopathic (20)	16	3	6	4	0	1
non-idiopathic (17)	0	–	–	17	10	1
Vesely et al[3]						
idiopathic (67)	20	4	7/16	47	7	3/40
non-idiopathic (118)	2	0	1/2	116	48	2/68

* Data from 5 case series describing the occurrence of death and relapse among patients distinguished by severe deficiency or lack of severe deficiency of ADAMTS13 activity.

Data from Veyradier et al[15] presented here are on 63 of 111 patients who were described as having TTP rather than hemolytic uremic syndrome (HUS). Deaths were not reported. Patients were distinguished as "sporadic" or "intermittent." For this table, sporadic was assumed to be a single episode while intermittent was assumed to indicate the occurrence of relapses.

Data from Mori et al[12] presented here are on 18 of 27 patients who were described as having TTP rather than HUS. Relapses were not reported.

Data from Raife et al[14] presented here are on 107 consecutive patients described as having thrombotic microangiopathy; syndromes resembling TTP or HUS were not distinguished. Relapses were not reported.

All 37 patients in the study of Zheng et al[13] were described as having TTP. Patients who were described as idiopathic had no apparent pre-existing illness. Patients described as non-idiopathic had had hematopoietic stem cell transplantation, were pregnant or postpartum, had systemic lupus erythematosis, or had taken FK506, mitomycin C, or clopidogrel.

Data from Vesely et al[3] presented here are on 185 patients who had ADAMTS13 measurements at the time of their initial presentation, representing 90% of the 206 patients enrolled in the Oklahoma TTP-HUS Registry, November 13, 1995-December 31, 2003. Data on 142 of these patients, up to June 30, 2002, have been previously published.[3] Patients defined as idiopathic had no apparent etiology or associated clinical conditions. Patients described as non-idiopathic had had hematopoietic stem cell transplantation, were pregnant or postpartum, had taken a drug associated with TTP-HUS, had a bloody diarrhea prodrome, or had an additional/alternative diagnosis. Deaths are reported if they occurred within 30 days of stopping plasma exchange treatment. Relapses are reported in patients who survived for more than 30 days following their plasma exchange treatment.

case series[3,12-14] have reported lower mortality among patients diagnosed with TTP who had severe ADAMTS13 deficiency (**Table 4**). In the two studies[3,13] that distinguished patients who had idiopathic TTP from patients who had potential etiologies and associated conditions that could have contributed to their illness, the mortality rates were less in the patients with idiopathic TTP. These reports suggested that the high rates of mortality among patients who did not have severe ADAMTS13 deficiency may have been caused by other serious conditions, such as systemic infections. Among the four case series reporting mortality in patients with severe ADAMTS13 deficiency,[3,12-14] overall mortality was 13% (**Table 4**). Among the 3 case series reporting relapses in patients with severe ADAMTS13 deficiency, the frequencies of relapse were 10 of 44 (23%),[15] 6 of 16 (38%),[13] and 7 of 16 patients (44%)[3] (**Table 4**).

Zheng et al[13] have reported a further distinction among patients with severe ADAMTS13 deficiency, between patients who had no demonstrable inhibitor at presentation and those patients who had a high titer of inhibitory activity. In patients with no demonstrable inhibitor, plasma exchange was effective in 8 of 9 patients; none of the 8 patients who recovered have subsequently relapsed. In contrast, 4 patients with severe ADAMTS13 deficiency and a high titer inhibitor had more prolonged courses with more frequent serious complications; all suffered relapses and 2 have died, in spite of additional intensive immunosuppressive treatment.

The demonstration of severe ADAMTS13 deficiency with a high titer inhibitor may be an appropriate indication for intensive immunosuppressive treatment. Current clinical practice is inconsistent regarding the use of glucocorticoids and other immunosuppressive agents.[1] Part of this inconsistency is related to the uncertain initial diagnosis in many patients, and the presumed lack of efficacy of immunosuppressive agents for patients with non-idiopathic TTP. However, even among patients with acquired idiopathic TTP and severe ADAMTS13 deficiency, some respond promptly and completely to short courses of plasma exchange, without additional glucocorticoid treatment.[3,13] Patients who respond quickly and completely with only plasma exchange treatment may have only minimal inhibitor

activity or an inhibitor that is not detectable by current methods. Patients with high-titer inhibitors may require not only glucocorticoids but also more intensive immunosuppressive treatment with agents such as rituximab or cyclophosphamide.

However, the correlation between the clinical course and ADAMTS13 levels is not consistent. Several patients have been described who had prolonged stable hematologic remissions in spite of persistent undetectable ADAMTS13 activity.[13,16] These observations, similar to observations of patients with congenitally absent ADAMTS13 activity who may live many years or even a lifetime without evidence of TTP,[4] demonstrate that severe ADAMTS13 deficiency alone is not sufficient to cause the clinical syndrome of TTP. The suggestions of clinical correlation are also tempered by observations that some assays for ADAMTS13 activity may not be consistent across different laboratories, although a multicenter study documented excellent agreement among most assay techniques.[17] Therefore management decisions should remain based upon the clinical course. Patients who respond promptly and completely to plasma exchange may require no additional treatment. Patients whose platelet count does not increase within several days, or in whom recurrent thrombocytopenia recurs when plasma exchange treatments are diminished or discontinued, will likely benefit from glucocorticoid treatment. Patients who have a more severe course, with more severe neurologic abnormalities, and who either do not respond or exacerbate in spite of continuing plasma exchange and glucocorticoid treatment may benefit from more intensive immunosuppressive treatment.

Risk for Relapse of Thrombotic Thrombocytopenic Purpura

The major concern of patients who achieve remission from TTP is the risk for relapse. Current data suggest that the risk for relapse is almost totally restricted to patients who have severe ADAMTS13 deficiency, and severe ADAMTS13 deficiency is usually, but not always, restricted to patients who have idiopathic TTP. Data from the Oklahoma TTP-HUS Registry are presented in **Table 5**. Relapse has not occurred among survivors who had TTP following stem cell transplantation or that was associated with dose-dependent drug toxicity, or who had a prodrome of bloody diarrhea. Two patients with an immune-mediated drug association have relapsed, but these recurrent episodes were caused by repeated ingestion of quinine. Three women whose initial episode of TTP was associated with pregnancy have relapsed. In 1 woman who had recurrent mid-trimester fetal losses, the diagnoses of both the initial and recurrent episode of TTP were uncertain. One woman had 2 episodes of TTP during the first trimester of each of her first 2 pregnancies, and then a third episode when she was not pregnant; ADAMTS13 ac-

Table 5. Relapses in 301 consecutive patients from the Oklahoma TTP-HUS Registry with a clinical diagnosis of thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP-HUS).*

Clinical Category	Patients (Total No.)	Survivors (No.)	Relapse (No.)	Comments Regarding Patients with Relapses
Stem cell transplantation	23	6	0	
Pregnant/postpartum	25	23	3 (13%)	1 patient, recurrent fetal loss, ? TTP. 1 patient, 2 relapses, 1 not with pregnancy. 1 patient, 4 relapses, none with pregnancy, initial ADAMTS13 < 5%.
Drug-association:				
immune-mediated	19	15	2 (13%)	Recurrent quinine use
dose-dependent	17	14	0	
Bloody diarrhea prodrome	19	13	0	
Additional/alternative diagnosis	79	34	2 (6%)	1 patient, scleroderma, 3 relapses, ADAMTS 13%–40%. 1 patient, HIV infection, 4 relapses, initial ADAMTS13 60%, 5th episode < 3%.
Idiopathic	119	96	19 (20%)	11 patients, 1 relapse; 6 patients, 2 relapses; 2 patients, 4 & 5 relapses ADAMTS13 assay on 12 patients; < 5% at initial or subsequent episode in 11 (92%)

* Data are presented on all 301 consecutive patients in the Oklahoma TTP-HUS Registry, 1989-2003. The total number of patients in each clinical category is presented; definitions of the clinical categories have been previously published.[3] Survival is defined as more than 30 days after the last plasma exchange treatment. Relapse is defined as a diagnosis of TTP-HUS in a patient who has survived more than 30 days following their last plasma exchange.

tivity was not measured. The third woman, whose initial episode of TTP was diagnosed at the time of delivery of her first child, had severe ADAMTS13 deficiency with a low titer inhibitor (**Figure 4**, Patient 7); she had 4 relapses during the following year when she was not pregnant; cholecystectomy and splenectomy were performed following her fourth relapse; she has been asymptomatic since that time and has had 2 subsequent uncomplicated pregnancies.

Two patients who had additional disorders that may have contributed to the development of TTP have had multiple relapses. One woman with scleroderma had 3 relapses; her ADAMTS13 activity was not measured initially but was 40% at the time of her first relapse. One man with characteristic features of TTP was incidentally diagnosed with HIV infection at the time of his initial episode when his ADAMTS13 activity was 60%. He subsequently has had 4 relapses and his ADAMTS13 activity has decreased with each episode; ADAMTS13 activity was undetectable at the time of his fourth relapse.

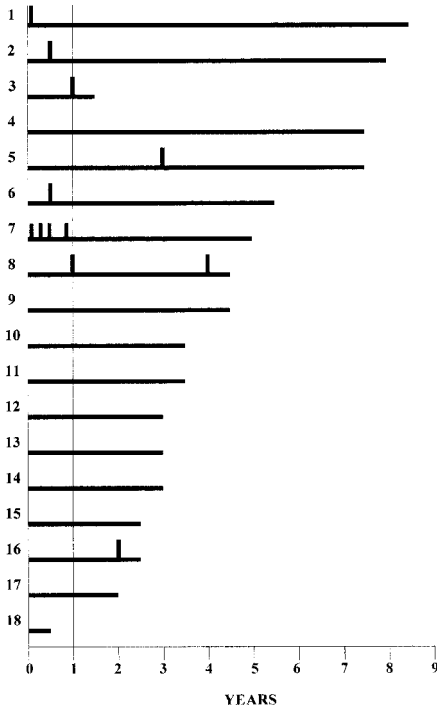

Figure 4. Occurrences of relapses among 18 surviving patients in the Oklahoma TTP-HUS Registry who had severe ADAMTS13 deficiency documented at the time of their initial diagnosis.

Patients are presented consecutively, from patient 1 diagnosed in December 1995 to patient 18 diagnosed in December 2003. During this time 4 patients with severe ADAMTS13 deficiency died during their first episode, before achieving remission and are not presented in this figure. Follow-up is complete to the present time on all patients. Patient 3 died of acute myocardial infarction during her first relapse. Patient 7 had a splenectomy after her fourth relapse. Horizontal bars indicate the duration of follow-up; vertical bars indicate the occurrence of a relapse. Median follow-up duration is 3.3 years; one year of follow-up is indicated by the vertical line.

Among patients with idiopathic TTP, 19 have relapsed (**Table 5**). Twelve of these patients have had ADAMTS13 activity measurements; 11 patients had undetectable activity either at the time of their initial diagnosis or at the time of a subsequent relapse. The exception is a woman with 3 episodes of TTP who had ADAMTS13 measured only at the time of her third episode; ADAMTS13 activity was 100%. Three of these 12 patients had ADAMTS13 activities of 5%–25% at the time of their initial episode and then undetectable ADAMTS13 activity at the time of a relapse. Their initial higher levels of ADAMTS13 activity may have been related to multiple red cell and platelet transfusions given prior to consideration of the initial diagnosis of TTP-HUS.

Figure 4 illustrates the course following recovery from the initial episode of TTP in 18 patients who initially had severe ADAMTS13 deficiency. Four additional patients had severe ADAMTS13 activity but died during their initial episode, 3 from unresponsive disease and 1 from acute hemorrhage related to central venous catheter insertion. Among the 18 survivors, 8 (44%) have had 1 or more relapses; the occurrence of relapse did not appear to be related to the strength of inhibitor activity. Among the patients who have relapsed, 1 (patient 5) had no demonstrable inhibitor, 3 (patients 6, 7, and 8) had trace or mild inhibitory activity, 3 (patients 1, 3, and 16) had moderate inhibitory activity, and 1 patient (patient 2) had strong inhibitory activity.[3] At the time of his relapse, patient 2 was diagnosed with systemic lupus erythematosus and this has been his continuing major problem. Of the 6 patients who had strong inhibitory activity (patients 2, 4, 9, 11, 12, 15), only 1 has relapsed. Although the follow-up of these patients is limited, with a median duration of 3.3 years, these observations suggest that in most patients who will relapse, the initial relapse will occur within the first year (6 of 8 patients). These observations also suggest that in patients who relapse, most will have only 1 relapse (6 of 8 patients). Therefore these observations demonstrate the difficulty of assessing the benefit of any treatment to prevent future relapses. For example, reports of success of splenectomy for preventing future relapses may only be observing the natural history of TTP, with diminishing risk for relapses over time from the initial diagnosis.[18,19]

The observations that essentially all patients who have recurrent episodes of TTP have severe ADAMTS13 deficiency, and that nearly all of these patients have

demonstrable inhibitory activity, suggests that it is appropriate to begin immunosuppressive therapy together with plasma exchange for management of patients with a recurrent episode.

Although almost all recurrent episodes of TTP are immediately recognizable, recent observations suggest the potential for diagnostic difficulty. Three women have been reported who had stroke symptoms following recovery from TTP without accompanying thrombocytopenia; recurrent TTP was considered and symptoms improved with plasma exchange treatment. Subsequently the diagnosis of TTP was supported by finding absent ADAMTS13 activity.[20,21]

Risk for Recurrent Thrombotic Thrombocytopenic Purpura with Subsequent Pregnancies

Since TTP primarily affects women, and since pregnancy appears to be associated with TTP,[22] the risks of a future pregnancy are a common concern among women who have recovered from TTP. Case reports suggest that the risk for recurrent TTP with a subsequent pregnancy is high; among 49 women who have been reported to have 70 subsequent pregnancies, 36 (73%) had recurrent TTP with a pregnancy.[23] However, these reports may be biased by descriptions of unusual patients with complex outcomes.[23] Our experience with 19 women who have had 30 pregnancies following recovery from TTP is more encouraging. Only 5 (26%) women have been diagnosed with recurrent TTP during a subsequent pregnancy, each only during 1 subsequent pregnancy.[23] In 3 of these 5 women, the diagnosis of recurrent TTP was uncertain because of coexisting complications: severe preeclampsia, intrauterine fetal death, and extreme hypertension in a woman with chronic renal failure. This experience emphasizes the difficulty of diagnosing TTP in the presence of other pregnancy-related complications, especially when anticipation is high because of a previous diagnosis of TTP. However, this experience, with complete documentation of all uncomplicated pregnancies, also suggests that recurrent TTP with a subsequent pregnancy is uncommon.

Other Long-Term Outcomes of Thrombotic Thrombocytopenic Purpura

Although the risk for relapse is the major concern of patients who have recovered from TTP, it is not the only concern. Many patients describe persistent cognitive abnormalities for many years following recovery that can be documented by tests of new learning and recent memory.[24] The etiology of these abnormalities is unclear. Perhaps successful treatment with plasma exchange and immunosuppressive therapy will reveal additional long-term sequelae that require further study and perhaps additional supportive care.

References

I. Idiopathic Thrombotic Thrombocytopenia Purpura

1. Moake JL. Thrombotic microangiopathies. N Engl J Med. 2002;347:589-600.
2. Moake JL, Rudy CK, Troll JH, et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med. 1982;307:1432-1435.
3. Furlan M, Robles R, Solenthaler M, et al. Deficient activity of von Willegrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. Blood. 1997;89:3097-3103.
4. Moake JL, Turner NA, Stathopoulos NA, et al. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. J Clin Invest. 1986;78:1456-1461.
5. Arya M, Anvari B, Romo GM, et al. Ultra-large multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet GP Ib-IX complex: studies using optical tweezers. Blood. 2002;99:3971-3977.
6. Moake JL, Byrnes JJ, Troll JH, et al. Effects of fresh-frozen plasma and its cryosupernatant fraction on von Willebrand factor multimeric forms in chronic relapsing thrombotic thrombocytopenic purpura. Blood. 1985;65:1232-1236.
7. Furlan M, Robles R, Galbusera M, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome. N Engl J Med. 1998;339:1578-1584.
8. Tsai HM, Lian EC-Y. Antibodies of von Willebrand factor cleaving protease in acute thrombotic thrombocytopenic purpura. N Engl J Med. 1998;339:1585-1594.
9. Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. Blood. 2001;98:1662-1666.
10. Levy GA, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488-494.
11. Zheng X, Chung C, Takayama TK, et al. Structure of von Willebrand factor cleaving protease (ADAMTS13), a metaloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem. 2001;276:41059-41063.
12. Dong J-f, Moake JL, Nolasco L, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100:4033-4039.
13. Dong J-f, Moake JL, Bernardo A, et al. ADAMTS-13 metalloprotease interacts with the endothelial cell-derived ultra-large von Willebrand factor. J Biol Chem. 2003;278:29633-29639.
14. Padilla A, Moake JL, Bernardo A, et al. P-selectin anchors newly released ultralarge von Willebrand factor multimers to the endothelial cell surface. Blood. 2004;103:2150-2156.
15. Bernardo A, Ball C, Nolasco L, et al. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultra-large von Willebrand factor multimers under

flow. Blood. 2004;104:100-106.
16. Bianchi V, Robles R, Alberio L, et al. Von Willebrand factor-cleaving protease (ADAMTS13) in thrombotic thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. Blood. 2002;100:710-713.
17. Klaus C, Plaimauer B, Studt JD, et al. Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. Blood. 2004;103:4514-4519.
18. Studt JD, Kremer Hovinga JA, Radonic R, et al. Familial acquired thrombotic thrombocytopenic purpura: ADAMTS-13 inhibitory autoantibodies in identical twins. Blood. 2004;103:4195-4197.
19. Mannucci PM, Canciani MT, Forza I, et al. Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. Blood. 2001;98:2730-2735.
20. Byrnes JJ, Khurana M. Treatment of thrombotic thrombocytopenic purpura with plasma. N Engl J Med. 1977;297:1386-1389.
21. Plaimauer B, Zimmermann K, Volkel D, et al. Cloning, expression, and functional characterization of the von Willebrand factor-cleaving protease (ADAMTS13). Blood. 2002;100:3626-3632.

II. New ADAMTS13 Assays and Clinical Applications

1. George JN, Sadler JE, Lämmle B. Platelets: thrombotic thrombocytopenic purpura. Hematology (Am Soc Hematol Educ Program). 2002:315-334.
2. Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in *ADAMTS13* gene responsible for von Willebrand factor-cleaving protease activity. Proc Natl Acad Sci U S A. 2002;99:11902-11907.
3. Zheng X, Nishio K, Majerus EM, Sadler JE. Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. J Biol Chem. 2003;278:30136-30141.
4. Soejima K, Matsumoto M, Kokame K, et al. ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. Blood. 2003;102:3232-3237.
5. Klaus C, Plaimauer B, Studt JD, et al. Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. Blood. 2004;103:4514-4519.
6. Majerus EM, Zheng X, Tuley EA, Sadler JE. Cleavage of the ADAMTS13 propeptide is not required for protease activity. J Biol Chem. 2003;278:46643-46648.
7. Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood. 1996;87:4223-4234.
8. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. Blood. 1996;87:4235-4244.
9. Obert B, Tout H, Veyradier A, Fressinaud E, Meyer D, Girma JP. Estimation of the von Willebrand factor-cleaving protease in plasma using monoclonal antibodies to vWF. Thromb Haemost. 1999;82:1382-1385.
10. Gerritsen HE, Turecek PL, Schwarz HP, Lämmle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF: a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). Thromb Haemost. 1999;82:1386-1389.
11. Böhm M, Vigh T, Scharrer I. Evaluation and clinical application of a new method for measuring activity of von Willebrand factor-cleaving metalloprotease (ADAMTS13). Ann Hematol. 2002;81:430-435.
12. Studt JD, Böhm M, Budde U, Girma JP, Varadi K, Lämmle B. Measurement of von Willebrand factor-cleaving protease (ADAMTS-13) activity in plasma: a multicenter comparison of different assay methods. J Thromb Haemost. 2003;1:1882-1887.
13. Kokame K, Matsumoto M, Fujimura Y, Miyata T. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. Blood. 2004;103:607-612.
14. Whitelock JL, Nolasco L, Bernardo A, Moake J, Dong JF, Cruz MA. ADAMTS-13 activity in plasma is rapidly measured by a new ELISA method that uses recombinant VWF-A2 domain as substrate. J Thromb Haemost. 2004;2:485-491.
15. Banno F, Kaminaka K, Soejima K, Kokame K, Miyata T. Identification of strain-specific variants of mouse *Adamts13* gene encoding von Willebrand factor-cleaving protease. J Biol Chem. 2004;279:30896-30903.
16. Zhou W, Tsai HM. An enzyme immunoassay of ADAMTS13 distinguishes patients with thrombotic thrombocytopenic purpura from normal individuals and carriers of ADAMTS13 mutations. Thromb Haemost. 2004;91:806-811.
17. Dong JF, Moake JL, Bernardo A, et al. ADAMTS-13 metalloprotease interacts with the endothelial cell-derived ultra-large von Willebrand factor. J Biol Chem. 2003;278:29633-29639.
18. Remuzzi G, Galbusera M, Noris M, et al. von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. Blood. 2002;100:778-785.
19. Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. N Engl J Med. 1998;339:1585-1594.
20. Bianchi V, Robles R, Alberio L, Furlan M, Lämmle B. Von Willebrand factor-cleaving protease (ADAMTS13) in thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. Blood. 2002;100:710-713.

III. Clinical Course and Long-Term Outcomes of Thrombotic Thombocytopenic Purpura

1. George JN. How I treat patients with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Blood. 2000;96:1223-1229.
2. Bianchi V, Robles R, Alberio L, Furlan M, Lammle B. Von Willebrand factor-cleaving protease (ADAMTS13) in thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. Blood. 2002;100:710-713.
3. Vesely SK, George JN, Lammle B, et al. ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. Blood. 2003;101:60-68.
4. Furlan M, Lammle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. Best Pract Res Clin Haematol. 2001;14:437-454.
5. George JN, Vesely SK, Terrell DR. The Oklahoma thrombotic thrombocytopenic purpura-hemolytic uremic syndrome (TTP-HUS) registry: a community perspective of patients with clinically diagnosed TTP-HUS. Semin Hematol. 2004;41:60-67.

6. Lattuada A, Rossi E, Calzarossa C, Candolfi R, Mannucci PM. Mild to moderate reduction of a von Willebrand factor cleaving protease (ADAMTS-13) in pregnant women with HELLP microangiopathic syndrome. Haematologia. 2003;88:1029-1034.
7. Barbot J, Costa E, Guerra M, et al. Ten years of prophylactic treatment with fresh-frozen plasma in a child with chronic relapsing thrombotic thrombocytopenic purpura as a result of a congenital deficiency of von Willebrand factor-cleaving protease. Br J Haematol. 2001;113:649-651.
8. Furlan M, Robles R, Galbusera M, et al. Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. New Engl J Med. 1998;339:1578-1584.
9. Veyradier A, Lavergne J-M, Ribba A, et al. Ten candidate ADAMTS13 mutations in six French families with congenital thrombotic thrombocytopenic purpura (Upshaw-Schulman syndrome). J Thromb Haemost. 2004;2:424-429.
10. Matsumoto M, Kokame K, Soejima K, et al. Molecular characterization of *ADAMTS13* gene mutations in Japanese patients with Upshaw-Schulman syndrome. Blood. 2004;103:1305-1310.
11. Hunt BJ, Lämmle B, Nevard CHF, Haycock GB, Furlan M. Von Willebrand factor-cleaving protease in childhood diarrhoea-associated haemolytic uraemic syndrome. Thromb Haemost. 2001;85:975-978.
12. Mori Y, Wada H, Gabazza EC, et al. Predicting response to plasma exchange in patients with thrombotic thrombocytopenic purpura with measurement of vWF-cleaving protease activity. Transfusion. 2002;42:572-580.
13. Zheng XL, Kaufman RM, Goodnough LT, Sadler JE. Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and non-idiopathic thrombotic thrombocytopenic purpura. Blood. 2004;103:4023-4049.
14. Raife TJ, Atkinson B, Montgomery RR, Vesely SK, Friedman K. Severe deficiency of VWF-cleaving protease (ADAMTS13) activity defines a distinct population of thrombotic microangiopathy patients. Transfusion. 2004;44:146-150.
15. Veyradier A, Obert B, Houllier A, Meyer D, Girma JP. Specific von Willebrand factor-cleaving protease in thrombotic microangiopathies: a study of 111 cases. Blood. 2001;98:1765-1772.
16. Studt J-D, Hovinga JK, Radonic R, et al. Familial acquired thrombotic thrombocytopenic purpura: ADAMTS13 inhibitory autoantibodies in identical twins. Blood. 2004;103: In press.
17. Studt J-D, Bohm M, Budde U, et al. Measurement of von Willebrand factor-cleaving protease (ADAMTS13) activity in plasma: a multicenter comparison of different assay methods. J Thromb Haemost. 2003;1:1882-1887.
18. Crowther MA, Heddle N, Hayward CPM, Warkentin T, Kelton JG. Splenectomy done during hematologic remission to prevent relapse in patients with thrombotic thrombocytopenic purpura. Ann Intern Med. 1996;125:294-296.
19. Aqui NA, Stein SH, Konkle BA, Abrams CS, Strobl FJ. Role of splenectomy in patients with refractory or relapsed thrombotic thrombocytopenic purpura. J Clin Apheresis. 2003;18:51-54.
20. Tsai HM, Shulman K. Rituximab induces remission of cerebral ischemia caused by thrombotic thrombocytopenic purpura. Eur J Haematol. 2003;70:183-185.
21. Downes KA, Yomtovian R, Tsai H-M, et al. Relapsed thrombotic thrombocytopenic purpura presenting as an acute cerebrovascular accident. J Clin Apheresis. 2004;19:86-89.
22. George JN. The association of pregnancy with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Curr Opin Hematol. 2003;10:339-344.
23. Vesely SK, Li X, McMinn JR, Terrell DR, George JN. Pregnancy outcomes after recovery from thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Transfusion. 2004;44:1149-1158.
24. Terrell DR, Perdue JJ, Kremer-Hovinga J, et al. Long-term follow-up of 21 patients with thrombotic thrombocytopenic purpura and severe ADAMTS13 deficiency: demonstration of persistant ADAMTS13 deficiency and neurocognitive abnormalities [abstract]. Blood. 2004;104: In press.