ADAMTS13 and anti-ADAMTS13 antibodies as markers for recurrence of acquired thrombotic thrombocytopenic purpura during remission

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ABSTRACT

Background
From 20 to 50% of patients who survive an acute episode of the acquired form of thrombotic thrombocytopenic purpura relapse but clinical and laboratory markers of recurrence are not well established.

Design and Methods
In 109 patients enrolled in an international registry we evaluated, in the frame of a retrospective cohort study, the predictive role of the metalloprotease ADAMTS13 as measured in plasma during remission. Anti-ADAMTS13 antibodies and von Willebrand factor were also evaluated in a smaller number of the same patients.

Results
Median values of ADAMTS13 activity and antigen were significantly lower in patients with recurrent thrombotic thrombocytopenic purpura than in those with no recurrence (activity: 12% *vs.* 41%; p=0.007; antigen: 36% *vs.* 58%; p=0.003). A severe deficiency of ADAMTS13 activity (10% or less) was associated with a higher likelihood of recurrence (odds ratio 2.9; 95% confidence interval 1.3 to 6.8; p=0.01). Anti-ADAMTS13 antibodies were also more prevalent in patients with recurrent thrombotic thrombocytopenic purpura (odds ratio 3.1; 95% confidence interval 1.4 to 7.3; p=0.006). The presence during remission of both severe ADAMTS13 deficiency and anti-ADAMTS13 antibodies increased the likelihood of recurrence 3.6 times (95% confidence interval 1.4 to 9.0; p=0.006). The presence of ultralarge von Willebrand factor multimers and of associated diseases or conditions did not increase recurrence.

Conclusions
Survivors of an acute episode of acquired thrombotic thrombocytopenic purpura with severely reduced levels of ADAMTS13 and/or with anti-ADAMTS13 antibodies during remission have an approximately three-fold greater likelihood of developing another episode of thrombotic thrombocytopenic purpura than patients with higher protease activity and no antibody.

Key words: thrombotic thrombocytopenic purpura, ADAMTS13, von Willebrand factor, risk factors, recurrence.

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Introduction

Acquired thrombotic thrombocytopenic purpura (TTP) is a rare but severe disease characterized by microangiopathic hemolytic anemia and consumptive thrombocytopenia leading to disseminated microvascular thrombosis that causes variable signs and symptoms of organ ischemia and damage.[1] The pathophysiology of TTP has become clearer after it was demonstrated that the plasma metalloprotease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin 1 repeats) cleaves the platelet-adhesive plasma protein von Willebrand factor (VWF) at the peptide bond Tyr1605-Met1606.[2-4] The clinical significance of this proteolysis rests on the property of uncleaved VWF of ultralarge molecular weight (ULVWF) to induce microvascular platelet aggregation under circulatory conditions of high shear stress,[5] and on the observation that TTP is often associated with the congenital or acquired deficiency of the VWF-cleaving protease ADAMTS13.[6-8] Acquired TTP is often due to anti-ADAMTS13 antibodies that inhibit the proteolytic activity of ADAMTS13 and/or bind the protease to accelerate its clearance from plasma.[7-12]

In 20 to 50% of patients who survive the acute initial episode, TTP does recur.[13-17] Recurrence, defined as the re-emergence of clinical and laboratory signs and symptoms of TTP after the remission of the initial acute episode (more frequently within the first year), occurs with a spectrum of presentations ranging from a single relapse to several episodes developing with variable frequency. Predicting which patients are at higher risk of recurrence is currently difficult. Although low plasma levels of ADAMTS13 activity and the presence of anti-ADAMTS13 antibodies were evaluated as predictors of the outcome of acquired TTP during an acute episode (14,18-20), the role of these markers as measured during remission to predict recurrence is uncertain. The need of predictive markers is more cogent during remission, when the patient is clinically stable, than during the acute episode, when most efforts are directed to achieve remission.

We chose to evaluate the predictive role for recurrence of laboratory markers such as ADAMTS13 activity and antigen, anti-ADAMTS13 antibodies (whether or not inhibiting protease activity), VWF antigen and ULVWF multimers as measured in patients with acquired TTP during the first remission and before any recurrence, comparing patients who experienced only a single episode with those who subsequently had at least one recurrence of TTP. The role of other variables, such as age, sex and the presence of associated conditions, was also evaluated.

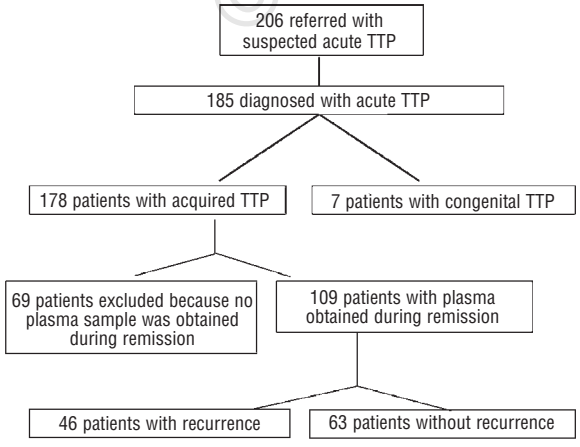

Figure 1. Selection of patients referred to the Hemophilia and Thrombosis Center (from 1994 to 2006) and diagnosed as having thrombotic thrombocytopenic purpura.

Design and Methods

Patients

During the period spanning from 1994 to 2006 clinical centers from eight countries (Italy, Hungary, Iran, Slovenia, Serbia/Montenegro, Greece, Turkey and Russia) had managed patients diagnosed with a first acute episode of TTP. The clinical centers also attempted to collect and store plasma samples, sometimes during the acute phase but more consistently during the period of follow-up after the clinical remission of the first acute episode. Since the year 1999, in the frame of an international registry, the same centers provided the Milan Hemophilia and Thrombosis Center with information on the clinical and laboratory data of these patients, using a specially-tailored collection form (available on request). The original diagnosis of acute TTP was confirmed in Milan when at least three of the following criteria were met: thrombocytopenia with no other apparent cause, Coombs-negative hemolytic anemia with schistocytes in the blood film, high serum levels of lactate dehydrogenase and signs and symptoms compatible with organ ischemia, mainly but not exclusively in the central nervous system.[21] Alternative diseases that present with similar signs and symptoms – such as the enterohemorrhagic form of the hemolytic uremic syndrome, the catastrophic antiphospholipid syndrome, disseminated intravascular coagulation, pre-eclampsia and related syndromes, collagen vascular diseases, metastatic cancer and Evans' syndrome – were excluded using currently accepted clinical and laboratory criteria. The rare atypical form of the hemolytic uremic syndrome cannot be distinguished from TTP using these criteria.

Figure 1 shows how the patients investigated in this study were selected. Of the 206 patients initially diagnosed with acute TTP by the referring clinical centers, 185 were included in the registry because the diagnosis of TTP was confirmed in Milan; 23 of these patients were included in a previous report dealing with patients who had plasma samples collected during the acute phase of TTP.[22] The cohort of the present study (n=109, all of Caucasian ethnicity) is smaller than the total cohort of 185 TTP patients enrolled in the registry because plasma samples could not

be obtained during remission from some patients and also because seven patients with genetically-confirmed congenital TTP were excluded, due the predictability of recurrent disease in them (Figure 1). In the great majority of patients blood samples were obtained on one occasion only, before any relapse and at variable times after the attainment of the first remission (Table 1), defined as the maintenance of full normalization of clinical and laboratory data lasting for least 30 days after stopping plasma therapy following the resolution of the first acute episode.[19] None of the 109 patients was on plasma therapy at the time of remission when samples were collected. Patients were analyzed comparing 46 patients with recurrence, i.e., those with at least two episodes of TTP (range, 2 to 13 episodes), and 63 patients with a single episode and no recurrence during at least 1 year of observation (Table 1). In the former group recurrence was defined as the re-emergence of clinical symptoms and/or laboratory criteria compatible with a diagnosis of TTP (*see above*) occurring at least 30 days or later after remission of the acute episode. This study was conducted with the patients' consent and the approval of the Institutional Review Board of the Milan Center.

Methods. Venous blood samples for the measurements of ADAMTS13 (activity, antigen and anti-ADAMTS13 antibodies) and VWF:Ag were collected into evacuated tubes containing trisodium citrate as anticoagulant and, for some patients only, into EDTA-anticoagulated tubes for multimeric analysis of VWF. Blood was then centrifuged at 2500 rpm for 15 min at 4°C and plasma samples were stored at -80°C. Plasma samples obtained during remission were sufficient and qualitatively adequate to measure ADAMTS13 activity in all the 109 cases. For the other measurements some of the patients' samples were quantitatively or qualitatively inadequate (*see below*).

ADAMTS13 activity. ADAMTS13 activity was measured in plasma from all 109 patients of the cohort using the collagen binding enzyme immunoassay described by Gerritsen *et al.*,[23] with some modifications. The source of VWF used as protease substrate was a purified VWF concentrate (Facteur Willebrand Humain Tres Haute Purité) provided by Laboratoire Francais du Fractionnement et des Biotechnologies (Lille, France). The product, which lacks endogenous ADAMTS13 activity, was reconstituted to a VWF concentration of 100 U/mL, aliquoted and stored at -30°C. The concentrate was then thawed, diluted 1 in 33 with 5 M urea in 5 mM Tris-HCl, pH 8, and incubated for 10 min at room temperature. Subsequently, 50 μL of the dilution of the VWF source were added to 100 μL of serial dilutions (1/10, 1/20, 1/40) of test plasma as a source of ADAMTS13. Test plasmas were diluted in 5 mM Tris-HCl, pH 8 containing 12.5 mM $BaCl_2$ and 1 mM Pefabloc SC (Roche, Mannheim, Germany). Values were expressed as percentages of pooled normal plasma. Our most recent laboratory evaluation of assay reproducibility yielded an intra-assay coefficient of variation of 9% and an inter-assay coefficient of variation of 12%. The lower limit of sensitivity was 6% of protease levels in pooled normal plasma taken as the reference standard. The lower value of the reference interval (46%) was set at the 5th percentile of the distribution of the values obtained in 200 healthy individuals. Severe ADAMTS13 deficiency was arbitrarily considered as levels of 10% or less of ADAMTS13 activity in plasma. Previous *in vitro* experiments showed that, using this assay, high plasma levels of VWF do not influence the measurement of ADAMTS13 activity to a major degree.[24]

Table 1. Clinical and laboratory data of patients with or without recurrence during remission after their first acute episode of thrombotic thrombocytopenic purpura.

	Patients without recurrence (n=63)	*Patients with recurrence (n=46)*
Females	43 (68%)	36 (78%)
Males	20 (32%)	10 (22%)
Age	38 (1-75)*	32 (16-69)*
With associated diseases/conditions†	35 (56%)	24 (52%)
infections	16 (30%)	6 (24%)
autoimmune diseases	13 (24%)	6 (24%)
pregnancy/delivery	4 (7%)	4 (16%)
liver disease	3 (6%)	1 (4%)
drug intake	3 (6%)	2 (8%)
major surgery	2 (4%)	0
hematologic malignancy	0	1 (4%)
others	12 (23%)	5 (20%)
Months from the acute episode [to first relapse	n.a.	21 (2-144)*
Months of observation time to [exclude a relapse	22 (13-134)*	n.a.
Number of episodes of TTP	1	2 (2-13)*
Months from remission [to sample collection	10 (1-96)*	12 (1-96)*
ADAMTS13 activity (n=109)	41% (<6-153)*	12% (<6-142)*
ADAMTS13 antigen (n=77)	58% (<2-122)*	36% (<2-101)*
Anti-ADAMTS13 antibody with [Western blotting (n=97)	36%	64%
Anti-ADAMTS13 antibody by [inhibitory activity (n=58)	36%	63%
VWF antigen (n=103)	154% (30-505)*	166% (44-310)*
Ultralarge VWF multimers (n=79)	14%	17%

**Data are shown as medians and, between parenthesis, ranges . †The sum of patients with different associated diseases/conditions exceeds the number of patients because of multiple associated diseases/conditions in one patient. n.a. denotes not applicable. n denotes the number of patients tested for that measurement.*

ADAMTS13 antigen. ADAMTS13 antigen was measured in 77 available samples (71%) using the enzyme-linked immunosorbent assay (ELISA) described by Feys *et al.*[25] In brief, the murine monoclonal anti-ADAMTS13 antibody 20A5 was used to immobilize the plasma protease on a microtiter plate. Bound ADAMTS13 was detected by using two biotinylated monoclonal antibodies (5C11 and 13F7 at 6.7 nM) and peroxidase labeled streptavidin (Roche, Mannheim, Germany). Four dilutions of test plasma (1/13, 1/26, 1/52 and 1/104) were measured in phosphate buffered saline (PBS) (supplemented with 0.3% skimmed milk) and compared with the reference standard. The lower limit of sensitivity of the assay was 2% of the reference standard. The intra- and inter-assay coefficients of variation were 4% and 8%, respectively. The lower limit of the normal laboratory range is 45%.

Anti-ADAMTS13 antibody. Anti-ADAMTS13 antibodies were searched for in 97 samples (89%) by western blot analysis. The supernatant of cells producing recombinant

ADAMTS13 as previously described[26] (100 ng/lane) was added to gel-loading buffer (50 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate, 0.1% bromophenol blue, 10% glycerol). Next, 7% sodium dodecyl sulphate polyacryl gel electrophoresis was performed in Tris-glycine buffer, pH 8.3 and migrated samples were transferred onto a pure nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blocking with skimmed milk, membranes were incubated with citrated plasma samples (diluted 1/100 in TBS, 5% skimmed milk, 0.05% Tween 20, pH 7.4) used as a possible source of anti-ADAMTS13 antibody. Bound antibodies were visualized using alkaline phosphatase-labeled anti-human immunoglobulin (diluted 1/2000) and an alkaline phosphatase conjugate substrate kit (Amersham Bioscences, Uppsala, Sweden). No attempt was made to render this method of antibody analysis quantitative.

ADAMTS13 inhibitor. Antibodies inhibiting ADAMTS13 protease activity were searched for in 58 of the 70 patients (83%) who had ADAMTS13 activity below normal plasma levels (46%) by measuring residual activity in a 50:50 mixture of heat-inactivated patient's plasma and normal pooled human plasma. Heat-inactivated plasma was obtained after incubation at 56°C for 60 minutes to completely neutralize endogenous ADAMTS13 activity.[12,18] A patient was considered to have ADAMTS13 inhibitors (1 Bethesda unit) if the residual activity of the protease in the plasma mixture was less than 50% of that in the control mixture (a 50:50 mixture of heat-inactivated and plain normal plasma).

VWF antigen. VWF antigen (VWF:Ag) was measured in 103 samples (94%) by an enzyme immunoassay using rabbit anti-human VWF polyclonal antibodies as primary and secondary antibodies (DAKO, Glostrup, Denmark). The reference intervals were calculated as for ADAMTS13 (42 to 151%).

VWF multimer analysis. VWF multimer analysis was carried out on 79 samples (72%) using low-resolution sodium dodecyl sulphate-agarose gel electrophoresis with 0.9% low gelling temperature agarose in order to optimally resolve larger multimers. After electrophoresis, gels were fixed, washed and exposed to human anti-VWF antibodies labeled with I-125 (Amersham, UK) and then transferred to intensifier cassettes for autoradiography. VWF multimers were scanned with a densitometer (Scanjet 5200 C; Hewlett Packard, Piscataway, NJ, USA), which resolved the multimers in peaks (OD × mm) and densitometric analysis was performed using a software program (Image Master; Amersham Pharmacia Biotech, Piscataway, NJ, USA). VWF multimers were defined as of low molecular weight (corresponding to bands 1-5), intermediate molecular weight (bands 6-10) and high molecular weight (bands >10). The corresponding areas were computed and expressed as percentage of the total area. ULVWF multimers were considered to be present when the percentage of high molecular weight multimers was higher than 34%, i.e. two standard deviations above the mean values found in the plasma from 50 healthy individuals (28.3±2.7%). A plasma sample obtained 30 min after the infusion of desmopressin to a normal volunteer and stored in large amounts was always run in each electrophoretic gel to enable a visual assessment of the presence of ULVWF multimers in patients' plasma, since these are consistently present in plasma from normal individuals after an infusion of desmopressin.

Statistical analysis

For continuous variables, differences between patients with and without recurrence were evaluated by the Mann-Whitney U test. For discrete variables, differences were evaluated by means of the χ^2 test or Fisher's exact test. The Kolmogorov-Smirnov test was used to test for normality of the distribution of continuous variables; Spearman's rank order correlation coefficient was used to investigate the correlation between variables. Logistic regression analysis was applied to compute odds ratios and 95% confidence intervals, which were used as an estimate of the likelihood of recurrence associated with the factors under investigation. Adjusted odds ratios were obtained from a linear logistic model that included the factor of interest and all of the remaining factors we wanted to adjust for; unadjusted odds ratios were obtained from a model that included only the factor of interest. p values less than 0.05 were considered statistically significant. Analyses were performed using the SPSS package version 14.0.

Results

Table 1 gives the main features pertaining to the 63 patients who did not relapse compared with the 46 patients who had recurrences. Even though TTP was twice more frequent in women than in men, gender was not a risk factor for recurrence (odds ratio 0.73; 95% confidence interval 0.31 to 1.73; p=0.5). The presence of associated conditions or diseases at remission and/or during the first acute episode was not associated with the likelihood of recurrence (odds ratio 1.1; 95% confidence interval 0.5 to 2.5; p=0.7).

ADAMTS13 activity

A severe deficiency of ADAMTS13 activity (10% or less) was found in 32% of patients at remission. Patients with recurrent TTP had significantly lower levels of ADAMTS13 activity at the time of the first remission than those without recurrence: median ADAMTS13 activity was 12% (range, <6% to 142%) *vs.* 41% (range, <6% to 153%), p=0.007 (Figure 2). Furthermore, the prevalence of patients with severe protease deficiency was higher in patients with recurrent TTP (46% *vs.* 22%, p=0.01). The unadjusted odds ratio, taken as an index of the likelihood of recurrence in patients with severe deficiency of ADAMTS13 activity at remission, was 2.9 (95% confidence interval 1.3 to 6.8) (Figure 3). The likelihood of recurrence associated with severe ADAMTS13 deficiency remained statistically significant when multivariate analysis was performed taking as co-variates sex (odds ratio 2.9; 95% confidence interval 1.3

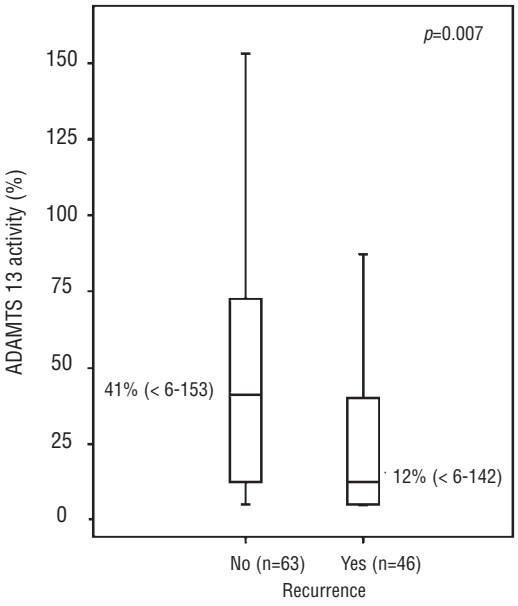

Figure 2. ADAMTS13 activity measured at remission in 46 patients with (Yes) or in 63 without (No) recurrence of acquired thrombotic thrombocytopenic purpura. Boxes represent the quartile range, and the solid horizontal lines the corresponding median values. Whiskers at the top and bottom of the boxes show the highest and lowest values.

to 6.8), age (odds ratio 2.8; 95% confidence interval 1.2 to 6.4) and associated conditions or diseases (odds ratio 3.0; 95% confidence interval 1.3 to 6.8), as well as when all covariates were included in the model (odds ratio 2.7; 95% confidence interval 1.2 to 6.4).

ADAMTS13 antigen

Plasma levels of ADAMTS13 activity and antigen measured in 77 patients during remission were positively correlated (Spearman's rho: 0.71), but some patients had antigen values higher than the corresponding activity values (Figure 4). Accordingly, among 21 patients (27%) with severe deficiency of protease activity (10% or less), three (14%) had normal antigen values (46% or more). Patients with recurrent TTP had significantly lower levels of ADAMTS13 antigen than those with no recurrence (median and ranges: 36%, <2% to 101%, *vs.* 58%, <2 to 122%; p=0.003) (Figure 5). Severe antigen deficiency (10% or less) was found in only 8% of them. When severe deficiency of ADAMTS13 antigen was used to evaluate the likelihood of recurrence, the unadjusted odds ratio was not statistically significant (odds ratio 1.4; 95% confidence interval 0.3 to 7.2) (Figure 3), and the same was also found after adjustment for sex, age and associated conditions or diseases.

Anti-ADAMTS13 antibodies

Of 97 plasma samples tested, 47 (48%) had anti-ADAMTS13 antibodies by western blot analysis, and among them 29 (62%) also had inhibitory activity. The prevalence of any anti-ADAMTS13 antibody (whether or not inhibiting protease activity) was significantly different (p=0.006) in patients with or without recurrence: 64% of patients with recurrent TTP had anti-ADAMTS13 antibodies during remission (of these, 70% had inhibitory activity), whereas only 36% of those without recurrence had anti-ADAMTS13 antibodies (50% had inhibitory activity). The unadjusted odds ratios for recurrence at remission were 3.1 (95% confidence interval 1.4 to 7.3) for patients with anti-ADAMTS13 antibodies by western blot and 3.1 (95% confidence interval 1.1 to 9.1) for those with inhibitors (Figure 3). Because all the patients with severe deficiency of ADAMTS13 activity also had anti-ADAMTS13 antibodies by western blot analysis, it was impossible to evaluate the independent role of each risk factor. However, because not all the patients with anti-ADAMTS13 antibodies had severe ADAMTS13 deficiency, the unadjusted odds ratio for recurrence in patients with the presence of both abnormalities during remission was 3.6 (95% confidence interval 1.4 to 9.0; p=0.006) (Figure 3).

VWF measurements

Higher VWF:Ag levels were observed in 103 patients at remission than in 100 healthy individuals comparable for sex and age (median: 160% *vs.* 104%, p<0.0001), with no significant difference between patients with or without recurrence (166% *vs.* 154%, p=0.3). ULVWF multimers were present in 12 of 79 tested patients (15%), five of whom had severe deficiency of ADAMTS13 activity and seven of whom had measurable levels. No difference was

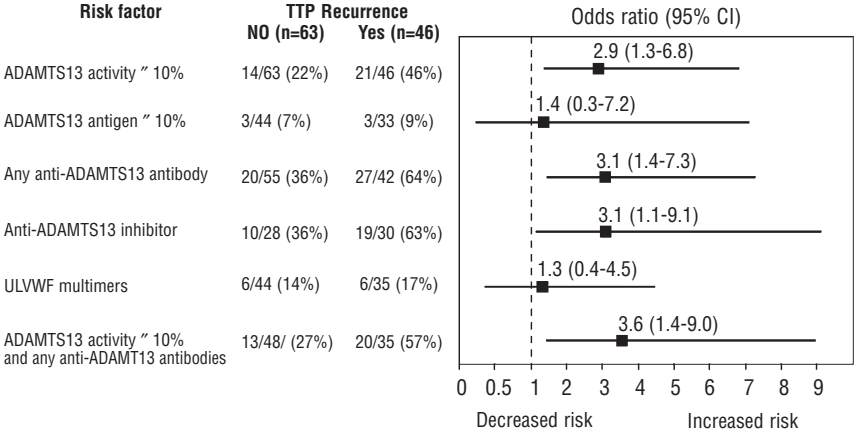

Figure 3. Markers of thrombotic thrombocytopenic purpura recurrence. Odds ratios are shown as solid squares with the 95% confidence intervals (95%CI) represented by horizontal bars. Odds ratios for the dichotomous variables are relative to a reference group of patients without the corresponding variable.

observed between patients with or without recurrence (17% *vs.* 14%; odds ratio 1.3; 95% confidence interval 0.4 to 4.5; p=0.7) (Figure 3).

Discussion

Even though the most common presentation of acquired TTP is a single acute episode, at least one third of the patients who survive the first episode relapse.[13-17] We retrospectively analyzed a cohort of 109 patients who met standard clinical and laboratory criteria for a diagnosis of a first acute episode of TTP, and compared those with or without recurrence (46 versus 63) for various measurements of ADAMTS13 and VWF obtained in plasma during the first remission, at a time when each patient was off therapy with plasma and clinically stable. Survivors of an acute episode of TTP with severely reduced levels of the VWF-cleaving protease ADAMTS13 during remission (10% or less) had a nearly 3-fold greater likelihood of developing another episode of TTP than those with higher protease activity. The search for predictors of TTP recurrence was also the focus of a study by Ferrari *et al.*,[27] who investigated 32 patients who had low plasma levels of ADAMTS13 activity at the time of the first acute episode of TTP and subsequently achieved remission. Persistence of low ADAMTS13 activity at remission was a predictor of recurrence, which occurred in six patients (19%) during the follow-up.[27] The study by Ferrari *et al.* and our own study both support the view that finding low levels of ADAMTS13 activity during first remission helps to predict recurrence. The predictive value of a severe deficiency of protease activity was also established in a large cohort of patients reported by Johnson *et al.*,[20] although the patients were tested during the acute phase of TTP, not during remission as done by Ferrari *et al.*[27] and ourselves.

ADAMTS13 was also measured as immunoreactive protein during remission in approximately two-thirds of the patients. Patients with recurrent TTP had, on average, lower levels of ADAMTS13 antigen than those without recurrence but, at variance with our findings pertaining to ADAMTS13 activity, a severe deficiency of the ADAMTS13 antigen was not associated with a higher likelihood of recurrence. This is perhaps due to the smaller sample size and/or to the fact that there were far fewer patients with severe deficiency of ADAMTS13 antigen than with severe activity deficiency. The discrepancy between activity and antigen levels in plasma might indicate that during remission at least some patients have ADAMTS13/anti-ADAMTS13 immune complexes that react in the immunoassay, as previously observed by Rieger *et al.*[28] in acute TTP. Another possible explanation for the discrepancy is that the high plasma levels of VWF found in the majority of our patients may have led to spuriously low levels of ADAMTS13 activity as measured by the collagen binding assay, whereas the immunoassay of ADAMTS13 is not affected by VWF levels. In the patients who had lower activity than antigen values, plasma VWF levels were indeed similar to those who had low values with both the assays (*data not shown*). Moreover, our previous *in vitro* studies carried out adding VWF to test samples showed that VWF interference in the collagen binding assay is not prominent, at least at VWF levels such as those measured in our patients.[24]

In this study, anti-ADAMTS13 antibodies were measured using two different assay methods: one based on the degree of neutralization of protease activity in a mixture of control and test plasma and another based upon western blot analysis, using recombinant ADAMTS13 as a source of antigen. In a preliminary study we showed that the qualitative western blot method is more sensitive that the inhibitor assay,

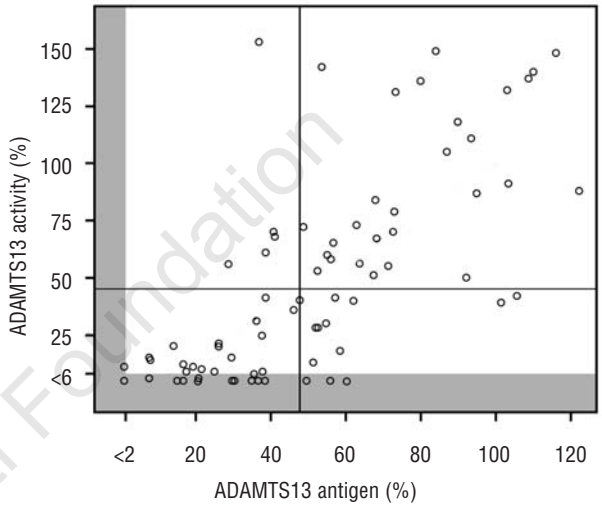

Figure 4. Comparison of ADAMTS13 activity and antigen in 77 patients with acquired thrombotic thrombocytopenic purpura during remission after the acute episode. The vertical and the horizontal lines indicate the lower limits of the normal range of ADAMTS13 antigen and activity. The gray areas indicate the sensitivity limits of the ADAMTS13 activity and antigen assays (see "*Methods*" section).

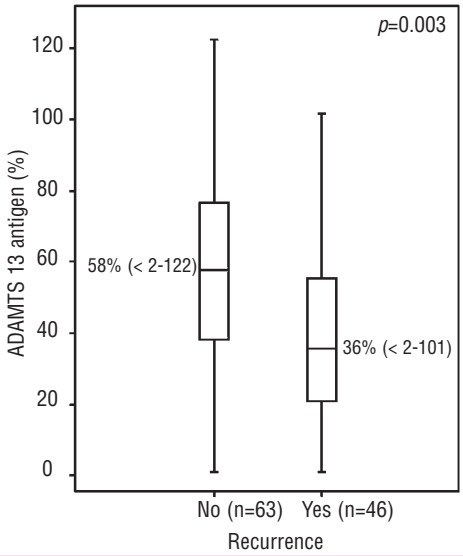

Figure 5. ADAMTS13 antigen measured at remission in patients with (Yes) or without (No) recurrence of acquired thrombotic thrombocytopenic purpura. Boxes represent the quartile range, and the solid horizontal lines the corresponding median values. Whiskers at the top and bottom of the boxes show the highest and lowest values.

because it was often positive in patients who tested negative in the inhibitor assays.[29] The presence of anti-ADAMTS13 antibodies, whether they did or did not inhibit protease activity, increased the likelihood of TTP recurrence by approximately 3-fold. The association of anti-ADAMTS13 antibodies with recurrence was also surmised by Luken *et al.*[30] who, in a patient with a long-standing remission of TTP, identified persisting oligoclonal B cells capable of producing antibodies to ADAMTS13, thereby implicating the proliferation of these residual B cells and the potential of the autoimmune process to relapse. ULVWF multimers are not a constant finding in patients with recurrent acquired TTP,[31] whereas they are in congenital cases.[32] We found that the presence of ULVWF multimers did not predict recurrence, and that a number of patients with normal ADAMTS13 had ultralarge multimers. The latter observation corroborates the views that abnormalities of VWF-cleaving proteases other than ADAMTS13 or of disulfide isomerases may be involved in the pathogenesis of some cases of TTP.[31] Unlike Johnson *et al.*,[20] we did not find that increasing age, male sex and the presence or absence of associated diseases or conditions at remission were markers of a greater likelihood of recurrence.

The limits of this study are its retrospective nature and the heterogeneity of a cohort referred to a tertiary care center from an array of different centers. No information was available on the occurrence of recurrence in approximately one-third of the original patients, who were excluded because no plasma sample was obtained during remission. The diagnoses of TTP made by the referring centers were scrutinized and adjudicated centrally using standard clinical and laboratory criteria, albeit based mainly on the exclusion of other diseases.[21] Given their referral to a tertiary care center, our patients likely form a cohort selected for the clinical severity of the disease; nevertheless, it is mainly in severe cases and in centers with limited experience and facilities for treatment that the possibility of predicting recurrence during remission is more cogent. The predictive value of severe ADAMTS13 deficiency and/or anti-protease antibodies as measured during remission was statistically significant but of limited robustness, because the confidence intervals of the odds ratios were wide. Not all the patients with these abnormalities had recurrences, and recurrences occurred also in patients without them. Hence, this study is unable to give a final answer on the best predictors of TTP recurrence during remission, and it is only hypothesis-generating. A large multinational prospective study based on ADAMTS13 and anti-ADAMTS13 measurements carried out at frequent and defined intervals after the acute episode is warranted. Despite these limitations, our results help in the consideration of strategies that might be adopted to prevent relapse, a risk that looms large in patients after acute TTP. Possible prophylactic strategies include the eradication of inhibitory autoantibodies by immunosuppressive agents and correction of low ADAMTS13 levels by means of replacement therapy. The most promising immunosuppressive treatment to date is rituximab (anti-CD20 monoclonal antibody), because, according to a survey of 12 publications reporting on 27 patients treated for chronic recurrent TTP and followed for 3-28 months, only three patients (11%) relapsed.[33] However, reporting bias is common and may skew the efficacy assessment of treatment based on case reports. Our findings, suggesting that anti-ADAMTS13 antibodies and low ADAMTS13 levels are associated with a greater likelihood of recurrence, set the stage for a randomized clinical trial, comparing during TTP remission the efficacy of replacement therapy, rituximab or both in the prevention of recurrence.

Authorship and Disclosures

FP designed the study and wrote the manuscript; SL and RP analyzed the results and contributed to writing the manuscript; HBF, KV, CV and MTC performed experimental work (ADAMTS13 antigen and activity measurement, determination of anti-ADAMTS13 antibody (with and without inhibiting activity, VWF antigen and multimers evaluation); TB performed the statistical analysis; FF, SZ, MR, DM, MK, LL and GG referred a large number of TTP patients; PMM critically revised the manuscript. All authors approved the final version of the manuscript.

The authors reported no potential conflicts of interest.

Appendix

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