Pathophysiology of thrombotic thrombocytopenic purpura : the "two-hit" paradigm

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CHAPTER 2

Residual plasmatic activity of ADAMTS13 is associated with phenotype severity in congenital thrombotic thrombocytopenic purpura

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Abstract

The quantification of residual plasmatic ADAMTS13 activity in congenital thrombotic thrombocytopenic purpura (TTP) patients is constrained by limitations in the sensitivity and reproducibility of commonly used assays at low levels of ADAMTS13 activity, blunting efforts to establish genotype-phenotype associations in the disease. In this study, the residual plasmatic activity of ADAMTS13 was centrally measured by SELDI-TOF mass spectrometry (limit-of-detection=0.5%) in 29 patients with congenital TTP. The results were used to study the correlations between ADAMTS13 genotype, residual plasmatic activity and the clinical phenotype of the disease. ADAMTS13 activity above 0.5% was measured in 26 (90%) patients and lower levels of activity were associated with earlier age of onset, more frequent recurrences and prescription of fresh frozen plasma prophylaxis. At receiver operating characteristic curve analysis, activity levels of less than 2.7% were discriminative of severe disease, i.e., age of onset <18 years, annual rate of TTP episodes >1, and use of prophylaxis. Mutations affecting the highly-conserved N-terminal domains of the protein were associated with lower residual ADAMTS13 activity and more severe disease phenotype in an allelic-dose dependent manner. Our results show that residual ADAMTS13 activity is associated with the severity of clinical phenotype in congenital TTP and provide insights into genotype-phenotype relationships.

Introduction

Congenital thrombotic thrombocytopenic purpura (TTP) (also known as Upshaw-Schulman syndrome, OMIM #274150) is a rare, recessively inherited thrombotic microangiopathy. The disease is characterized by the congenital severe deficiency of ADAMTS13 plasmatic activity caused by mutations in the *ADAMTS13* gene.[1-6] The phenotype of congenital TTP is variable in its severity. Some patients present with the disease in the neonatal period, while others have an adult disease-onset. Moreover, patients may experience only a few isolated episodes of TTP, whereas others have frequent recurrences leading to the prescription of fresh frozen plasma (FFP) prophylaxis.[5-8] A recent review of the ~100 published cases of congenital TTP showed that patients carrying the same *ADAMTS13* gene mutations develop their first disease episode at a similar age.[9] This observation suggests that different *ADAMTS13* mutations may influence the severity of clinical phenotype, probably by determining different levels of residual plasmatic activity of ADAMTS13. However, the quantification of residual ADAMTS13 activity in congenital TTP patients is blunted by limitations in the analytical sensitivity and performance in the low-end of ADAMTS13 activity distribution (i.e. activity below 6%) of the commonly used ADAMTS13 activity assays.[10-13] A recently described method, based on surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry, is able to accurately measure ADAMTS13 plasmatic activity with high analytical sensitivity (limit of detection of 0.5%, i.e. 5- to 10-fold higher sensitivity than most commercially available assays).[14, 15]

In this study, we measured the residual activity of ADAMTS13 by SELDI-TOF mass spectrometry in a cohort of 29 patients with congenital TTP and studied the relationships between *ADAMTS13* genotype, residual plasmatic activity and the clinical phenotype of the disease.

Patients and Methods

Patients

Patients registered between 2000 and 2010 in four European TTP registries, the Milan TTP registry (Milan, Italy),[16, 17] the Southeast England TTP registry (London, UK)[18], the International Registry for HUS and TTP (Bergamo, Italy),[19] the TMA Registry of the French Reference Center for the management of thrombotic microangiopathies (Paris, France),[20] were evaluated for study eligibility. Inclusion criteria were: (a) history of at least one episode of TTP (defined by the presence of thrombocytopenia and microangiopathic Coombs negative hemolytic anemia with signs of red blood cell fragmentation), (b) severe deficiency of ADAMTS13 with activity <6%, measured by collagen binding assay[10] or fluorescence resonance energy transfer[11], (c) absence of anti-ADAMTS13 autoantibodies, searched for by western blotting[21] or ELISA[17-20] (d) documented mutations of *ADAMTS13* after sequencing of the protein coding area of the gene by PCR and Sanger sequencing (e) availability of 100 µL of citrated plasma collected during remission at least 20 days from the last infusion of FFP or any other blood-derived products. A total of 29 patients were included in the study (Milan, n=12; UK, n=7; Bergamo, n=5; France, n=5). The disease histories of 16 patients had already been described elsewhere,[9, 22-29] whereas 13 are

presented here for the first time. For each patient, phenotypic data were retrieved including: age at last follow up and sex, ethnicity, age at first TTP episode (in order to minimize misclassification, the age of onset was adjudicated on the basis of the first disease episode that required FFP infusion), lifetime and annual frequency of TTP episodes, use of regular FFP prophylaxis, history of neonatal jaundice or thrombocytopenia and presence of renal or neurological damage, defined as presence of chronic renal failure and persistence of neurological deficit during remission. Individual clinical and genetic information are reported in Supplementary Material S1. The study was approved by the Institutional Review Boards of the participating centers and all subjects gave informed consent.

Measurement of ADAMTS13 activity

All samples were shipped in dry ice to the Hematology laboratory at Department of Pathology, Ohio State University, Columbus, OH (USA) for centralized measurement. ADAMTS13 activity was determined in this central laboratory using a SELDI-TOF mass-spectrometer-based method.[14] The laboratory personnel was unaware of the clinical features of the patients. ADAMTS13 activity was determined by mixing patient plasma with the enzyme substrate VWF73 containing a 6XHis tag. The cleavage product was enriched by IMAC ProteinChip and then quantified using SELDI-TOF mass spectrometry.[14, 15] An internal control was generated by cleaving recombinant-6×His-tagged human VWF73 by PreScissionTM Protease (Amersham Biosciences), as described before.[14] Briefly, 10 µL of patient plasma were mixed with 30 µL buffer (5 mM Tris HCl, 5 mM NaCl, 1 mM BaCl2, pH 7.5) containing 2.5 µg of 6×His tagged human VWF73 (D1596-R1668). The cleavage reaction was performed for 16

hours at 37 °C and then terminated by boiling the samples at 100 °C for 2 min. Each experiment included a standard curve performed under identical conditions except that the plasma sample was replaced by pooled normal plasma (PNP) diluted at 7.5%, 5%, 3.5%, 2.5%, 1.5%, 1% and 0.5% in 100 mM NaCl containing 0.1% bovine serum albumin (BSA) (Figure 1). Following the cleavage reaction, 40 µL internal control (0.01 µg/ µL) was mixed with 35 µL reaction sample from each patient on the corresponding IMAC ProteinChip spot. After incubation for 30 min at room temperature with constant shaking, each spot was washed five times with 200 µL of washing buffer. This was followed by one quick wash with 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0. Finally, 1 µL of energy absorbing molecule (EAM) solution (100% saturated sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was added to each spot. The cleavage products on the IMAC ProteinChips were analyzed by PCS4000 SELDI-TOF mass spectrometer (Vermillion Inc). In order to evaluate the test reproducibility in the lower analytical range, two external controls (two TTP patients with their plasma samples aliquoted and frozen at -80C) were tested repeatedly over time and by different operators. The ADAMTS13 activity levels obtained (mean ± 2SD) for these two patients were 1.8%±0.2% and 4.8%±0.8%, respectively, indicating good test reproducibility. The coefficient of variation for those two controls was 6.8% and 7.8%, respectively.

Mutation analysis and annotation

In keeping with the Human Genome Variation Society (HGVS) instructions, nucleotide numbering for reported mutations refers to cDNA numbering with the

A of the ATG codon numbered as +1. According to new instructions, stop codons were designed as "*" instead of "X". NM_139025.3 was used as a reference for nucleotide changes. NP_620596.2 was used as protein reference sequence. The review of *ADAMTS13* mutations associated with congenital TTP was based on published articles found through searches on PubMed (updated to July 2011), using "ADAMTS13 mutation", "ADAMTS13 genotype", and "congenital thrombotic thrombocytopenic purpura" as queries. Polymorphisms of *ADAMTS13* were searched for on dbSNP. As suggested by the HGVS, the accuracy of nucleotide change for reported mutations was verified by Mutalyzer 2.0 software (URL http://www.mutalyzer.nl/2.0/). Annotation of genetic variants was performed on dbSNP131 (URL:http://www.ncbi.nlm.nih.gov/projects/SNP), 1000Genomes (URL:www.1000genomes.org) databases (used as databases of common genetic variation), and Polyphen 2 (URL: http://genetics.bwh.harvard.edu/pph2/) and SIFT (URL:http://sift.jcvi.org/) software, (used to predict the effect of aminoacid changes on protein function). A list of all the proteins used in the SIFT alignment to determine aminoacid conservation is provided in Supplementary Material S2. Splicing mutations were annotated on NetGene2 and their effect on protein translation predicted by Expasy (URL: http://web.expasy.org/translate/). Annotation was performed either manually or by automated submission of mutation batches with custom scripts written in Perl programming language. Sequencing of *ADAMTS13* in 99 control individuals free from TTP was performed in the frame of the DVT Milan Study, a study on deep vein thrombosis predisposing genetic variants. In these subjects, *ADAMTS13* exons were sequenced by DNA target capture on NimbleGen

Custom Human Sequence Capture 2.1M Array chips followed by next-generation DNA sequencing on ABI SOLiD 4 platforms. Sequencing was performed at the Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX (USA). *ADAMTS13* sequence coverage was above an average of 20X per base in each of the sequenced individuals, providing enough coverage for confident variant calls.

Genotype risk classification

To study the relationship between mutation localization and clinical phenotype, a genotype classification was constructed considering N-terminal mutations as a risk factor for more severe disease. N-terminal mutations were mutations affecting ADAMTS13 aminoacid 1-714, C-terminal mutatons were mutations affecting aminoacid 715-1427. One point was added to the score for each missense mutation affecting the N-terminal of the ADAMTS13 protein. Mutations at the C-terminal (either missense or truncating mutations affecting C-terminal domains only) did not add points to the score. Patients with splice-site or truncating mutations affecting both N- and C-terminal domains were excluded from analysis. The resulting scores were: genotype score=0 (i.e. a 'mild genotype') for patients with compound heterozygous or homozygous mutations both affecting the C-terminal of ADAMTS13, genotype score=1 (i.e. a 'moderate genotype') for compound heterozygous patients with one mutation affecting the N-terminal and one affecting the C-terminal of ADAMTS13, genotype score=2 (i.e. a 'severe genotype') for patients with compound heterozygous or homozygous mutations both affecting the N-terminal of ADAMTS13 and 'unclassified genotype' for patients who had at least one splice-site mutation or a

truncating mutation affecting both N- and C-terminal domains of ADAMTS13. Mutation and genotype score classification data for all patients are provided in the Supplementary Material S1.

Statistical analysis

Descriptive statistics are presented as medians (interquartile range [IQR]) and percentages. Bivariate associations between categorical variables were done using the Fisher's exact test. Linear and logistic regression analysis were used to calculate estimates and 95% confidence intervals (CI) of the associations between plasmatic ADAMTS13 activity, the genotype risk score and clinical outcomes. Receiver operating characteristic (ROC) curve analysis was used to determine the ADAMTS13 activity levels that could discriminate patients who had severe clinical outcomes with the highest sum of sensitivity and specificity.

Results

Patients characteristics and SELDI-TOF based measurement of ADAMTS13 activity

The features of the 29 patients with congenital TTP included in this study are presented in Table 1. Residual plasmatic activity of ADAMTS13 was measured in a central laboratory on samples collected during remission at least 20 days after the last infusion of FFP or other blood-derived products. Measurements were carried out in two duplicates of the same sample, the median difference in the measurements between the two duplicates being 0.13% (IQR: 0.43%). Raw data on duplicate measurement results are available in the Supplementary Material S1. A total of 26 patients (90%) had measurable residual activity of ADAMTS13,

whereas three patients had activity <0.5%. Activity was below 10% in all patients confirming the previous diagnosis of severe deficiency of ADAMTS13. The median plasmatic ADAMTS13 activity was 3.08% (IQR: 4.17%; range: <0.5-6.77%). In a few patients with multiple remission samples available for measurement, residual ADAMTS13 activity was similar in the different samples (Supplementary Material S3).

ADAMTS13 mutations

ADAMTS13 genetic analysis in the 29 congenital TTP patients revealed 31 mutations (2 mutations of the splice site, 7 indels, 3 nonsense and 19 missense), of which 8 (1 splice site mutation and 7 missense) are novel. All identified mutations and their functional annotations are reported in Table 2. Support for the causal role of the newly identified *ADAMTS13* mutations was sought for by annotation on databases of common genetic variation and sequencing of the *ADAMTS13* gene in 99 healthy Caucasian individuals. None of the mutations were present in dbSNP131, in the 1000Genomes database or in 198 alleles from 99 controls, enabling us to exclude that the identified variants were common polymorphisms. All the novel missense mutations were also annotated on SIFT and Polyphen 2 software for the prediction of the functional effect of protein changes (predicted to be damaging vs predicted to be benign) (Table 2). As a critical test of the utility of these annotations, we determined their ability to distinguish common from disease-associated variants of *ADAMTS13*. To estimate the sensitivity and specificity of each software, we used as positive controls 55 missense mutations of *ADAMTS13* found through a review of all congenital TTP cases reported in the literature and as negative controls 20 missense single

nucleotide polymorphisms (SNPs) of *ADAMTS13* reported by dbSNP. Polyphen 2 was the most sensitive software, with sensitivity of 95% and specificity of 60%. SIFT was the most specific software, with sensitivity of 71% and specificity of 95%. Annotation of all *ADAMTS13* missense mutations and SNPs is presented in Supplementary Material S4. All 7 newly identified missense mutations were predicted to be damaging for protein function by Polyphen 2 software. Only p.Q436H was predicted by SIFT to be tolerated. The mutation is a c.1308G>C nucleotide substitution located in the donor splice site of exon 11. In addition to the Q-->H aminoacid change, this nucleotide change was also predicted by NetGene 2 to result in a reduction of splicing efficiency providing further support to its causal role. The novel c.106-1G>C mutation, located at the acceptor splice site of in intron 1, was predicted by NetGene2 to result in a 2 base pair shift of the splice site. This splice shift is in turn expected to determine a deletion of 2 aminoacid residues (S36 and C37) with a frameshift of protein translation and formation of a premature stop codon after 102 aminoacid residues.

Association between residual ADAMTS13 activity and the severity of clinical phenotype in congenital TTP

The associations between residual ADAMTS13 activity and the phenotypic features of congenital TTP are outlined in Table 3. A lower residual ADAMTS13 activity was associated with earlier age of onset (Figure 2A). Lower levels of plasmatic ADAMTS13 were also associated with a higher annual rate of TTP episodes (Figure 2B) and with higher odds of regular FFP prophylaxis prescription. We used receiver operating characteristic curve analysis to determine the levels of residual ADAMTS13 activity that best discriminated

patients at risk for unfavorable clinical endpoints (i.e. age of onset below 18 years, annual rate of TTP episodes greater than 1 and prescription of FFP prophylaxis). An ADAMTS13 activity <2.74% discriminated patients who had a disease onset below 18 years and those who had prescription of regular FFP prophylaxis, whereas an activity <1.61% discriminated patients who had an annual rate of TTP episodes greater than 1 (Table 4).

Relationships between ADAMTS13 mutations, residual plasmatic activity of ADAMTS13 and clinical outcomes of congenital TTP

Because patients with the same *ADAMTS13* genotype were found to have similar age of onset,[9] we hypothesized that *ADAMTS13* gene mutations could influence the clinical phenotype of congenital TTP, by determining different patterns of residual ADAMTS13 activity. We plotted *ADAMTS13* genotypes that occurred more than once in the study cohort against the residual ADAMTS13 activity and the age of onset of the corresponding patients (Figure 3). A clustering of both age of onset and residual ADAMTS13 activity in patients with the same genotype was observed, suggesting that ADAMTS13 mutations influence both the amount of residual ADAMTS13 activity and the clinical features of the disease. In order to identify the relationships between specific ADAMTS13 mutations and the clinical features of congenital TTP we studied the type and distribution of congenital-TTP-causing *ADAMTS13* mutations on the ADAMTS13 protein domains. Including the 8 novel mutations, 121 mutations of *ADAMTS13* have been described to date in congenital TTP patients. Of these, 62 (51%) are missense. Notably, 76% (n=47) of the missense mutations of *ADAMTS13* described in TTP patients localize in the N-terminal of the protein (binomial

probability, $p=2.01x10^{-5}$), which is the area with the highest degree of evolutionary conservation[9] and where the domains necessary for ADAMTS13 catalytic activity are located.[30, 31] In contrast to this, missense single nucleotide polymorphisms (SNPs) of *ADAMTS13* included in dbSNP131, which are likely not all to be related to disease, do not show evidence of skewed distribution (p=0.14). We also evaluated the evolutionary conservation at the sites of all reported missense mutations of *ADAMTS13* (only mutations of *ADAMTS13* reported in association with congenital TTP were considered). Mutations of the C-terminal end of ADAMTS13 occurred at aminoacid residues that are highly conserved across species (SIFT score below 0.05), whereas causal mutations of congenital TTP in the N-terminal domains of ADAMTS13 were also found at aminoacid residues that are less conserved (SIFT score >0.05) (Figure 4A) (Fisher's exact test, p=0.006). All these analyses indicated that mutations at the N-terminal domains of ADAMTS13 are more severe than C-terminal domain mutations. In order to investigate whether mutations affecting different domains of ADAMTS13 were associated with variable degrees of clinical severity, we compared the residual plasmatic activity of study participants carrying mutations of the N-terminal domains of ADAMTS13 with that of patients with C-terminal domain mutations. Only patients with homozygous mutations were considered for analysis, to avoid confounding that in compound heterozygous patients derives from the coexistence of two different mutations. Patients with homozygous mutations at the N-terminal domains (n=4) displayed lower residual activity of ADAMTS13 and earlier age of disease onset than those with homozygous C-terminal domain mutations (n=8) (Figure 4B). To further study the relationship

between mutation localization and clinical phenotype of congenital TTP, we grouped patients in three classes considering N-terminal mutations as a risk factor for more severe disease. In this grouping (see Methods section for details), 9 patients had a 'mild genotype' (i.e., patient with compound heterozygous or homozygous mutations both affecting the C-terminal of ADAMTS13), 5 had a 'moderate genotype' (i.e., compound heterozygous patients with one mutation affecting the N-terminal and one affecting the C-terminal of ADAMTS13), 7 had a 'severe genotype' (i.e., patients with compound heterozygous or homozygous mutations both affecting the N-terminal of ADAMTS13) and 8 patients were not classifiable in any of the group (i.e., they either had at least one splice-site mutation or a truncating mutation affecting both N- and C-terminal domains of ADAMTS13). On linear regression analysis, the residual plasmatic activity of ADAMTS13 decreased with increasing genotype severity (Figure 5A). On logistic regression analysis, patients with a moderate genotype were 2-times more likely (OR: 2.0; 95% CI: 0.1-41) to receive regular FFP prophylaxis, while patients with a severe genotype were 11-times more likely (OR 10.7, 95% CI: 0.8-138) to receive regular FFP prophylaxis compared with patients with a mild genotype. The associations were attenuated when we adjusted for residual ADAMTS13 activity (moderate genotype: odds ratio [OR] 1.0 [95% CI 0.03-30] and severe genotype: OR 4.8 [95% CI: 0.2-91] compared with mild genotype). On linear regression analysis, there was a negative association between the genotype severity score and age of disease onset (Figure 5B). Moreover, there was a positive association between genotype severity and the annual rate of TTP

episodes (Figure 5C). Results remained unchanged when patients carrying truncating mutations at the C-terminal domains were excluded form the analysis.

Discussion

A new SELDI-TOF mass spectrometry-based method was used to measure the residual plasmatic activity of ADAMTS13 in 29 congenital TTP patients, with the aim of investigating the relationships between *ADAMTS13* genotype, residual plasmatic activity and the clinical phenotype of the disease. Our main study findings were the following: (a) a residual plasmatic activity of ADAMTS13 was measurable by SELDI-TOF mass-spectrometry in 90% of the study participants; (b) the amount of residual plasmatic activity of ADAMTS13 measured by SELDI-TOF mass spectrometry was inversely associated with the clinical severity of the phenotype; (c) residual plasmatic activity levels below levels of 2-3% identified patients with adverse clinical outcomes; (d) *ADAMTS13* mutations were associated with activity levels and clinical severity, with N-terminal domain mutations being associated with lower activity and severe disease in an allele-dosage dependent way. The existence of some degree of ADAMTS13 activity in the majority of congenital TTP patients was documented in this study for the first time. Previous case reports and case series reported the detection of a residual activity only in few patients. Because many of these studies adopted immunoblotting and collagen binding assay for the identification of severe ADAMTS13 deficiency, this result is not surprising in light of the high limit of detection and scarce reproducibility of these assays at low ADAMTS13 concentrations.[12, 13] Recently, a Japanese group reported a large cohort of

congenital TTP patients in whom ADAMTS13 was measured by an ELISA method with a reportedly low limit of detection (i.e, 0.5%), finding a residual activity only in a minority of patients. Because limits of detection are calculated against different calibration curves with different reference samples, a formal comparison of the limits of detection of that assay with ours cannot be made unless both assays are tested against a common 'standard'. However, we note that several proofs of the reliability and validity of our measurements were provided in this study, arguing against random or biased results. First, a calibration curve using calibrators with concentrations of ADAMTS13 <10% was calculated before each assay, showing the linearity of the method at very low concentrations (see Figure 1). Second, repeated measurements on the same sample (including duplicate measurements and two external controls repeated over time by different operators) and multiple samples of the same patient consistently produced similar activity results. Third, the detection method of the assay used in this study (i.e. mass spectrometry) is considered to be the 'gold standard' for protein detection, providing high analytical sensitivity and specificity for very tiny amounts of cleavage products. Decreasing levels of residual ADAMTS13 activity were associated with earlier age of onset, more frequent disease recurrences and prescription of FFP prophylaxis to prevent further recurrences. These results are consistent with the view that congenital TTP patients with a higher residual plasmatic activity of ADAMTS13 are protected from disease onset until they come across a strong challenge to their fragile ADAMTS13-VWF balance (e.g., pregnancy, which is often a triggering factor of TTP in women with adult-onset disease), whereas patients with low or no residual activity are vulnerable to

environmental challenges, developing early-onset disease and frequent recurrences that prompt caring physicians to prescribe FFP prophylaxis. We identified levels below 1.6-2.7% as levels of ADAMTS13 activity predictive of clinical endpoints of disease severity. An activity of ADAMTS13 below these levels identified patients with early disease-onset, frequent recurrences and the need for preventive FFP infusion with high specificity, suggesting that patients with low residual ADAMTS13 activity are unlikely to have mild disease.

In this study, we also reported 8 novel mutations of *ADAMTS13* associated with congenital TTP. A detailed analysis of the annotation, functional consequences and distribution on ADAMTS13 of these novel and of previously reported mutations revealed that mutations in the highly conserved N-terminal domains of ADAMTS13, which have been shown to be required for the catalytic activity of this enzyme, are more likely to be associated with congenital TTP, lower residual ADAMTS13 activity and earlier age of disease onset than those of C-terminal domains. The association of *ADAMTS13* genotype with the clinical phenotype was attenuated by adjusting for ADAMTS13 plasmatic activity, confirming that *ADAMTS13* genotype influences the disease severity at least in part through the residual plasmatic activity of ADAMTS13. Limitations of this study include that individual ADAMTS13 activity was measured once for the majority of patients and that clinical outcomes were ascertained some years after their occurrence. Both would have led to random misclassification – since neither mutations nor levels were known at the time – and hence an underestimation of actual differences. Serial measurements of ADAMTS13 would have been interesting, but were not feasible in this study with patients from different regions of different

continents. However, by measuring ADAMTS13 in more than one sample of several patients we showed that residual ADAMTS13 activity during remission is likely constant in a given patient. Since the prevalence of congenital TTP is estimated to be ~1/1 million,[32] prospective studies will rarely be feasible. In addition, the clinically relevant endpoints of the study (i.e. age of onset, rate of episodes, use of FFP prophylaxis) can be considered "hard" endpoints, unlikely to be misclassified. To further minimize subjectivity in age of onset definition, we required that the first disease episode warranting plasma infusion be considered as the first episode of the disease.

In conclusion, we found that residual ADAMTS13 activity is associated with the phenotype severity of congenital TTP and that mutations affecting the evolutionary conserved N-terminal domains of the protein are associated with more severe clinical phenotype. This study identified a disease-severity biomarker of potential clinical relevance (i.e. residual plasmatic activity of ADAMTS13) and provides insights into genotype-phenotype associations in this rare, life-threatening disease.

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Author contributions
LAL designed the research, carried out part of the analyses, interpreted the results, and wrote the manuscript. FP designed the research, interpreted the results, and critically reviewed the manuscript. HMW, AH and SY performed ADAMTS13 measurement by SELDI-TOF mass spectrometry and critically reviewed the manuscript. HMW also participated in the study design. MAS, MN, AV, GR, PC, RL, RD, CL, LAL and FP enrolled patients, collected and standardized clinical and laboratory information, critically reviewed the manuscript. RAG was responsible for next-generation sequencing and mutation annotation, and critically reviewed the manuscript. KMM conducted statistical analyses and contributed to writing the manuscript.

Conflict of interests
None relevant to this study.

Figures

Figure 1. Linearity of SELDI-TOF based measurements of plasmatic activity of ADAMTS13 at concentrations below 10%. The figure shows a representative example of the calibration curve that was prepared before each experiment to confirm the linear behavior of the assay and the detection of cleavage product.

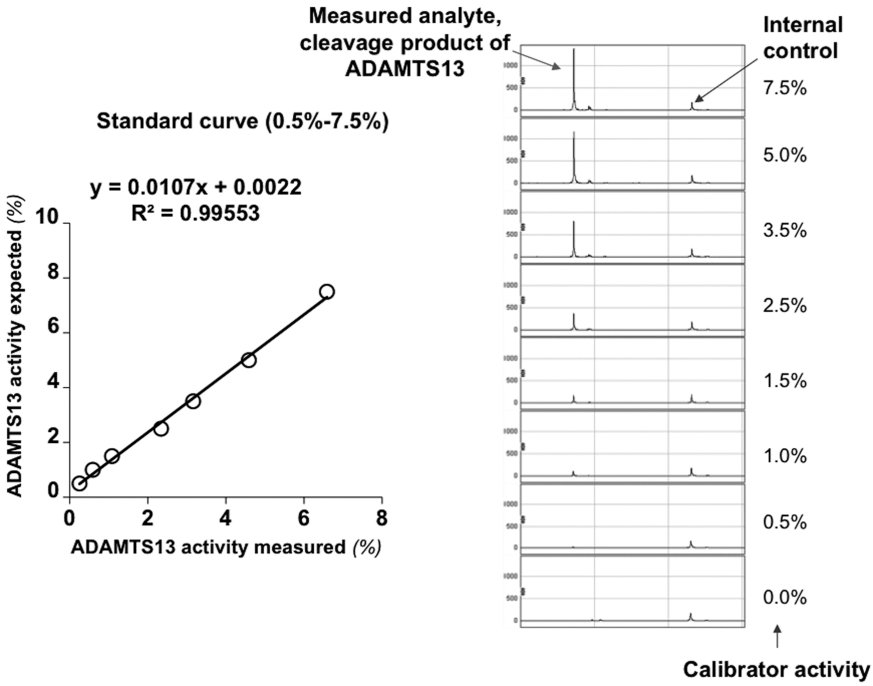


Figure 2. Relationship between residual ADAMTS13 activity and clinical features of congenital TTP. Panel A: association between residual ADAMTS13 deficiency and age of disease onset in 29 patients with congenital TTP at linear regression analysis. Only a few outliers were detected. A patient from the Milan cohort with adult age of onset and residual activity below 2.74% and 3 from the French cohort with neonatal onset and activity above 2.74%. Panel B: association between residual ADAMTS13 deficiency and the annual rate of TTP episodes.

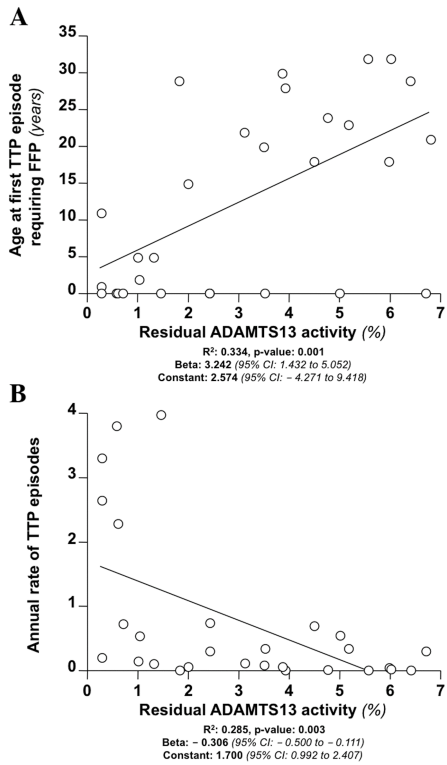


Figure 3. Relationship between *ADAMTS13* genotype, residual activity and age of disease onset. Age of disease onset and residual ADAMTS13 activity in multiple patients carrying the same mutations of *ADAMTS13*. A clustering of both the age of onset and the plasmatic activity of ADAMTS13 can be observed for patients with a given *ADAMTS13* genotype. For each patient ADAMTS13 activity (triangle) and age of onset (circle) are aligned.

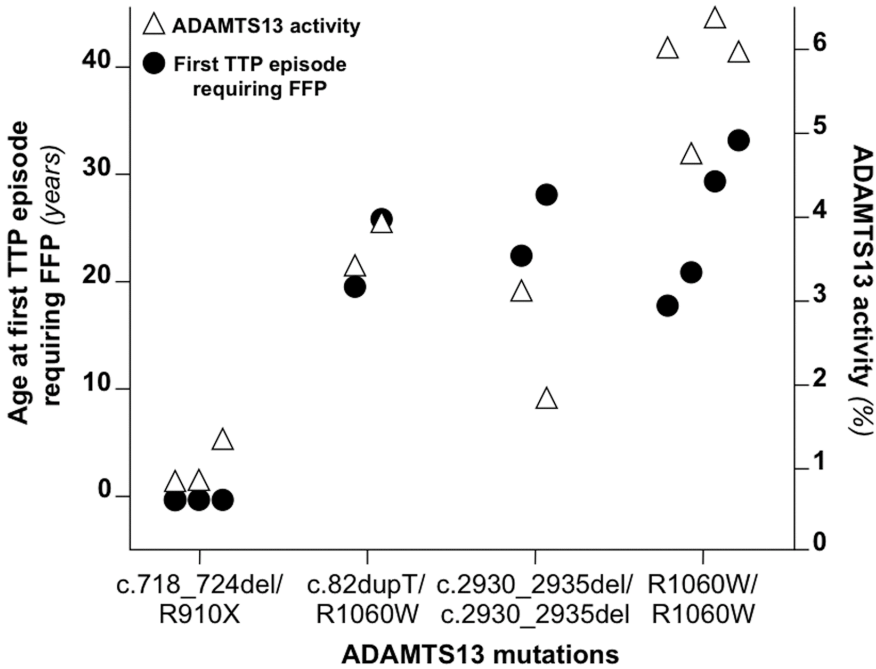


Figure 4. Evolutionary conservation, associated ADAMTS13 plasmatic activity and age of disease onset of N- and C-terminal mutations of ADAMTS13. Panel A: evolutionary conservation of amino acid residues affected by missense mutations of *ADAMTS13* associated with congenital TTP in all cases reported in the literature. Conservation was estimated using SIFT. Panel B: residual activity of ADAMTS13 and age of onset of patients with different C- and N-terminal mutations. Only mutations in homozygosis were considered.

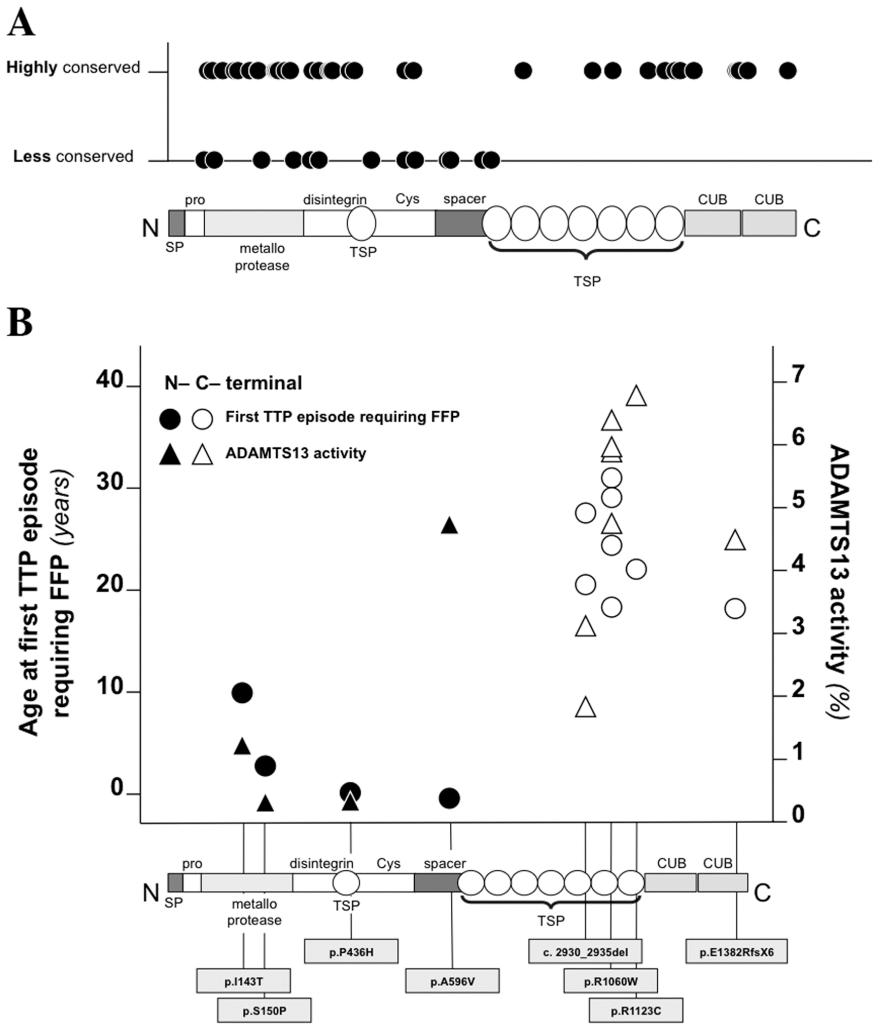
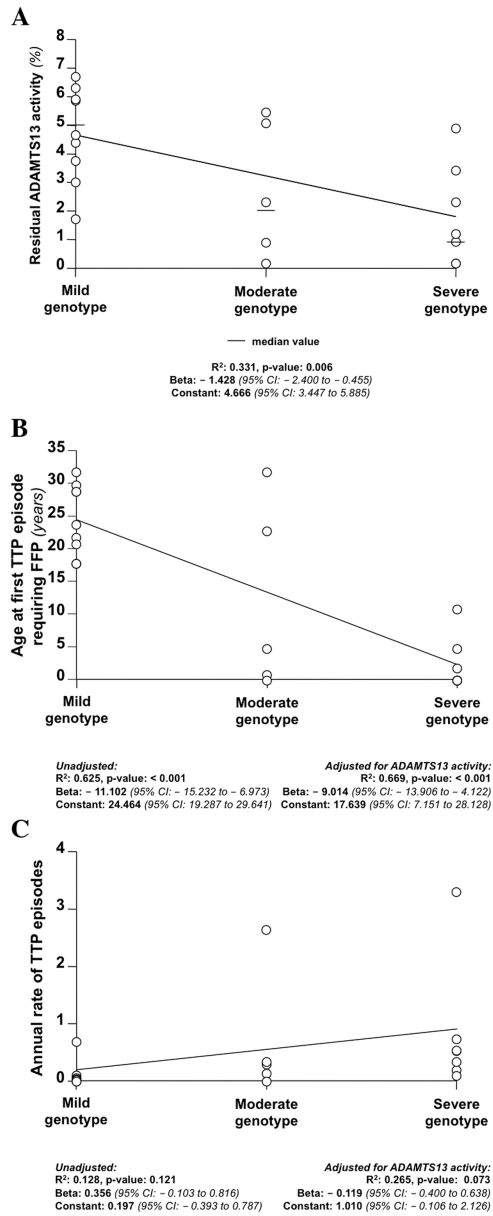


Figure 5. Association between genotype severity score, plasmatic activity of ADAMTS13, age of disease onset and annual rate of TTP episodes. Increasing severity in the genotype score (i.e. an increasing number of N-terminal mutations; 'mild genotype' = 2 C-terminal mutations, 'moderate genotype' = 1 C- and 1 N-terminal mutations; 'severe genotype' = 2 N-terminal mutations) was associated with lower plasmatic activity of ADAMTS13 (Panel A), earlier age of onset (Panel B) and higher annual rate of TTP episodes (Panel C).



Tables

Table 1. Patient characteristics (n=29).

Variable	**Value**
Median age (IQR), years	27 (24)
Male, n (%)	13 (45)
Ethnicity, n (%)	
Caucasian	22 (83)
Arab	4 (14)
Caribbean	3 (10)
Median age of disease onset (IQR), years	11 (23)
Neonatal jaundice and/or thrombocytopenia, n (%)	11 (38)
Persistence of renal/neurological damage, n (%)	3 (10)
Median total number of lifetime TTP episodes (IQR)	5 (8)
Multiple TTP episodes, n (%)	23 (79)
Median annual rate of TTP episodes (IQR)	0.28 (0.67)
Regular FFP prophylaxis, n (%)	12 (42)

FFP, fresh frozen plasma; TTP, thrombotic thrombocytopenic purpura; IQR, interquartile range.

Table 2. Mutations of *ADAMTS13* found in patients included in the study and their functional annotations. None of the mutations were found in dbSNP131 and 1000Genomes study databases.

DNA	Location	Predicted effect	Domain	Polyphen 2	SIFT	Reference
Splicing mutations						
c.106-1G>C	Int 1	p.(S36_C37delfs*102)	-	-	-	Novel
c.1309-?G>A	Int 11	-	-	-	-	Ref 30
Indels						
c.82dupT	Ex 1	p.W28Lfs*111	Signal peptide	-	-	Ref 23
c.4143dupA	Ex 29	p.E1382Rfs*6	CUB-2	-	-	Ref 5
c.291_319del	Ex 3	p.E98Pfs*31	Metalloprotease	-	-	Ref 29
c.825-?_?del	Int8_ex8	-		-	-	Ref 30
c.718_724del	Ex 7	p.S240Afs*7	Metalloprotease	-	-	Ref 24
c.2930_2935del	Ex 23	p.C977_R979delinsW	TSP1-6	-	-	Ref 28
c.3254-3255del	Ex 25	p.S1085Cfs*12	TSP1-8	-	-	Ref 30
Nonsense						
c.2728C>T	Ex 21	p.R910*	TSP1-5	-	-	Ref 5
c.3047G>A	Ex 24	p.W1016*	TSP1-7	-	-	Ref 23
c.3616C>T	Ex 26	p.R1206*	CUB-1	-	-	Ref 8
Missense						
c.237C>G	Ex 3	p.I79M	Metalloprotease	Ben	Tolerated	Ref 30
c.262G>A	Ex 3	p.V88M	Metalloprotease	Prod	Affect	Ref 27
c.304C>T	Ex 3	p.R102C	Metalloprotease	Prod	Tolerated	Ref 2
c.428T>C	Ex 5	p.I143T	Metalloprotease	Prod	Affect	Novel
c.448T>C	Ex 5	p.S150P	Metalloprotease	Prod	Affect	Novel
c.578G>A	Ex 6	p.R193Q	Metalloprotease	Prod	Affect	Novel
c.607T>C	Ex 6	p.S203P	Metalloprotease	Prod	Tolerated	Ref 30
c.703G>T	Ex 7	p. D235Y	Metalloprotease	Prod	Affect	Novel
c.706G>T	Ex 7	p.G236C	Metalloprotease	Prod	Affect	Novel
c.803G>C	Ex 7	p.R268P	Metalloprotease	Ben	Tolerated	Ref 3
c.1308G>C	Ex 11	p.Q436H	TSP1-1	Prod	Tolerated	Novel
c.1520G>A	Ex 13	p.R507Q	Cysteine-rich	Prod	Tolerated	Ref 30
c.1787C>T	Ex 16	p.A596V	Spacer	Prod	Tolerated	Ref 30
c.2272T>C	Ex 19	p.C758R	TSP1-3	Prod	Affect	Ref 30
c.3178C>T	Ex 24	p.R1060W	TSP1-7	Prod	Affect	Ref 25
c.3251G>A	Ex 25	p.C1084Y	TSP1-8	Prod	Affect	Novel
c.3283C>T	Ex 25	p.R1095W	TSP1-8	Prod	Affect	Novel
c.3367C>T	Ex 25	p.R1123C	TSP1-8	Prod	Affect	Ref 23
c.3716G>T	Ex 27	p.G1239V	CUB-1	Prod	Affect	Ref 27

Ben, predicted benign; Prod, predicted to be probably damaging.

Table 3. Association between residual ADAMTS13 activity and phenotypic outcomes.

Phenotype	**ADAMTS13 activity, %**
Age of disease onset, years[a]	
beta (95% CI)	3.2 (1.4 to 5.1)
R^2	0.3
p-value	0.001
Total number of lifetime TTP episodes[a]	
beta (95% CI)	-2.1 (-4.5 to 0.3)
R^2	0.1
p-value	0.082
Annual rate of TTP episodes[a]	
beta (95% CI)	-0.3 (-0.5 to -0.1)
R^2	0.3
p-value	0.003
Multiple TTP episodes[b]	
OR (95% CI)	0.8 (0.5 to 1.2)[c]
p-value	0.302
Neonatal jaundice and/or thrombocytopenia[b]	
OR (95% CI)	0.9 (0.6 to 1.2)[c]
p-value	0.478
Persistence of renal/neurological damage[b]	
OR (95% CI)	2.0 (0.9 to 4.7)[c]
p-value	0.105
Regular FFP prophylaxis[b]	
OR (95% CI)	0.6 (0.4 to 0.9)[c]
p-value	0.030

a On linear regression analysis.

b On logistic regression analysis.

c Per 1% increase in ADAMTS13 activity.

FFP, fresh frozen plasma; TTP, thrombotic thrombocytopenic purpura; OR, odds ratio; CI, confidence interval.

Table 4. Receiver operating characteristic curve analysis.

Phenotype	Age of disease onset <18 years	Annual rate of TTP episodes >1	Regular FFP prophylaxis
ADAMTS13 activity cut-off, %	<2.74	<1.61	<2.74
Area under the curve (95% CI)	0.88 (0.75-1.01)	0.93 (0.83-1.03)	0.75 (0.56-0.94)
p-value	0.001	0.003	0.023
Sensitivity	92.3%	78.3%	70.6%
Specificity	81.3%	100%	75.0%
OR (95% CI)	52 (4.7-570.5)	NC	7.2 (1.3-38.3)

FFP, fresh frozen plasma; TTP, thrombotic thrombocytopenic purpura; OR, odds ratio; CI, confidence interval; NC, non-calculable.

Supplementary Material

Supplementary Material S1. Individual clinical and genetic information on study participants.

Part 1: *ADAMTS13* mutations and genotype score.

Cohort	Patient number	Mutation	Mutation classification N-/C-terminal	Mutation severity score (=1 for N-terminal and =0 for C-terminal)	Genotype severity score (sum of mutation severity scores)
Bergamo	1	p.E1382Rfs*6	C	0	0
		p.E1382Rfs*6	C	0	
Bergamo	2	p.R1123C	C	0	0
		p.R1123C	C	0	
Bergamo	3	p.W1016*	C	0	1
		p.G236C	N	1	
Bergamo	4	p.W28Lfs*111	Not classified	NA	NA
		p.R1060W	C	0	
Bergamo	5	p.W28Lfs*111	Not classified	NA	NA
		p.R1060W	C	0	
UK	6	p.R1060W	C	0	0
		p.R1060W	C	0	
UK	7	p.R1206*	C	0	0
		p.R1060W	C	0	
UK	8	p.R1060W	C	0	0
		p.R1060W	C	0	
UK	9	p.R1060W	C	0	0
		p.R1060W	C	0	
UK	10	p.S240Afs*7	Not classified	NA	NA
		p.R910*	C	0	
UK	11	p.S240Afs*7	Not classified	NA	NA
		p.R910*	C	0	
UK	12	p.S240Afs*7	Not classified	NA	NA
		p.R910*	C	0	
France	13	p.R507Q	N	1	2
		p.A596V	N	1	
France	14	c.825-?_?del	Not classified	NA	NA
		p.C758R	C	0	
France	15	p.I79M	N	1	2
		p.R268P	N	1	
France	16	p.A596V	N	1	2
		p.A596V	N	1	
France	17	p.S1085Cfs*12	C	0	1
		p.S203P	N	1	
Milan	18	p.V88M	N	1	1
		p.G1239V	C	0	
Milan	19	p.E98Pfs*31	Not classified	NA	NA
		p.E1382Rfs*6	C	0	
Milan	20	p.C977_R979delinsW	C	0	0
		p.C977_R979delinsW	C	0	
Milan	21	p.I143T	N	1	2
		p.I143T	N	1	
Milan	22	p.Q436H	N	1	2
		p.Q436H	N	1	
Milan	23	p.R1060W	C	0	0
		p.R1060W	C	0	
Milan	24	p.R102C	N	1	2
		p. D235Y	N	1	
Milan	25	p.C977_R979delinsW	C	0	0
		p.C977_R979delinsW	C	0	
Milan	26	p.R193Q	N	1	1
		p.R1095W	C	0	
Milan	27	p.(S36_C37delfs*102)	Not classified	NA	NA
		p.(S36_C37delfs*102)	Not classified	NA	
Milan	28	p.S150P	N	1	2
		p.S150P	N	1	
Milan	29	p.A596V	N	1	1
		p.C1084Y	C	0	

Part 2: ADAMTS13 measurement by SELDI-TOF mass spectrometry.

Cohort	Patient number	ADAMTS13 Activity°, %	A13:Act Replicate 1, %	A13:Act Replicate 2, %	A13:Act Difference in the two replicates, %
Bergamo	1	4.46	4.00	4.91	0.91
Bergamo	2	6.77	6.04	7.49	1.45
Bergamo	3	1.01	0.97	1.04	0.07
Bergamo	4	3.47	3.73	3.20	0.53
Bergamo	5	3.89	3.64	4.14	0.50
UK	6	4.73	4.66	4.79	0.13
UK	7	3.83	3.82	3.84	0.02
UK	8	6.37	5.82	6.93	1.11
UK	9	5.98	5.82	6.14	0.32
UK	10	0.54	0.52	0.56	0.04
UK	11	0.57	0.57	0.56	0.01
UK	12	1.42	1.45	1.39	0.06
France	13	3.49	3.34	3.65	0.31
France	14	6.67	6.27	7.07	0.80
France	15	2.39	2.16	2.61	0.45
France	16	4.97	4.71	5.23	0.52
France	17	2.39	2.47	2.31	0.16
Milan	18	5.14	5.27	5.02	0.25
Milan	19	1.96	1.96	1.96	0.00
Milan	20	3.08	3.07	3.08	0.01
Milan	21	0.25	0.25	0.25	0.00
Milan	22	0.25	0.25	0.25	0.00
Milan	23	5.94	5.89	5.98	0.09
Milan	24	1	0.94	1.06	0.12
Milan	25	1.79	1.71	1.87	0.16
Milan	26	5.53	5.69	5.38	0.31
Milan	27	0.67	0.65	0.70	0.05
Milan	28	1.28	1.27	1.29	0.02
Milan	29	0.25	0.25	0.25	0.00

° Measured by SELDI-TOF mass spectrometry.

Part 3: Clinical phenotype.

Cohort	Patient number	Gender	Age of onset	Annual Rate of TTP episodes	FFP prophylaxis
Bergamo	1	M	18	0.72	1
Bergamo	2	M	21	Na*	0
Bergamo	3	M	5	0.17	0
Bergamo	4	F	20	0.11	0
Bergamo	5	F	28	0.03	0
UK	6	F	24	0.04	0
UK	7	F	30	0.09	0
UK	8	F	29	0.03	0
UK	9	F	32	0.05	0
UK	10	M	0.16	3.83	1
UK	11	M	0.16	2.31	1
UK	12	M	0.16	4	1
France	13	F	0.16	0.37	1
France	14	M	0.16	0.33	1
France	15	F	0.16	0.77	1
France	16	F	0.16	0.57	0
France	17	F	0.16	0.33	0
Milan	18	M	23	0.37	0
Milan	19	F	15	0.09	1
Milan	20	M	22	0.14	0
Milan	21	M	11	0.23	0
Milan	22	M	0.16	3.33	1
Milan	23	F	18	0.07	0
Milan	24	F	2	0.56	0
Milan	25	M	29	0.03	0
Milan	26	F	32	0.03	0
Milan	27	F	0.16	0.75	1
Milan	28	F	5	0.13	Na**
Milan	29	M	1	2.57	1

* Chronic jaundice and thrombocytopenia.

** Current prophylaxis status unknown.

Supplementary Material S2. List of protein sequences, functionally related with ADAMTS13, used by SIFT to predict the weighted probability of aminoacid substitutions.

gi16306598, gi355567362, gi296191110, gi332833255, gi355752956, gi332255397, gi335281186, gi354499351, gi301770673, gi47076321, gi76671947, gi348574536, gi334311967, gi351702668, gi291416170, gi337298368, gi301627601, gi47214646, gi170587999, gi327277760, gi324499880, gi312073463, gi341881902, gi187036667, gi189310657, gi308481215, gi194225985, gi344276566, gi224073646, gi326930462, gi345305489, gi363740569, gi291233410, gi293348901, gi156554461, gi307177515, gi242016083, gi326670284, gi317419046, gi156393647, gi348516300, gi115961217, gi297674911, gi208967601, gi328712328, gi332022171, gi340729183, gi350417726, gi221130399, gi328791471, gi307204909, gi157110122, gi198434768, gi241752359, gi229286595, gi125980875, gi195162497, gi270002066, gi347965338, gi321479334, gi195043400, gi195131919, gi195340560, gi281359911, gi195396533, gi194888962, gi194764322, gi195448967, gi55249587, gi195501264, gi357614481, gi157278173, gi313245795, gi34452244, gi170069658, gi195996845, gi112983432, gi268580489, gi195574509, gi6164595, gi290886119, gi13346812, gi256071138, gi170791244, gi358338697, gi339237895, gi312373041, gi301130834, gi322787488, gi218780434, gi320169775, gi307111205, gi340378441, gi323650036, gi226457552, gi146271971, gi110735379, gi139689701, gi339521865, gi13928546.

Supplementary Material S3. Multiple measurements in the same patients on different samples. Similar residual activity was measured in samples collected months apart from the same patients.

Patient NO (see Supplementary Material S1)	ADAMTS13 activity*	A13:Act Replicate 1	A13:Act Replicate 2
9	5.98	5.82	6.14
	5.19	4.97	5.41
26	5.53	5.69	5.38
	6.57	6.81	6.33
27	0.67	0.65	0.70
	0.58	0.58	0.59

***** Measured by SELDI-TOF mass spectrometry.

Supplementary Material S4. ADAMTS13 missense mutations and polymorphisms and their functional annotation.

Protein	SIFT	Polyphen 2	PMut	Align GVGD
TTP associated mutations				
p.I79M	T	BEN	NEUTRAL	C0
p.V88M	A	PROD	NEUTRAL	C15
p.H96D	A	PROD	NEUTRAL	C65
p.R102C	T	PROD	NEUTRAL	C65
p.S119F	A	PROD	NEUTRAL	C65
p.I178T	A	PROD	NEUTRAL	C65
p.R193W	A	PROD	PATHOLOGICAL	C65
p.T196I	A	PROD	NEUTRAL	C65
p.S203P	T	PROD	PATHOLOGICAL	C65
p.L232Q	A	PROD	NEUTRAL	C65
p.H234Q	A	PROD	NEUTRAL	C15
p.D235H	A	PROD	NEUTRAL	C65
p.G236C	A	PROD	NEUTRAL	N/D
p.A250V	A	PROD	NEUTRAL	C65
p.S263C	A	PROD	NEUTRAL	C65
p.R268P	T	BEN	NEUTRAL	C65
p.Y304C	T	PROD	NEUTRAL	C65
p.C311Y	A	PROD	PATHOLOGICAL	C65
p.R312Q	T	PROD	NEUTRAL	C35
p.C322G	A	PROD	NEUTRAL	C65
p.T323R	T	PROD	PATHOLOGICAL	C65
p.F324L	T	PROD	NEUTRAL	C15
p.C347S	A	PROD	NEUTRAL	C65
p.R349C	A	PROD	NEUTRAL	C65
p.P353L	A	PROD	NEUTRAL	C65
p.G385E	A	PROD	PATHOLOGICAL	C65
p.W390C	A	PROD	PATHOLOGICAL	C65
p.R398C	A	PROD	NEUTRAL	C35
p.R398H	A	PROD	PATHOLOGICAL	C25
p.C438S	A	PROD	NEUTRAL	C65
p.R507Q	T	PROD	PATHOLOGICAL	C35
p.C508Y	A	PROD	PATHOLOGICAL	C65
p.G525D	A	PROD	PATHOLOGICAL	C65
p.R528G	T	PROD	PATHOLOGICAL	C65
p.G550R	A	PROD	PATHOLOGICAL	C65
p.A596V	T	PROD	NEUTRAL	C65
p.A606P	T	PROD	PATHOLOGICAL	C25
p.P671L	T	PROD	PATHOLOGICAL	C65
p.I673F	T	PROD	NEUTRAL	C15
p.R692C	T	PROD	NEUTRAL	C65
p.Q723K	T	BEN	PATHOLOGICAL	C45
p.C758R	A	PROD	PATHOLOGICAL	C65
p.C908S	A	PROD	PATHOLOGICAL	C65
p.C908Y	A	PROD	PATHOLOGICAL	C65
p.G909R	A	PROD	PATHOLOGICAL	C65
p.C951G	A	PROD	PATHOLOGICAL	C65
p.C1024R	A	PROD	PATHOLOGICAL	C65
p.C1024G	A	PROD	PATHOLOGICAL	C65
p.R1060W	A	PROD	PATHOLOGICAL	C65
p.R1123C	A	PROD	PATHOLOGICAL	C65
p.C1213Y	A	PROD	PATHOLOGICAL	N/D
p.I1217T	A	PROD	PATHOLOGICAL	N/D
p.R1219W	A	PROD	PATHOLOGICAL	N/D
p.G1239V	A	PROD	PATHOLOGICAL	N/D
p.R1336W	A	PROD	PATHOLOGICAL	N/D
ADAMTS13 polymorphisms				
p.R7W	T	BEN	PATHOLOGICAL	C65
p.T339R	T	PROD	NEUTRAL	C65
p.Q448E	T	BEN	NEUTRAL	C25
p.Q456H	A	BEN	NEUTRAL	C15
p.P457L	T	PROD	NEUTRAL	C65
p.P475S	T	BEN	PATHOLOGICAL	C65

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