Online Supplemental Material

ADAMTS13 secretion and residual activity among congenital TTP patients with and without renal impairment

Supplemental Methods

TTP diagnosis

TTP was diagnosed in all cases reported to have one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of hematocrit (Ht)<30%, hemoglobin (Hb)<10g/dl, platelet count <150,000/µl, serum lactate dehydrogenase (LDH)>460 IU/l, undetectable haptoglobin and presence of fragmented erythrocytes in the peripheral blood smear, with or without neurological symptoms (coma, convulsions, motor deficits, headache, migraine, visual disorders and/or altered mental states) and with or without acute renal impairment during bouts, including acute renal injury (diagnosed in the presence of serum creatinine >0.5 mg/dl in 1-5 years old children, >0.8 mg/dl in 5-10 years old children, and >1.2 mg/dl in >10 years old children and in adults or when serum urea >16 mg/dl in children till 1 year old, and >20 mg/dl in all the other patients), need for dialysis and/or urinary abnormalities (diagnosed in the presence of hematuria and/or proteinuria) in patients with previously normal renal function. Congenital TTP was diagnosed in patients with a severe deficiency of ADAMTS13 activity [<6% as measured by the Collagen Binding Assay (CBA, (1)) or <3% by Fluorescence Resonance Energy Transfer (FRETS-rVWF73, (2))] in remission and absence of inhibitory anti-ADAMTS13 auto-antibodies as tested by CBA, Western Blotting and/or ELISA(3,4). Congenital TTP patients were screened for ADAMTS13 mutations and patients with one homozygous or two heterozygous mutations were included in the study. The onset of the disease was defined as the first episode of thrombocytopenia and microangiopathic hemolytic anemia requiring or not plasma infusion. Neonatal and childhood records were examined to find out possible episodes of early thrombocytopenia that were excluded in patients with adult onset.

Genetic screening

Coding sequences and intronic flanking regions of *ADAMTS13*, *CFH*, *MCP* and *CFI* were genotyped by automatic DNA sequencing (AB-3730 DNA Analyzer), as previously described (5,6).

ADAMTS13 antigen

Plasma ADAMTS13 antigen levels were evaluated by commercial (IMUBIND® ADAMTS13 ELISA, American Diagnostica Inc.) or home-made ELISA (4).

Construction of rADAMTS13 plasmid

Experimental studies on rADAMTS13 mutants were performed at the IRCCS-Istituto di Ricerche Farmacologiche "Mario Negri" laboratories with the exception of the I143T mutant (studies performed at the IRCCS-Fondazione Ca' Granda Ospedale Maggiore Policlinico laboratories).

Two micrograms total human liver RNA (Stratagene) were reverse transcribed using the SuperScript First-Strand synthesis system (Invitrogen) and random primers, as required by manufacturer's instructions. A cDNA construct covering the complete ADAMTS13sequence was amplified using high fidelity PfuTurbo Hotstart DNA polymerase (Stratagene) as previously described (7). The PCR product was recombined with plasmid pDONR 201 and BP clonase to prepare entry clone according to standard protocol (Invitrogen). Mammalian expression vector coding Wild-type rADAMTS13 (WT-rADAMTS13) was prepared by recombination of entry clone with destination vector pcDNA-DEST40 and LR Clonase (Invitrogen).

To create the I143T mutant a pcDNA 3.1/V5-His TOPO®TA vector (Invitrogen, Carlsbad, CA, USA) containing the complete ADAMTS13 cDNA (kindly provided by Dr. F. Scheiflinger, Baxter Bioscience, Vienna, Austria) was used.

ADAMTS13 mutagenesis

To create I143F, I143T, D235Y, Q429X, G761S, R1060W, R1095Q and D1345Tfs14X mutants, single nucleotide point mutations were introduced by the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). WT-rADAMTS13 cloned in pcDNA-DEST40 or in pcDNA3.1/V5-His (for I143T) expression vectors was used as template.

Each mutant plasmid was subjected to DNA sequence analysis in order to confirm the mutation.

***In vitro* expression of rADAMTS13**

Each expression vector was transfected into Human Embryonic Kidney 293T (HEK293T) cells using Lipofectamine 2000 (Invitrogen). HEK293T cells were cultured in DMEM with 10% FBS, 2 mM L-Glut and 100 U/ml-100 μg/ml Pen-Strep. Five hours after transfection the medium was changed and serum-free DMEM was applied for 48 hours. Conditioned medium was collected and concentrated 20-fold using Amicon Ultra-4 100 kDa MWCO (Millipore). Cytoplasmatic proteins were extracted using NE-PER® Extraction Reagents according to standard protocols (Pierce/Celbio). Untransfected cells were used as negative controls. For each rADAMTS13 mutant, three transfections were performed (7).

In order to characterize the I143T mutant the vector was expressed as previously described (8) with minor modifications. HEK 293T cells were transfected with 33 μg of WT- or mutant rADAMTS13

DNA and with 1µl of the pRL-TK plasmid to normalize the transfection efficiency. Cells were transfected using 10 mM polyethylenimine (PEI) (Polysciences Inc.). Supernatant samples were concentrated 20-fold.

Characterization of rADAMTS13

Cytoplasmatic extracts or culture media of HEK293T cells transfected with WT or mutant rADAMTS13 were subjected to reducing 6% SDS-PAGE and transferred to a poly(vinylidenedifluoride) membrane (GE Healthcare). After blocking with 5% non-fat dry milk, the membranes were incubated with polyclonal goat anti-ADAMTS13 antibody A300-002A (1:5,000, Bethyl Laboratories) and then with HRP-labeled rabbit anti-goat IgG, 1:10,000 (Sigma-Aldrich). Samples of I143T mutant were loaded onto 4-12% Bis-Tris polyacrylamide gels (Bio-Rad Laboratories). Protein was then transferred onto 0.45µm nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% milk for 1 hour and then incubated with a mouse anti-V5 monoclonal antibody (1:2,000, Invitrogen) against the C-terminal V5 tag. Membranes were then incubated with an ECL anti-mouse IgG HRP antibody (1:500, GE Healthcare).

Membranes were developed using ECL detection systems (Pierce or Thermo Scientific). The luminograms were scanned, and the relative amount of recombinant proteins detected was estimated by densitometry using NIH Image J (NIH). Expression levels of mutant proteins were evaluated by dividing the densitometric value of the mutant band by the densitometric value of WT band x 100 and by ELISA.

Recombinant ADAMTS13 activity assays

Recombinant ADAMTS13 activity was evaluated by CBA, FRETS-rVWF73, and by cleavage of recombinant VWF (rVWF) fragments. A mixture of purified rVWF fragment (10 ng/µl, f.c.) comprising A1-A2-A3 domains (residues 508 to 1111) of the mature VWF subunit (7) and HEK293T cell supernatants containing equal amounts of mutant or WT-rADAMTS13 was incubated and processed as described (3). The amount of proteolytic fragment was calculated by densitometric analysis. Mutant rADAMTS13 activity levels were obtained by dividing the densitometric value of the rVWF A1-A2-A3 cleavage fragment formed by the mutants by the densitometric value of the rVWF A1-A2-A3 cleavage fragment formed by the WT x 100. The results were expressed as the mean of n=3 experiments.

Residual ADAMTS13 activity

Residual rADAMTS13 activities in cell supernatant was calculated multiplying the activity of each mutant (percentage of activity in respect to WT protein) in the rVWF A1-A2-A3 cleavage test for the corresponding percentage of secretion (with respect to WT protein). For compound heterozygous patients half percentage of secretion was considered for the mutants to reflect their expression by a single allele and the sum of residual activities of the two mutants was calculated. As an example, for the mutations found in patient F48#002 the calculated residual activity was 6% considering 0% for the p.G1239V + 6% [40% activity x (30% secretion/2)] for the p.V88M.

Cleavage of rVWF A1-A2-A3 by patients' serum ADAMTS13

This is a modification of a method reported by Tripodi et al. (3,9). Purified A1-A2-A3 domains of rVWF (30 ng/µl) and 13 µl of patients' serum or serial dilutions of Normal Human Serum (NHS) pool were incubated for 6 hours at 37°C in 5 mM Tris saline buffer with 1.5 M Urea, pH 8.0, and 3 mM $BaCl_2$ (80 µl final volume). The cleavage was stopped by 5.7 µl of 0.825 M Na_2SO_4. Fifteen microliters of the reaction were subjected to 10% SDS-PAGE and transferred to a poly(vinylidenedifluoride) membrane. After blocking with 0.5% non-fat dry milk, the membrane was incubated with a mAb against the rVWF carboxy-terminal (kindly provided by Dr. Z.M. Ruggeri, The Scripps Research Institute, CA) (10) and then with HRP-labeled rabbit anti-mouse IgG (1:2,000, Histoline Laboratories) (3). The membrane was developed using 3,3'-diaminobenzidine (Merck).

The patients' ADAMTS13 activity was determined by standardization with a reference NHS pool defined as having 100% protease activity (3). The standard curve was obtained for each cleavage assay by testing serial dilutions (from 1:4 to 1:128) of the reference NHS pool, which correspond to 25% to 0.78% ADAMTS13 activity (Supplemental Fig.3A).

The amount of proteolytic fragment formed (about 30 KDa) from rVWF A1-A2-A3 cleavage by either patient's serum or NHS pool was calculated by densitometric analyses with NIH Image J. The plotting of densitometric values of the proteolytic fragment obtained by cleavage with serial dilutions of NHS pool resulted in a sigmoid curve with an R^2=0.96 (Supplemental Fig.3B). The lowest limit of sensitivity of the assay with intra- and inter-assay coefficients of variation (CV)<20% (15% and 17%, respectively, according to the standard formula: %CV=SD/mean x 100) was 0.78% activity in NHS pool.

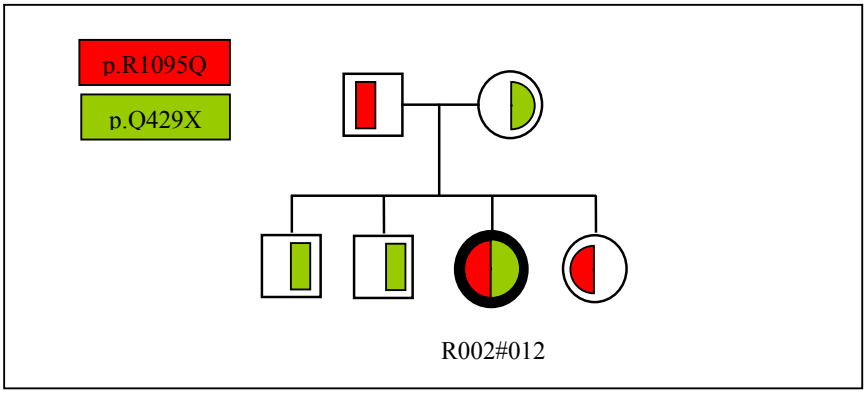
ADAMTS13 activity in patients' serum was extrapolated from the sigmoid curve and the results were expressed as the mean of n=3 experiments.

Patients' case report

Patients developing acute renal impairment during bouts

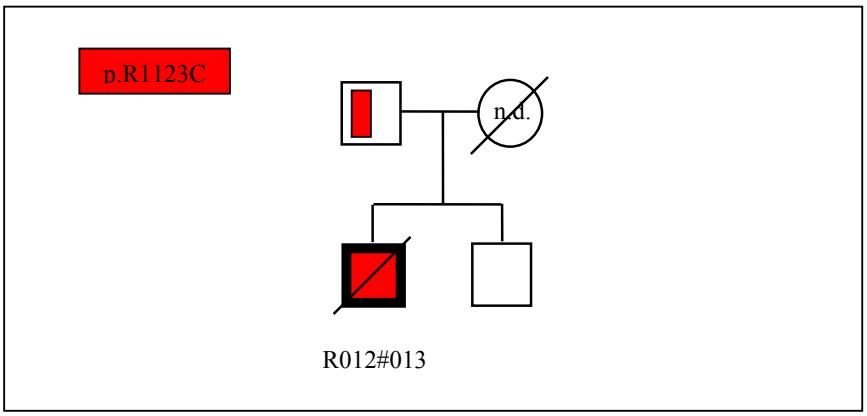
Patient R002 is a 20-year-old woman from North Italy, previously described (7). At birth, the patient had neonatal jaundice treated with whole-blood exchange transfusion. At 3 months she had her first episode of TTP, triggered by bronchiolitis, that improved with plasma infusion. Subsequently, she had repeated relapses accompanied by acute renal impairment (microhematuria, gross hematuria, proteinuria and mild renal failure; creatinine 0.5-0.7 mg/dl), which became monthly since the age of 5 years. Neurological symptoms were not apparent during the first episode, but became dominant subsequently needing prophylaxis with anti-epileptic drugs. At the age of 10 years a regimen of regular fresh frozen plasma (rFFP) infusions every 2-3 weeks was established to maintain clinical and hematological remission. Attempts to lengthen the FFP infusion interval resulted in prompt relapse of thrombocytopenia and neurological/renal symptoms. At the last follow-up the patient was hematologically stable under rFFP infusion every 2 weeks with normal renal findings, but with mild neurological sequelae (paresthesias). Her parents are unrelated and healthy.

The patient carries two heterozygous ADAMTS13 mutations: the p.R1095Q and the p.Q429X inherited from the father and the mother, respectively. The p.R1095Q was also found in the healthy sister, while two healthy brothers carry the p.Q429X.



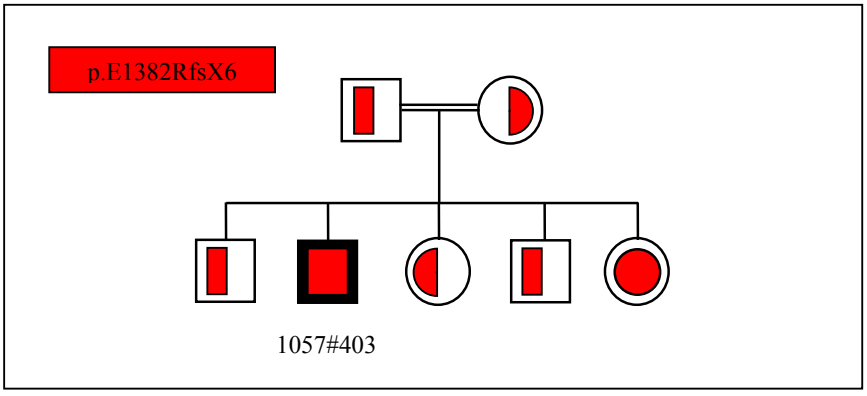
Patient R012 was previously described (7). At birth, the patient had jaundice and fever with a platelet count of 37,000/µl and hematocrit 20%, diagnosed as idiopathic neonatal jaundice and treated with whole-blood exchange transfusion. At the age of 3, 4, 5 and 15 years, he experienced 4 similar episodes of thrombocytopenia and hemolytic anemia, triggered by infection and associated with acute renal insufficiency (serum creatinine greater than 1 mg/dl) and proteinuria. Neurological symptoms were not apparent during these episodes, but became dominant after the age of 21 years when TTP episodes occurred at monthly intervals. Hematological remission was always achieved with plasma exchange or infusion. Since the age of 27 years the patient developed chronic kidney disease (creatinine 1.4-1.8 mg/dl). Regular FFP infusions every two weeks started at the age of 30 years preventing other bouts; nevertheless renal function progressively deteriorated (creatinine range 2.4-3.7 mg/dl) and at the age of 32 years the patient reached end stage renal failure. At the age of 37 years, the patient was at imminent risk of death since he had no further vascular access for extracorporeal dialysis. Peritoneal dialysis was contraindicated for intestinal localization of microangiopathic lesions and also gastrointestinal symptoms, during relapses. In view of the fact that the liver is the major source of ADAMTS13, a combined kidney and liver transplant was performed with the aim to correct the deficiency of ADAMTS13 and prevent relapse of the disease. In the pre-operative period the patient underwent a prophylactic treatment with daily plasma infusion. Nevertheless, the post-operative course was dramatic: the liver and kidney grafts never functioned, the patient continued to be treated with hemodialysis and plasma infusion but he rapidly developed cerebral complications and coma and died within a few days. The autoptic examination showed widespread thrombi disseminated in all organs, cerebral edema, ischemic necrosis of the kidney and hemorrhagic necrosis of the liver.

His parents were unrelated. The patient carried the p.R1123C homozygous ADAMTS13 mutation that was also found in heterozygosity in the healthy father. The mother died after car accident at the age of 32 years and DNA was not available for genetic analysis. Uniparental isodisomy was excluded by finding heterozygosity in the D9S290 and in the D9S1838 microsatellite markers flanking to ADAMTS13 gene (7). Gross gene deletions were excluded by fluorescence in situ hybridization analysis.



Patient R1057 (11) is a 40-year-old man (from North Italy), who was firstly hospitalized at the age of 7 years with a diagnosis of idiopathic thrombocytopenic purpura and tonsillitis. Since the age of 18 years he developed a relapsing form of TTP, often triggered by upper respiratory tract infections, with acute renal failure and neurological involvement but prompt response to plasmapheresis. However, renal function deteriorated during relapses and since the age of 30 years the patient developed chronic kidney disease (creatinine ranging 1.74-2.8 mg/dl) with proteinuria. At the age of 32 years he also manifested ischemic ictus and seizures during TTP episodes, needing prophylaxis with anti-epileptic drugs. At the age of 36 years, after the thirtieth relapse, a regimen of rFFP infusions every 2-3 weeks was established to maintain clinical and hematological remission. Attempts to lengthen the rFFP infusion interval resulted in further relapses. At the last follow-up the patient was hematologically stable under FFP infusion every 3 weeks, but with chronic kidney disease (creatinine 3.28 mg/dl) and neurological sequelae.

The patient carries the p.E1382RfsX6 homozygous ADAMTS13 mutation, found in heterozygosity both in the healthy father and in the healthy mother that are first cousins. The p.E1382RfsX6 mutation was found in homozygosity in his youngest sister (22 years old) who did not manifest acute episode of TTP up to now. Of note, she has not been pregnant yet. All the other siblings of the patient are healthy and carry the heterozygous p.E1382RfsX6 mutation.

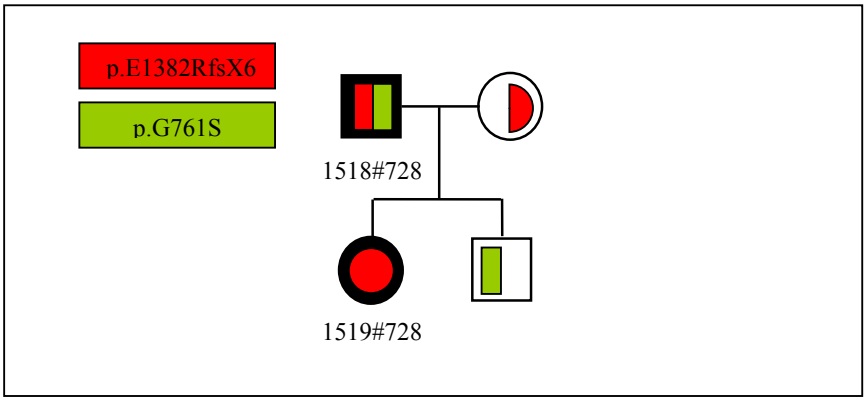


Patient F1519#728 is a 19-year-old girl from Poland and ***patient F1518#728*** is her 53-year-old father. The F1519#728 patient had neonatal jaundice at birth without need of whole-blood exchange transfusion and developed her first episode of TTP at 3.5 years of age, triggered by upper respiratory tract infection and fever, with neurological signs (aphasia and disturbances of consciousness) and acute renal failure (creatinine 1.9 mg/dl) needing dialysis treatment for one week. She manifested also proteinuria (about 1 g/24h) and microhematuria. Plasma infusion

induced normalization of hematologic parameters and clinical signs. Thereafter a regimen of rFFP infusions every 3 weeks was established to maintain clinical and hematological remission. Nevertheless the patient experienced other 3 episodes of TTP, always triggered by infections. After the last episode, at the age of 10 years, the rFFP infusion interval was shortened at 2 weeks without other relapses. At the last follow-up, the patient was in complete remission.

Consanguinity of the parents was denied. Patient F1519#728 carries a homozygous p.E1382RfsX6 mutation that was inherited from the heterozygous parents. Interestingly, also the father (F1518#728)was affected by TTP but was found to be compound heterozygous for the p.E1382RfsX6 and the p.G761S ADAMTS13 mutations. He manifested firstly the disease at the age of 35 years and since that time he had 15 relapses (almost one every year), always successfully treated with plasma infusions. Since the last episode in 2010, he was treated with rFFP every 2 weeks. Of note, in 2003 he had acute renal failure (creatinine 2.1-2.3 mg/dl), proteinuria (1.2-1.4 g/24h) and microhematuria during bout, and in subsequent relapses (2008/2010) he manifested aphasia, hemiparesis and episodes of stroke. At the last follow-up, he showed chronic kidney disease (creatinine 2 mg/dl).

The healthy brother of the proband carries the p.G761S in heterozygosity.



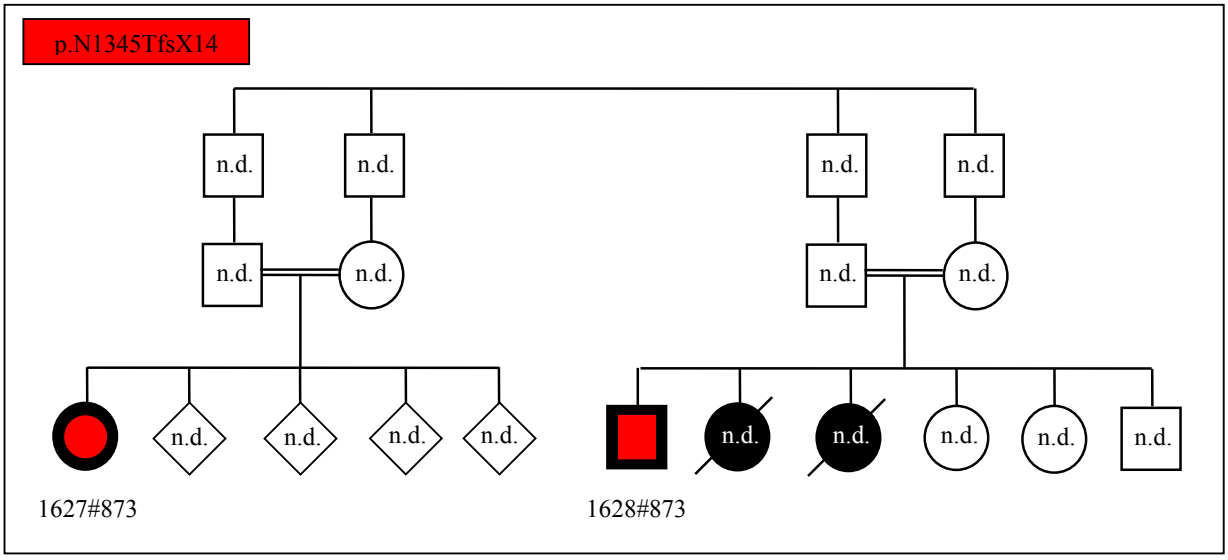
Patient F1627#873 (9 year-old female) and ***F1628#873*** (24 year-old male) are cousins of Arab origin from Israel.

Patient F1627#873was firstly admitted at the age of 22 months with hemolytic anemia, thrombocytopenia and hematuria, without neurological signs. TTP was diagnosed and she started weekly rFFP infusions with good response. She had a relapse one year later with hematuria and proteinuria. Since that time she receives weekly rFFP without recurrent events. She has 4 healthy siblings. At the last follow-up, she was in complete remission.

Patient F1628#873 had recurrent episodes of hemolytic anemia, thrombocytopenia, macroscopic hematuria and mildly elevated creatinine since birth. He suffered also neonatal jaundice at birth that was treated with whole-blood exchange transfusion. The episodes occurred twice a year. Therapy with intravenously immunoglobulin failed. At the age of 7 years he was admitted with a severe episode of hemolytic anemia, thrombocytopenia and acute renal failure. The diagnosis of TTP was established on the basis of pathologic blood smear and response to FFP therapy. Since that time he is receiving weekly rFFP therapy without recurrent events of TTP.At the last follow-up, he was in complete remission.

He has two healthy sisters and one healthy brother. Two sisters died 12 hours after delivery with a clinical picture of severe hemolytic anemia.

Parents of both patients are first degree cousins. Both the patients carry the p.N1345TfsX14 homozygous ADAMTS13 mutation. The biological samples of any other member of the two families were not available for the genetic screening.

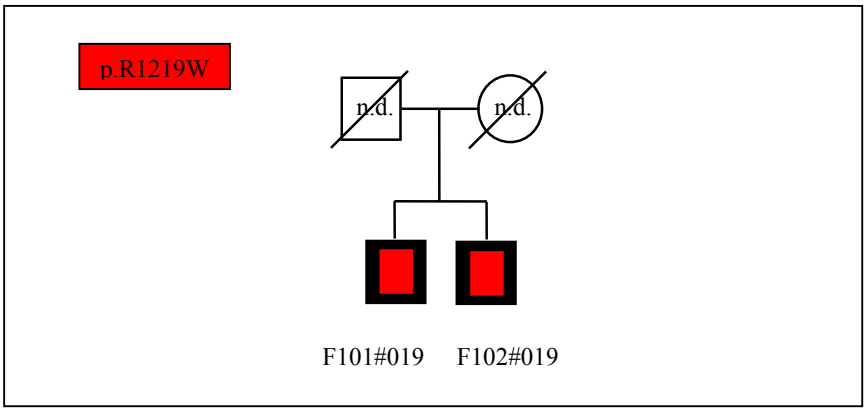


Patients F101#019 and ***F102#019*** are adult brothers from South Italy, previously described (7).

Patient F101#019 manifested his first episode at the age of 35 years, with generalized weakness, vomiting, diarrhea, abdominal pain and severe acute renal failure (creatinine 11.3 mg/dl). Plasma-exchange induced normalization of hematologic parameters and of renal function. Ten years later he had two relapses (without renal impairment: creatinine 1 mg/dl), both treated with plasma exchanges. At the last follow-up, the patient was in complete remission and no further relapse was reported.

Patient F102#019 had the first episode of TTP at the age of 28 years with loss of consciousness, seizures and coma, renal function was normal. The patient achieved remission with plasma exchanges. Six years later he had a relapse with severe acute renal failure (creatinine 12.8 mg/dl) and also neurological involvement, however he recovered with plasma exchange. He was discharged with anti-platelet therapy. He had no further relapse nor sequelae until the last follow-up, when the patient was in complete remission.

The two patients carry the homozygous p.R1219W ADAMTS13 mutation. Their parents are unrelated. The DNA samples of the parents were not available for genetic analysis.

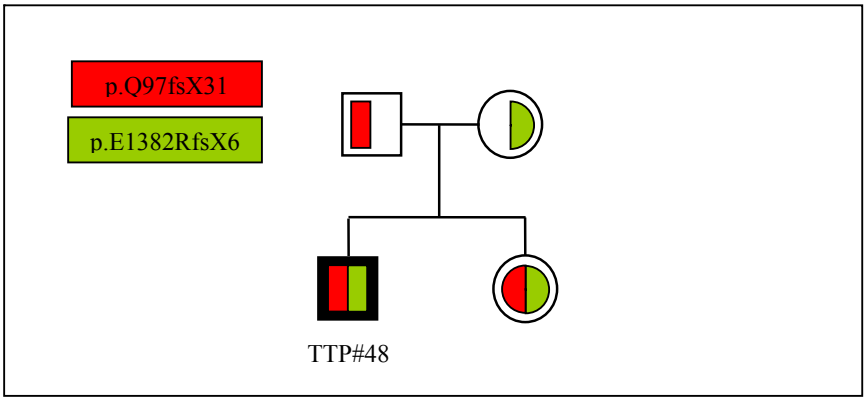


Patient TTP#48 was previously described (8).

A Turkish male patient was hospitalized at the age of 15 years because of a gastrointestinal infection in association with abdominal pain, fever and vomiting. Since that time the patient had a chronic disease state and five years later, he was admitted to the hospital with acute symptoms (purpura, renal failure and decreased platelet counts). Laboratory data on admission were as follows: Coombs-negative hemolytic anemia with schistocytes in the blood smear, hemoglobin 11.7 g/dL, low platelet count (11×10^9/L), high serum levels of lactate dehydrogenase (1809 UI/L), total bilirubin 2.6 mg/dL and creatinine 4.6 mg/dL. Laboratory results and the clinical symptoms confirmed the diagnosis of TTP. Six additional episodes occurred, usually in association with triggers such as infections or alcohol consumption. He was successfully treated with FFP (10 mL/kg) and now receives prophylaxis with one infusion every 3 weeks. His parents are unrelated and healthy.

The patient carries two heterozygous ADAMTS13 mutations: the p.Q97fs31X and the p.E1382RfsX6 inherited from the father and the mother, respectively. Both ADAMTS13 mutations

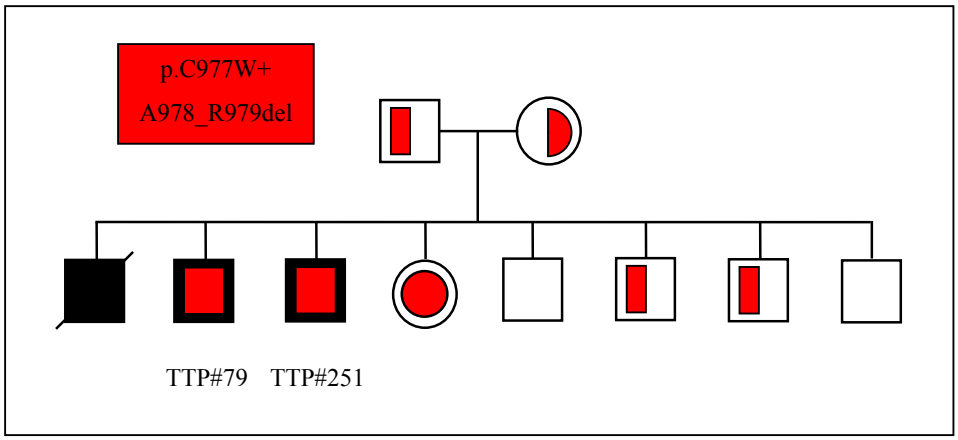
carried by the patient were found in the younger sister of 22 years without an acute episode of TTP up to now.



Patient TTP#79 and TTP#251 were previously described (12).

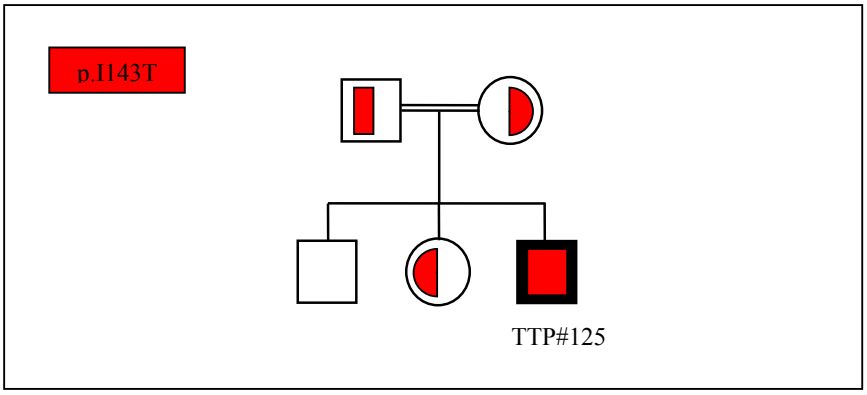
An Iranian male (TTP#79) had his first episode of TTP at the age of 23 years and 6 subsequent recurrent episodes with no precipitating event or triggering agent. Bleeding symptoms such as purpura and petechiae were present at each episode, accompanied by fever and vomiting, whereas mild neurological symptoms (visual disorders and drowsiness) were observed only during the first episode. The clinical diagnosis was established at the time of the first episode by the presence of thrombocytopenia (platelet count no higher than 20×109/L), Coombs negative hemolytic anemia (Hb 10.3 g/L), fragmented erythrocytes and high serum level of lactate dehydrogenase (LDH 1055 IU/L). The patient was successfully treated during each acute episode with plasma exchange and high dose of corticosteroids, before the molecular diagnosis. After his sixth episode of TTP, to prevent further relapses, the patient underwent prophylactic treatment with FFP (30 ml/kg) every three weeks for a period of two years. However, due to his gipsy life-style, he now receives FFP infusion only at disease manifestation. During a recent bout, the patient experienced severe acute renal failure and at the last follow-up he had mild chronic kidney disease. The younger brother of this patient, TTP#251 is also affected by TTP. He developed his first episode at the age of 29 years in association with an episode of pneumonia. Daily FFP infusions (30 mL/kg) were effective as reflected by a progressive increase in the platelet count. The patient manifested several subsequent episodes in two years. Unfortunately, he could not be treated with prophylactic FFP because of his gipsy life-style. Since the onset, he received FFP infusion when his platelet count fell below 100×10^9/L. His clinical information is scanty. This patient has been lost at follow-up, thus he was excluded from this study. According to the family history, the oldest brother had a TTP episode at the age of 23 years and died because of multiorgan failure. Genetic analysis resulted that both patients TTP#79 and TTP#251 carry the p.C977W+A978_R979del ADAMTS13 mutation in

homozygosity. DNA sample from the died oldest brother was not available for genetic analysis. Also the patients' sister (24 years old) carries the p.C977W+A978_R979del in homozygosity, but she remained asymptomatic until now. Of note, she has not been pregnant yet. The same mutation has been found in heterozygosity in the healthy parents and in two out of their four healthy siblings.



Patient TTP#125

Patient TTP#125 is a 26 year-old Turkish male patient. A detailed case report of this patient's clinical history has been described (13). He first presented with a lung infection at 7 years and was diagnosed with pulmonary tuberculosis. The patient's medical history at the time revealed that he had easy bruising and anemia since birth. During a 9 month follow-up for tuberculosis the patient hemoglobin and thrombocyte count remained low and urinalysis revealed the presence of hematuria (8-10 erythrocytes) and trace of proteinuria. At a routine follow-up at the age of 13 years anemia, unconjugated hyperbilirubinemia, thrombocytopenia and the presence of schistocytes on a peripheral blood smear and an elevated level of blood urea (99 mg/dl) were observed and the patient was treated with FFP. During follow-up the presence of a persistently low haptoglobin level of <1.61 mg/dL led to start of prophylactic FFP infusions. The patient is currently treated with regular FFP infusions every 4 weeks. The patient carries the p.I143T homozygous ADAMTS13 mutation. The same mutation was found in heterozygosity in the sister and in both parents that are second cousins. Patient's brother did not carry the mutation.

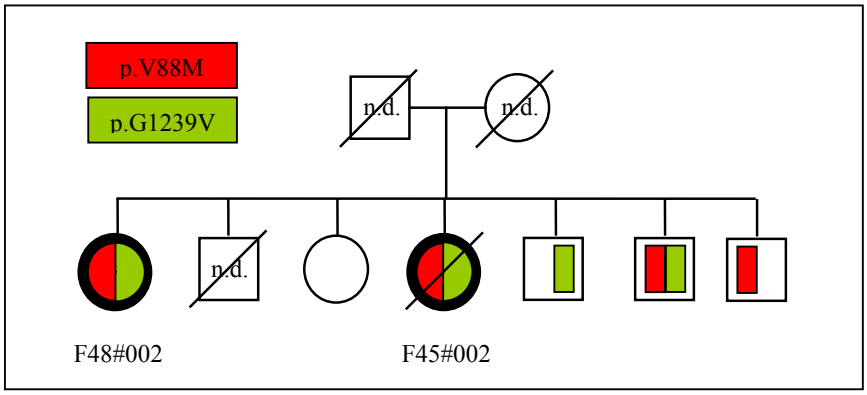

p.I143T
TTP#125

Patients without acute renal impairment during bouts

Patient F48#002, a woman now 69 years old from North Italy, and her younger sister ***F45#002***, experienced their first episode of TTP at the age of 23 (F48#002) and 22 (F45#002) years, respectively triggered by their first pregnancy. They subsequently experienced 10 disease relapses concomitant with precipitating events such as pregnancy, spontaneous abortion in the first to second trimester, or infection. In patient F48#002, neurological symptoms (dysarthria, dyslalia, aphasia, and facial paralysis) were dominant and renal function always remained normal(she had only traces of proteinuria and hematuria but values remained within normal range), while in patient F45#002 the course of the disease was more severe and was associated with neurological signs and acute renalfailure (creatinine 2.3 mg/dl). Transient improvement was achieved by plasma infusions, but then renal function progressively deteriorated (creatinine increased up to 10 mg/dl). At the age of 44 yr, the patient started chronic dialysis. She died at the age of 55 yr because of a cerebrovascular event.

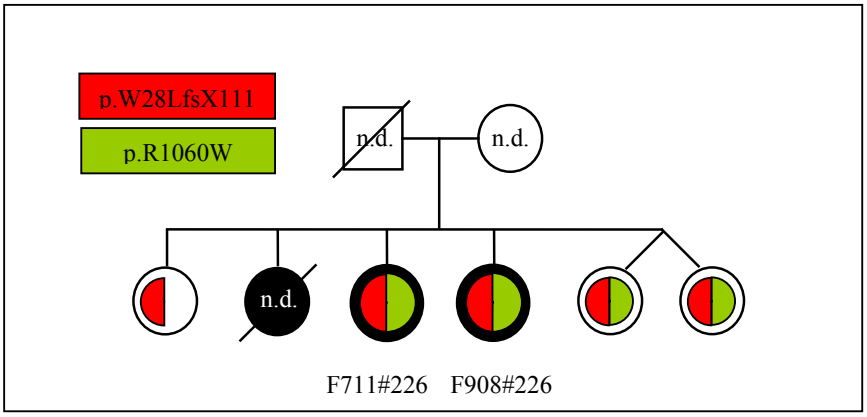
The two patients carry two heterozygous ADAMTS13 mutations: the p.V88M and the p.G1239V. Moreover, the patient F45#002 carried the heterozygous missense variant p.S890I in CFH as previously described in detail (5).

The ADAMTS13 mutations p.V88M and p.G1239V were found in heterozygosity also in a brother who had never manifested the disease up to now. The other two brothers were healthy and were found to carry a single heterozygous mutation, one the p.G1239V and the other the p.V88M. Their only healthy sister does not carry any ADAMTS13 mutation. The DNA samples of the parents and those of a brother who died at the age of 15 years of leukemia were not available for genetic analysis.



Patients F711#226 and ***F908#226*** are 41 and 39 year-old sisters, from Spain, previously described (7). Both experienced TTP during pregnancy: F711#226 had two episodes of TTP during her two pregnancies that resolved after miscarriage; F908#226 had one episode during her first and unique pregnancy resolved after delivery. Both patients developed migraine and mental confusion during the episodes. An older sister who manifested TTP at the age of 26 years during her first pregnancy died post-partum from a massive hemorrhage.

The two patients carry two heterozygous ADAMTS13 mutations: the p.W28LfsX111 and the p.R1060W. The two twin sisters were found to carry both mutations in heterozygosity. However, they did not manifest the disease up to now, probably because they have not been exposed to pregnancy, the triggering event associated with all the TTP episodes of their affected sisters. The oldest sister carries the p.W28LfsX111 in heterozygosity. Their parents were unrelated. The father died at the age of 56 years because of diabetes and cirrhosis. The mother is healthy. The DNA samples of the parents and those of the affected sister died were not available for genetic analysis.

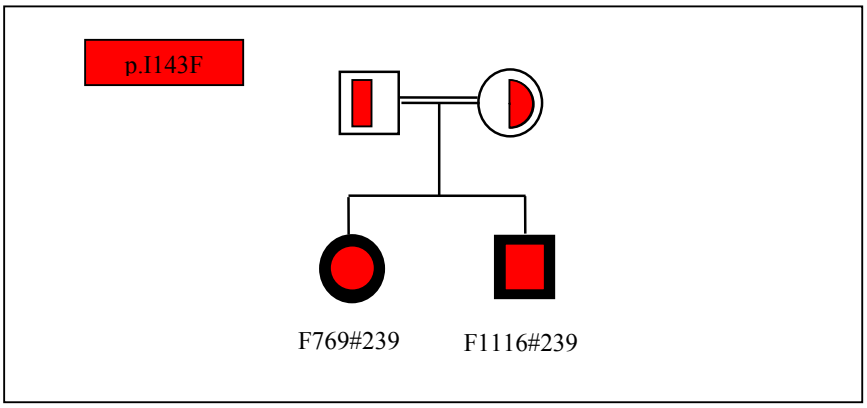


Patients F769#239 and ***F1116#239*** are 37 and 27 year-old siblings, from North Italy. F769#239 was first hospitalized at the age of 21 years because of severe anemia and thrombocytopenia with metrorrhagia and severe neurological signs (coma), developed some days after upper respiratory tract infection and fever, which were treated with antibiotics. Remission was achieved with plasma infusions and blood transfusions. At the age of 29 years, the patient had a relapse of TTP at the fifteenth week of her first pregnancy, forthwith treated with plasma infusions. During the following weeks of pregnancy, the patient was maintained under therapy with weekly and then daily plasma infusions until delivery at the thirty-sixth week, with the birth of a healthy baby. Thereafter, the patient remained in good health without any therapy for at least one year. Subsequently she had

other 4 relapses in 7 year follow-up triggered by gastroenteritis, always treated with plasma infusions. At the last follow-up, she was in complete remission.

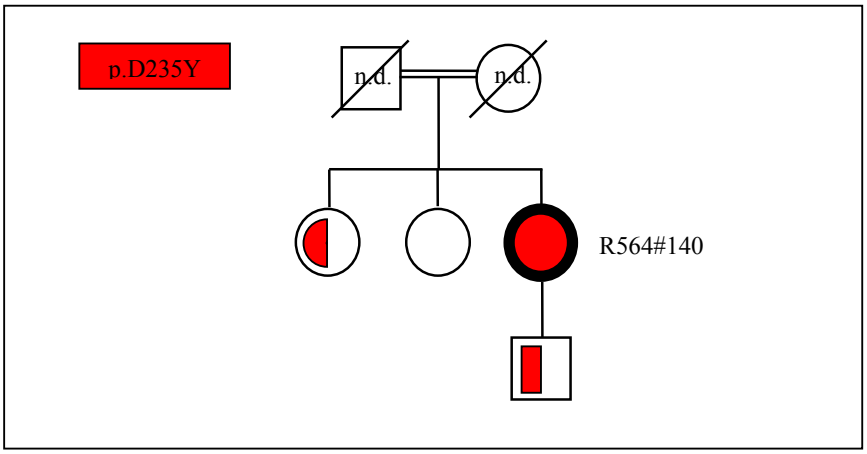
The brother F1116#239 manifested his first episode of TTP at the age of 18 years, triggered by upper respiratory tract infection and fever. Thereafter he had three relapses in 8 year follow-up triggered by gastroenteritis or upper respiratory tract infection and quickly improved by plasma infusions. He manifested neurological signs (paresthesias) during relapses. At the last follow-up, he was in complete remission.

The two patients carry the homozygous p.I143F ADAMTS13 mutation inherited from the healthy father and the healthy mother that are first cousins and carry the mutation in heterozygosity.



Patient R564 is a 42-year-old-woman (from South Italy). She had her first episode of thrombocytopenia and hemolytic anemia at the age of 24, triggered by her first and unique pregnancy and associated with mild neurological signs (cephalea). Remission was achieved with plasma infusions. After the onset, the patient underwent a maintenance therapy with periodic plasma infusions for two years. Thereafter, the patient remained in good health without any therapy until she experienced two relapses at the age of 32, triggered by gastroenteritis and again associated with mild neurological signs, quickly improved by plasma infusions. Since this time she had other four relapses in 9 year follow-up always treated with plasma infusions obtaining complete remission.

The patient carries the p.D235Y homozygous ADAMTS13 mutations. Her parents were first cousins; they died for unknown reasons and their DNA was not available for genetic analysis. In one out of two patient's healthy sisters and in her healthy son the p.D235Y mutation was found in heterozygosity.


p.D235Y
n.d.
n.d.
R564#140

Supplemental Results

Mutation screening results

Here we report the genetic ADAMTS13 defect identified in the eight patients not previously screened for *ADAMTS13* (R002, F1518#728, F1519#728, F1627#873, F1628#873, F769#239, F1116#239, and R564).

A nonsense c.1285C>T mutation in exon 11 causing a p.Q429X (TSP1-1) was found in heterozygosity in patient R002. The mutation was inherited from the healthy mother. In this patient an additional missense mutation (c.3284G>A) was inherited from the father, causing the p.R1095Q amino acid change in the TSP1-8. Another mutation, c.4143insA which causes protein interruption in the CUB2 domain (p.E1382RfsX6), was identified in two related patients, F1518#728and F1519#728. The latter carried the c.4143insA in homozygosity, while her father, patient F1518#728, carried it in heterozygosity in combination with the c.2281G>A mutation that causes an amino acid change in the TSP1-3 domain (p.G761S). The c.4034delA mutation which causes a premature protein interruption in the CUB2 domain (p.N1345TfsX14) was indentified in homozygosity in two first-degree cousins, F1627#873 and F1628#873. A homozygous missense c.427A>T mutation in exon 5, causing the p.I143F change in the metalloprotease domain, was found in two siblings (F769#239 and F1116#239). Their parents are consanguineous and carry the same mutation in heterozygosity. In patient R564 we found a homozygous c.703G>T mutation in exon 7, which predicts the p.D235Y change in the metalloprotease domain. Of note the p.I143F, the p.G761S, the p.Q429X, and the p.N1345TfsX14 are novel.

Mutation phase was determined when possible by studying available relatives, as detailed in Patients' case report.

None of the mutations were found in any of 100 Caucasian unrelated healthy subjects sequenced, in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/snp/), in the 1000Genomes database (http://browser.1000genomes.org/Homo_sapiens/Info/Index), or in the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS) with the exception of the p.R1060W (MAF<$1x10^{-3}$), the p.R1095Q (MAF<$8x10^{-5}$) and the c.4143insA (MAF<$8x10^{-5}$).

References

1. **Gerritsen HE, Turecek PL, Schwarz HP, Lammle B, Furlan M: Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF. *Thrombosis and Haemostasis,* 82: 1386-1389, 1999**
2. **Palla R, Valsecchi C, Bajetta M, Spreafico M, De Cristofaro R, Peyvandi F: Evaluation of assay methods to measure plasma ADAMTS13 activity in thrombotic microangiopathies. *ThrombHaemost,* 105: 381-385, 2011**
3. **Remuzzi G, Galbusera M, Noris M, Canciani MT, Daina E, Bresin E, Contaretti S, Caprioli J, Gamba S, Ruggenenti P, Perico N, Mannucci PM: von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Blood,* 100: 778-785, 2002**
4. **Bettoni G, Palla R, Valsecchi C, Consonni D, Lotta LA, Trisolini SM, Mancini I, Musallam KM, Rosendaal FR, Peyvandi F: ADAMTS-13 activity and autoantibodies classes and subclasses as prognostic predictors in acquired thrombotic thrombocytopenic purpura. *J ThrombHaemost,* 10: 1556-1565, 2012**
5. **Noris M, Bucchioni S, Galbusera M, Donadelli R, Bresin E, Castelletti F, Caprioli J, Brioschi S, Scheiflinger F, Remuzzi G: Complement factor H mutation in familial thrombotic thrombocytopenic purpura with ADAMTS13 deficiency and renal involvement. *Journal of the American Society of Nephrology,* 16: 1177-1183, 2005**
6. **Fremeaux-Bacchi V, Dragon-Durey MA, Blouin J, Vigneau C, Kuypers D, Boudailliez B, Loirat C, Rondeau E, Fridman WH: Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J Med Genet,* 41: e84, 2004**
7. **Donadelli R, Banterla F, Galbusera M, Capoferri C, Bucchioni S, Gastoldi S, Nosari S, Monteferrante G, Ruggeri ZM, Bresin E, Scheiflinger F, Rossi E, Martinez C, Coppo R, Remuzzi G, Noris M: In-vitro and in-vivo consequences of mutations in the von Willebrand factor cleaving protease ADAMTS13 in thrombotic thrombocytopenic purpura. *ThrombHaemost,* 96: 454-464, 2006**
8. **Garagiola I, Valsecchi C, Lavoretano S, Oren H, Bohm M, Peyvandi F: Nonsense-mediated mRNA decay in the ADAMTS13 gene caused by a 29-nucleotide deletion. *Haematologica,* 93: 1678-1685, 2008**
9. **Tripodi A, Chantarangkul V, Bohm M, Budde U, Dong JF, Friedman KD, Galbusera M, Girma JP, Moake J, Rick ME, Studt JD, Turecek PL, Mannucci PM: Measurement of von Willebrand factor cleaving protease (ADAMTS-13): results of an international**

collaborative study involving 11 methods testing the same set of coded plasmas. *J ThrombHaemost,* 2: 1601-1609, 2004

10. Galbusera M, Noris M, Rossi C, Orisio S, Caprioli J, Ruggeri ZM, Amadei B, Ruggenenti P, Vasile B, Casari G, Remuzzi G: Increased fragmentation of von Willebrand factor, due to abnormal cleavage of the subunit, parallels disease activity in recurrent hemolytic uremic syndrome and thrombotic thrombocytopenic purpura and discloses predisposition in families. The Italian Registry of Familial and Recurrent HUS/TTP. *Blood,* 94: 610-620, 1999

11. Lotta LA, Wu HM, Mackie IJ, Noris M, Veyradier A, Scully MA, Remuzzi G, Coppo P, Liesner R, Donadelli R, Loirat C, Gibbs RA, Horne A, Yang S, Garagiola I, Musallam KM, Peyvandi F: Residual plasmatic activity of ADAMTS13 is correlated with phenotype severity in congenital thrombotic thrombocytopenic purpura. *Blood,* 120: 440-448, 2012

12. Palla R, Lavoretano S, Lombardi R, Garagiola I, Karimi M, Afrasiabi A, Ramzi M, De Cristofaro R, Peyvandi F: The first deletion mutation in the TSP1-6 repeat domain of ADAMTS13 in a family with inherited thrombotic thrombocytopenic purpura. *Haematologica,* 94: 289-293, 2009

13. Metin A, Unal S, Gumruk F, Palla R, Cairo A, Underwood M, Gurgey A: Congenital thrombotic thrombocytopenic purpura with novel mutations in three unrelated Turkish children. *Pediatr Blood Cancer,* 61: 558-561, 2014

Supplemental table 1. Prevalence of acute renal impairment during bouts in cTTP

	Abnormal serum creatinine	***Need of dialysis***	***Proteinuria***	***Hematuria***	***Overall acute renal signs°***
Childhood onset (<18 years)	7/8 (87%)	3/8 (37%)	7/8 (87%)	8/8 (100%)	100%
Adult onset (≥18 years)	4/10 (40%)	2/10 (20%)	2/9* (22%)	4/9* (66%)	40%

* In one adult patient, proteinuria and hematuria were never checked during bouts.

° Percentage of patients with at least one renal sign.

Supplemental table 2.Renal parameters of patients during acute bouts and in hematological remission (last follow-up)

Patient	*Age at TTP onset (years)*	*SCr (mg/dl)*		*Need of dialysis*		*Proteinuria*		*Hematuria (number of erytrocytes)*	
		Acute bouts (rangesCr)	*Remission*	*Acute bouts*	*Remission*	*Acute bouts*	*Remission*	*Acute bouts*	*Remission*
R002	0.25	0.5-0.7	0.8	no	no	+++*	negative	30-50 er	negative
R012	3	1-6.8	13.4	yes	yes	> 250 mg	+*	+++*	negative
R1057	7	1.9-6	3.28	yes	no	1-2 g	++*	5-10 er	negative
F1518#728	35	2.1-2.3	2	no	no	1.2-1.4 g	negative	50-60 er	negative
F1519#728	3.5	0.9-1.9	0.8	yes	no	1 g	negative	30-40 er	negative
F1627#873	1.83	0.35-0.38	0.45	no	no	> 300 mg	negative	++*	negative
F1628#873	birth	0.7-0.9	0.83	no	no	negative	negative	> 50 er	negative
F101#019	35	1.1-11.3	1.07	yes	no	0.3-1 g	negative	++*	negative
F102#019	28	1.2-12.8	0.77	yes	no	negative	negative	+*	negative
TTP#48	15	1.1-4.6	1.3	no	no	++++*	negative	++++*	negative
TTP#79	23	8	1.3	no	no	negative	negative	+++*	+*
TTP#125	7	0.9	normal	no	no	+*	negative	8-10 er	negative
F48#002	23	0.64-1.05	0.91	no	no	20-30 mg	negative	1-4 er	negative
F711#226	20	0.59	0.5	no	no	70-80 mg	negative	negative	negative
F908#226	28	normal	normal	no	no	negative	-	negative	-
F769#239	21	normal	0.83	no	no	negative	negative	negative	negative
F1116#239	18	normal	0.94	no	no	negative	negative	negative	negative
R564	24	0.6-0.8	0.7	no	no	-	-	-	-

Normal values. Serum creatinine: children <1-5 years, 0.3-0.5 mg/dl; children 5-10 years, 0.5-0.8 mg/dl, children >10 years/adults, 0.5-1.2 mg/dl. Proteinuria: < 200 mg/24h or negative dipstick. Hematuria: < 5 erytrocytes or negative dipstick. SCr: serum creatinine; er:erytrocytes. -: data not available; *dipstick was used for these evaluations.

Supplemental table 3. Results of *in vitro* expression studies on the new secreted rADAMTS13 mutants and comparison between activity levels measured by cleavage test on rVWF A1-A2-A3, CBA and FRETS-rVWF73.

Mutation	*In vitro* rADAMTS13 secretion	*In vitro* rADAMTS13 activity	*In vitro* rADAMTS13 residual activity in cells supernatant		
			Cleavage test*	CBA**	FRETS-rVWF73**
I143F	10±5%	5±2.6%	0.5%	<6%	<3%
D235Y	10±3.5%	0%	0%	<6%	<3%
R1095Q	7.5±3.5%	0%	0%	<6%	<3%
R1060W	30±3%	46±10.8%	13.8%	11.4%	11.5%

Data expressed as percentage in respect to WT-rADAMTS13. *Mean of three determination, **mean of two determinations.

Legend to Supplemental figures

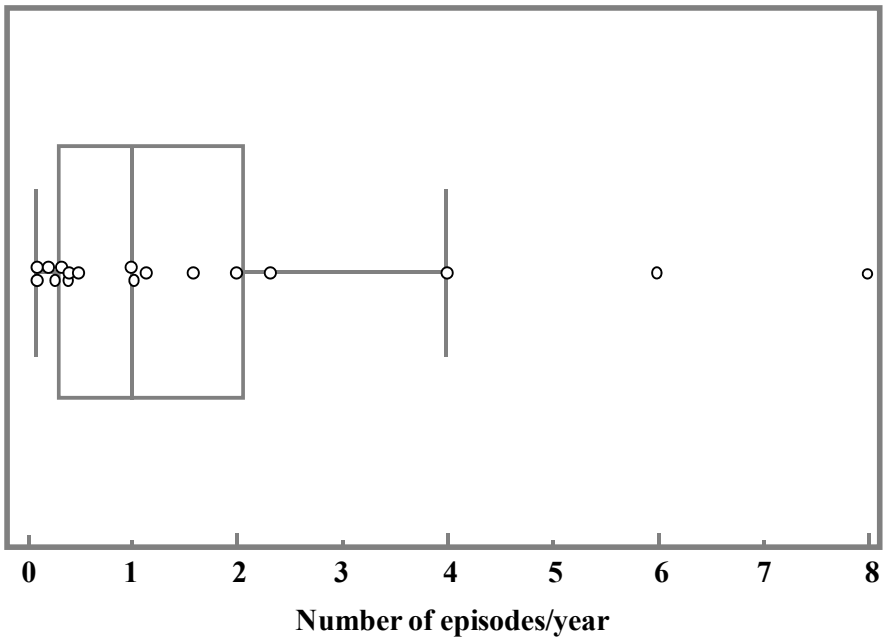
Supplemental figure 1. Distribution of patients' number of episode per year. All data, median value, 25 and 75 percentiles are plotted.

Supplemental figure 2. Localization of ADAMTS13 mutations found in cTTP patients. *Domain localization of the mutations refers to the localization of the stop codon.

Supplemental figure 3. Recombinant VWF A1-A2-A3 cleavage test by ADAMTS13 in normal human serum (NHS) pool. Panel A: Western Blot analysis of rVWF A1-A2-A3 cleaved by ADAMTS13 in 1:4 to 1:128 different dilutions of NHS pool corresponding to ADAMTS13 activity from 25% to 0.78%, respectively. Panel B: Sigmoid curve produced after the plotting of rVWF A1-A2-A3 proteolytic fragment densitometric values.

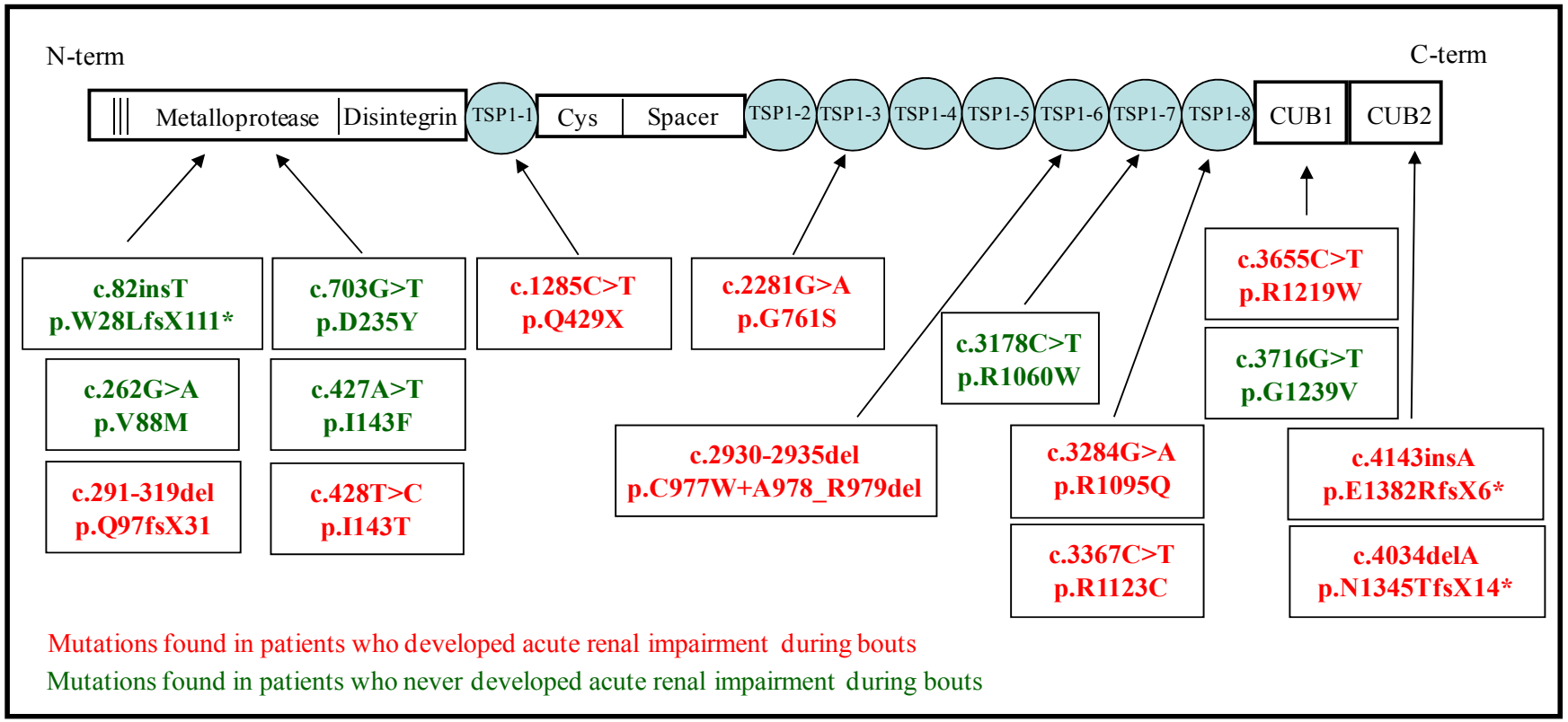
Supplemental figures

Supplemental figure 1. Distribution of patients' number of episode per year



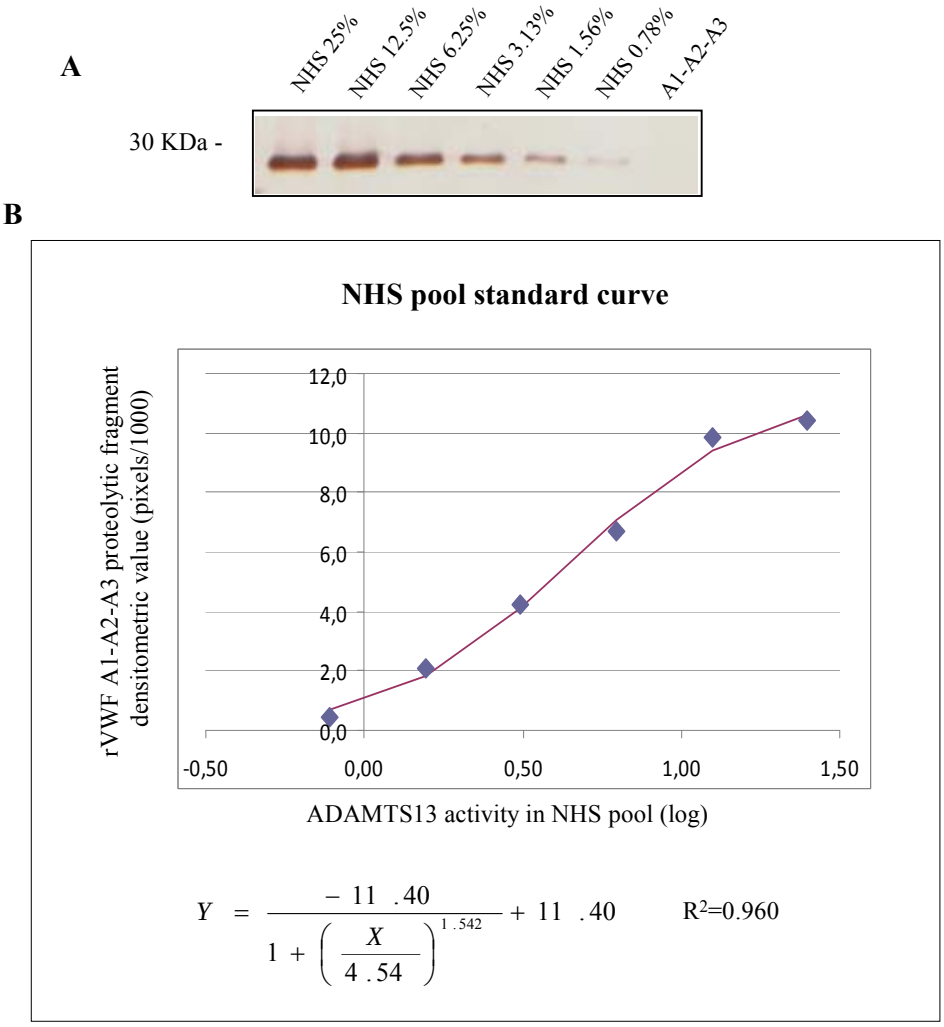
Supplemental figure 1

Supplemental figure 2. Localization of ADAMTS13 mutations found in cTTP patients



Supplemental figure 2

Supplemental figure 3. Recombinant VWF A1-A2-A3 cleavage test by ADAMTS13 in normal human serum (NHS) pool



Supplemental figure 3