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Pathophysiology of Thrombotic Thrombocytopenic Purpura: the "Two-Hit" paradigm

Pathophysiology of Thrombotic Thrombocytopenic Purpura: the “Two-Hit” paradigm

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CONTENTS

INTRODUCTION

Thrombotic microangiopathies

Thrombosis is the pathologic formation of a blood clot within the vascular lumen. Intravascular clotting should always be regarded as pathologic, because blood clots physiologically form only at the sites of vascular wall injury. Thrombotic diseases constitute the main primary cause of death and disability worldwide and are the second most frequent secondary cause of death in patients with cancer.[1] Thrombosis may occur in virtually any vessel of the blood circulation and therefore thrombotic diseases are classified according to the type and location of the vessel that is occluded (Figure 1). Thrombosis in the vessels of microscopic caliber (terminal arterioles and capillaries) is the defining characteristic of thrombotic microangiopathies. Thrombotic microangiopathies are a group of diseases or clinical syndromes with overlapping clinical characteristics and heterogeneous etiology, highlighting the multicausal nature of microvascular thrombotic disease.[2] Classifying thrombotic microanciopathies is difficult. There are many possible classification criteria (by clinical symptoms, by associated clinical condition, by pathophysiology, by any other characteristic), and we propose a reasonable classification in Table 1. Idiopathic thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS) are the two most important forms of idiopathic thrombotic microangiopathy. From a clinical standpoint, the two diseases are characterized by acute episodes of thrombocytopenia and microangipathic hemolytic anemia (Coombs negative hemolytic anemia with signs of red blood cell fragmentation).

Atypical HUS has prominent renal involvement with acute renal failure (which is a required criterion for the diagnosis of HUS), whereas TTP frequently (but not invariably) displays neurological manifestations. From a pathophysiological standpoint, aHUS is characterized by hyperactivation of the alternative complement pathway.[3] Hyperactivation of the alternative pathway is usually caused by rare nonsynonymous mutations in complement factor or complement regulation genes.[3] The patterns of inheritance of aHUS are articulated and characterized by variable penetrance, considerable locus heterogeneity and epistasis. Idiopathic TTP is characterized by a severe deficiency of the von Willebrand factor (VWF) cleaving protease, ADAMTS13. This form of microvascular thrombosis is the main focus of this thesis.

Thrombotic thrombocytopenic purpura and severe ADAMTS13 deficiency

Thrombotic thrombocytopenic purpura (TTP) is a rare thrombotic microangiopathy characterized by acute episodes of widespread microvascular thrombosis causing severe ischemic organ damage.[4] In the late 1990s it was discovered that the plasmatic activity of the von Willebrand factor (VWF) cleaving protease, ADAMTS13, is severely deficient in individuals with TTP. This discovery represented a turning point in the understanding of the pathophysiology of the disease.[5] ADAMTS13 (**a** **d**isintegrin **a**nd **m**etalloproteinase with a **t**hrombo**s**pondin type 1 motif, member **13**) is the thirteenth member of the ADAMTS family of metalloproteases. Severe ADAMTS13 deficiency (i.e., a plasmatic activity of the protease below 10% of normal) is either due to circulating anti-ADAMTS13 autoantibodies (i.e. acquired deficiency)[6] or, less frequently, to recessively inherited mutations of *ADAMTS13*

(i.e. congenital deficiency).[7] Although not all of the patients with a clinically defined TTP present with severely reduced ADAMTS13, the finding of severe ADAMTS13 deficiency has been shown by several studies to define a distinct etiologic subgroup of the disease.[5] In TTP patients with severe ADAMTS13 deficiency, the pathologic presence in the circulation of uncleaved ultralarge VWF multimers is considered as the mechanism responsible for VWF-mediated platelet aggregation and thrombosis.[4] The clinical severity of TTP is heterogeneous both in terms of short and long term prognosis. The studies presented in this thesis investigate the pathophysiology of TTP with ADAMTS13 deficiency, in an effort to better understand the clinical heterogeneity that characterizes TTP patients.

We studied both congenital (Section I, Chapters 1-4) and acquired (Section II, Chapters 5-9) forms of TTP in order to identify factors influencing clinical heterogeneity.

In **Chapter 1**, we sought to compile a catalog of all genetic variants in the *ADAMTS13* gene that are associated with congenital TTP. We systematically studied the type and location of these variants, the results of functional studies and their association with disease phenotype. The aim was to discover whether genotype-phenotype relationships existed. We found that *ADAMTS13* genotype is a determinant of clinical heterogeneity.

In **Chapter 2**, we expanded these observations and conducted a study measuring residual ADAMTS13 activity in 29 patients with congenital TTP. The goal was to find whether a residual ADAMTS13 activity exists in congenital TTP patients and if that activity is associated with phenotype severity. We found that residual

ADAMTS13 activity is a determinant of clinical heterogeneity and we described genotype-phenotype relationships.

In **Chapter 3**, we described a case of congenital TTP. By measuring residual ADAMTS13 we sought to determine if ADAMTS13 activity is abolished during acute TTP in patients with congenital TTP who have measurable activity in remission.

In **Chapter 4**, we summarized all recent studies on residual ADAMTS13 activity in TTP, discussing their pathophysiologic implications.

In **Chapter 5**, we introduced acquired TTP, summarizing knowledge on the role of anti-ADAMTS13 autoantibodies in the disease.

In **Chapter 6**, we compared the clinical severity of acquired TTP in first episodes and recurrences, in order to determine whether clinical episode number is associated with disease severity. We found that episode number is a determinant of clinical heterogeneity in TTP.

In **Chapter 7**, we studied VWF-related measurements during acute disease and remission of TTP. The goal was to see if changes of VWF properties (e.g. its conformation) were associated with the onset of thrombosis in patients with TTP and if these properties were associated with the disease severity. We found that multimeric VWF pattern is associated with acute severity of TTP.

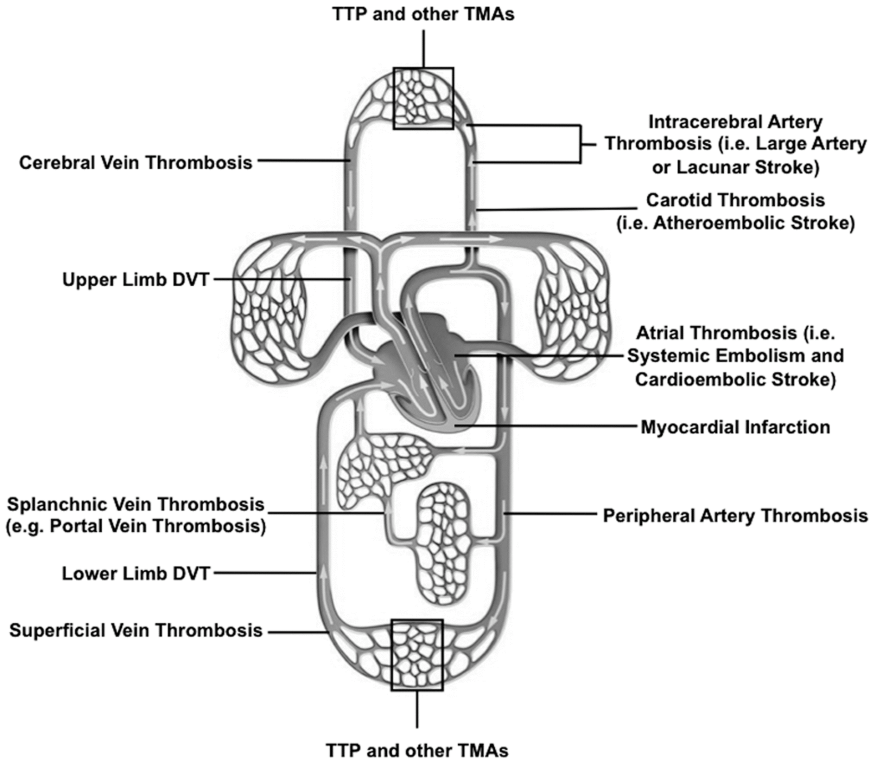
In **Chapter 8**, we studied several ADAMTS13- and anti-ADAMTS13 autoantibody-related measurements in acquired TTP patients, in order to determine their association with acute disease severity and with the occurrence of disease recurrence. We found several associations between study measurements

and clinically relevant endpoints, such as number of plasma exchange procedures needed to achieve remission or the development of recurrence.

In **Chapter 9**, we reported the case of a patient with a history of acquired TTP who was safely treated with thienopyridines (i.e. drugs associated with the development of drug-induced-TTP). The report demonstrates that these drugs do not necessarily induce TTP in patients with a history of the disease.

Figures

Figure 1. Localization of thrombotic diseases.
Adapted from *http://www.humanbody.dke-explore.com/clipart/human/image_human056.htm*.



TTP, thrombotic thrombocytopenic purpura; TMA, thrombotic microangiopathy; DVT, deep vein thrombosis.

Tables

Table 1. Classification of thrombotic microangiopathies.

Name	Localization of thrombosis	Pathophysiology	Clinical notes
Idiopathic			
Thrombotic thrombocytopenic purpura	Widespread Brain, heart, bowel typically involved	Severe ADAMTS13 deficiency (*ADAMTS13* mutations or anti-ADAMTS13 autoantibodies) Ultralarge VWF mediated thrombosis	Fever, neurological and cardiac involvement are frequent Purpura is the most common manifestation
Atypical hemolytic uremic syndrome	Widespread Renal circulation prominently involved	Hyperactivation of the alternative complement activation pathway (mutations in complement factor or regulator genes or auto-antibodies against complement regulators)	Prominent renal involvement with acute renal failure
Catastrophic antiphospholipid antibody syndrome	Widespread (including large vessels)	Anti-phospholipid autoantibodies	Positive test for anti-phospholipid antibodies Thrombosis may occur also in large vessels
Secondary			
Secondary thrombotic thrombocytopenic purpura			
Cancer	Widespread	Cancer-related endothelial damage and activation Metastatic cell embolization Cancer-related hypercoagulability	Poor response to PEX
Bone marrow transplantation	Widespread	Endothelial damage and activation	Poor prognosis
Drug induced (dose independent)	Widespread	Development of acute disease after use of the drug (quinine, ticlopidine, clopidogrel) Anti-ADAMTS13 autoantibodies may be present	Acute disease days/weeks after the use of the drug
Drug induced (dose dependent)	Widespread	Dose- and duration-dependent toxic effect of chemotherapy (e.g. mitomycine, gemcitabine, etc.)	Insidious onset of symptoms
HIV infection	Widespread	HIV-mediated endothelial damage and activation	May be sensitive to anti-retroviral therapy

Typical hemolytic uremic syndrome	Widespread Renal circulation prominently involved	Infection by enterohemorrhagic *E. Coli* O157:H7	Bloody-diarrhoea prodrome. Prominent renal involvement with acute renal failure
Hemolysis elevated liver enzymes low platelet (HELLP) syndrome[a]	Widespread Liver is affected	Unknown The disease is considered a severe form of pre-eclampsia	May have mild symptoms Typical manifestations are epigastric pain and malaise
Disseminated intravascular coagulation[b]	Widespread	Hyperactivation of the coagulation cascade in response to a variety of diseases	Prominent hemorrhagic symptoms
Autoimmune-disease associated thrombotic microangiopathy	Widespread	Manifestation of the primary autoimmune disorder	Insidious onset Frequent renal involvement

a HELLP is classified as secondary TMA because it develops in pregnant women with preeclampsia

b Disseminated intravascular coagulation is secondary to many different disease/conditions

VWF, von Willebrand factor; PEX, plasma exchange.

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SECTION I

CHAPTER 1

ADAMTS13 mutations and polymorphisms in congenital thrombotic thrombocytopenic purpura

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Adapted from Human Mutation 2010; 31: 11-9.

Abstract

Congenital thrombotic thrombocytopenic purpura (TTP) (also known as Upshaw-Schulman syndrome, USS) is a rare, life-threatening disease characterized by thrombocytopenia and microangiopathic hemolytic anemia. The disease is caused by a deficiency of the von Willebrand factor-cleaving protease (ADAMTS13) due to mutations in the corresponding gene. The spectrum of clinical phenotypes in congenital TTP is wide, encompassing neonatal-onset disease and adult-onset disease, forms with a single disease episode and chronic-relapsing forms.

In this article, we review *ADAMTS13* gene variants associated with inherited ADAMTS13 deficiency and congenital TTP. To date, 76 mutations of *ADAMTS13* are reported in the literature. Missense mutations, which constitute nearly 60% of *ADAMTS13* mutations, preferentially localize in the 5'-half of the gene encoding the N-terminal half of the protein, where the domains that are indispensable for ADAMTS13 catalytic function are situated. *In vitro* expression studies in cell cultures have shown that defects in protein secretion and catalytic activity are the main mechanisms responsible for the deficiency of ADAMTS13 in congenital TTP patients. Even though data from the literature suggest the existence of genotype-phenotype associations, a clear relationship between the type and the effect of *ADAMTS13* genetic defects with disease manifestations remains to be established.

Background

Congenital thrombotic thrombocytopenic purpura (TTP), also known as Upshaw-Schulman syndrome (OMIM accession number 274150), is a rare, life-threatening disease characterized by single or recurrent episodes of thrombocytopenia, microangiopathic haemolytic anaemia and widespread microvascular thrombosis, which leads to ischemic damage of multiple organs (mainly kidney, heart and brain).[1, 2] Congenital TTP has an autosomal recessive inheritance and is caused by mutations in *ADAMTS13* (OMIM gene accession number: MIM# 604134).[3] The gene encodes a plasma zinc metalloprotease of the ADAMTS family (a disintegrin-like and metalloprotease with thrombospondin type 1 motif), responsible for the cleavage of von Willebrand factor (VWF).

VWF is a large multimeric glycoprotein synthesized in vascular endothelial cells, stored in Weibel–Palade bodies, and secreted upon endothelial cell activation or injury into plasma, where it plays two main functions in hemostasis. First, it is essential for platelet-plug formation as an adhesion protein that diverts circulating platelets to the sites of vascular injury, particularly through multimers of ultralarge size (ULVWF).[4] Second, it forms a noncovalent complex with coagulation factor VIII in plasma, protecting the latter from inactivation and clearance.[5] In physiologic conditions, ADAMTS13 cleaves ULVWF as soon as this adhesive protein is released by endothelial cells.[6] When ADAMTS13 activity is deficient, uncleaved ULVWF causes heightened platelet adhesion and aggregation in the microcirculation, resulting in platelet-rich thrombi that are responsible for the fragmentation of circulating erythrocytes (microangiopathic hemolytic anemia) and for signs and symptoms of organ ischemia and

dysfunction.[7] ADAMTS13 deficiency may be due to the production of anti-ADAMTS13 auto-antibodies (acquired) or to mutations in *ADAMTS13* (congenital).[3, 8-10] Congenital disease accounts for no more than 5% of all TTP cases associated with ADAMTS13 deficiency, the incidence of which is 2/1.000.000 person/year.[11] The exact incidence of congenital TTP has not been established, but it is less than 1/1.000.000 person/year. Congenital TTP was originally described as a form of thrombocytopenia dramatically responsive to plasma infusion, that Schulman, who first described the disease in 1960, ascribed to the congenital deficiency of a "humoral regulator of thrombopoiesis".[2] The mechanism of the disease remained unclear until 2001, when Levy et al. by means of linkage analysis performed on 4 pedigrees with congenital TTP, demonstrated that mutations in the *ADAMTS13* gene resulting in severely reduced VWF-cleaving protease activity in plasma are responsible for the autosomal recessive inheritance of the disease.[3] *ADAMTS13* spans 29 exons and ~37 kb and is located at chromosome 9q34. From its N-terminus, the encoded metalloprotease ADAMTS13 comprises a signal peptide domain, a propeptide domain, a metalloprotease domain, a disintegrin like domain, a thrombombospondin type 1 repeat (TSP1) domain, a cysteine-rich domain, a spacer domain, 7 additional TSP1 repeats and two terminal complement C1r/C1s, Uegf, Bmp1 (CUB) domains.[12]

We hereby review *ADAMTS13* gene variants associated with inherited ADAMTS13 deficiency and congenital TTP. The features of these variants pertaining to their type, their localization on the gene and their effect on amino acid residues are presented. The results of *in vitro* expression studies of

ADAMTS13 mutations and polymorphisms are also summarized. Finally, the potential relevance of the results of these studies to the attribution of genotype-phenotype associations is discussed. According to the Human Genome Variation Society (HGVS) instructions, nucleotide numbering refers to cDNA numbering with the A of the ATG codon numbered as +1. NM_139025.3 was used as reference sequence for nucleotide changes. NP_620596.2 was used as protein reference sequence. The review was based on published articles found through web-based searches on PubMed (updated at July 2009), using "ADAMTS13 mutation", "ADAMTS13 genotype", "ADAMTS13 polymorphism", "congenital thrombotic thrombocytopenic purpura" as queries.

Clinical features of the disease

The spectrum of clinical phenotype in congenital TTP is wide. While many patients develop thrombocytopenia and microangiopathic hemolytic anemia soon after birth, others have their first disease episode in adulthood, during the second or the third decade of life.[13] The clinical severity of disease episodes varies from asymptomatic episodes of thrombocytopenia and anemia to multi-organ failure that threatens patients' life.[14] In addition, after the first disease episode patients may undergo one or more relapses, and in some cases progressive organ failure develops, likely as a result of the accumulation of ischemic damage.[15] Aspects of disease phenotype severity might not be always consistent. For instance, it has been reported that adult-onset patients may also die of the disease[16] or develop relapsing disease after their first TTP episode[13, 17, 18] as well as early-onset patients do.

Supplementary Material S1 summarizes the 89 cases with congenital ADAMTS13 deficiency reported in the literature. Patients were included on the basis of a diagnosis of inherited severe deficiency of ADAMTS13 (ADAMTS13 activity <10% of normal with concomitant absence of anti-ADAMTS13 autoantibodies) and presence of mutations on both alleles of *ADAMTS13*. There are three cases reported in the literature[16, 19] of patients with TTP episodes and a laboratory pattern suggestive of inherited severe deficiency of ADAMTS13, in whom it was possible to identify only one *ADAMTS13* mutation after gene sequencing by PCR and Sanger sequencing. The phenotype of these patients may have been caused by a combination of *ADAMTS13* single nucleotide polymorphisms (SNPs), which can have a profound effect on ADAMTS13 secretion and activity, or by a mutation localized in the non-coding areas of *ADAMTS13*, which are not routinely analyzed. However, it cannot be excluded that genetic variants in genomic areas other than *ADAMTS13* are responsible for the peculiar pattern of these patients. When the history of a patient was reported in more than one publication, the patient was included only once in the Table. Cross-references between studies were examined to avoid inclusion of the same patient more than once. Of the 89 reported cases, 84 had episodes of TTP, while 5 reached adult age without developing TTP episodes, in spite of their severe ADAMTS13 deficiency. These individuals were siblings of patients with congenital TTP. For as many as 78 individuals with congenital ADAMTS13 deficiency information was provided on disease onset, ranging from neonatal to 35 years (median: 1,3 years). Of the 78 patients, 45% (n=35) had neonatal disease onset, 29% (n=23) intermediate onset (spanning from 2 months to 18 years), 20%

(n=15) adult onset (>18 years) and 6% (n=5) reached adulthood without developing TTP, in spite of undetectable ADAMTS13 activity and TTP-causing mutations. Information on other features of the disease phenotype was difficult to obtain from published cases and was available for a limited number of patients only.

Genetic variants of *ADAMTS13*

Since 2001, when *ADAMTS13* mutations were first discovered to be associated with congenital TTP, 76 *ADAMTS13* mutations have been reported (Table 1). These include: 45 missense mutations (59% of all reported mutations), 10 nonsense mutations (13%), 10 deletions (13%), 4 insertions (6%) and 7 splice site mutations (9%). The majority of reported congenital TTP cases (64%) are compound heterozygous, while only 36% of patients were homozygous for *ADAMTS13* mutations, making it difficult to identify genotype-phenotype associations. Figure 1 shows a linear map of the localization of *ADAMTS13* missense mutations on the gene. Of 45 missense mutations, 33 (73%) localize in the 5' half of the gene encoding the N-terminal half of the protein where the domains essential for ADAMTS13 specific activity (metalloprotease through spacer domains) are located. No missense mutation has been described in exons 1, 2, 11, 14, 15, 18, 20, 23, 29.

The geographic distribution of all *ADAMTS13* mutations as established from the country of birth of the patients (available for 55% of all reported mutations) is shown in Table 1. Congenital TTP cases have been reported in all continents. Haplotype analyses of patients carrying c.4143dupA, the most frequently reported

ADAMTS13 mutation, revealed a common genetic background, suggesting of the existence of a common ancestor in Central Europe.[15]

Nineteen non-synonymous SNPs of *ADAMTS13* are recorded in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). Eight of them have also been reported in patients with congenital TTP. Two additional SNPs (c.1016C>G leading to p.T339R and c.2708C>T leading to p.S903L) were reported in Japanese patients with congenital TTP and confirmed to have minor allele frequency above 1% in the Japanese population,[20, 21] but were not included in dbSNP. Notably, in dbSNP, there are 3 polymorphisms (a nonsense SNP, one insertion and one deletion) that are predicted to result in the truncation of ADAMTS13. Table 2 summarizes nonsynonymous polymorphisms of the coding area of *ADAMTS13*.

Biological relevance: effect of *ADAMTS13* mutations on the encoded protein

ADAMTS13 mutations affect the metalloprotease, the disintegrin, the TSP1-1, the cysteine-rich, the spacer, the TSP1-2, TSP1-3, TSP1-5, TSP1-6, TSP1-7, TSP1-8, CUB-1 and CUB-2 domains (Table 1). No mutations affecting the signal peptide, the propeptide and the TSP1-4 have been reported as yet. Two SNPs of *ADAMTS13* (c.19C>T leading to p.R7W and c.2494G>A leading to p.V832M) localize in the propeptide and in the TSP1-4 domain, respectively, so that genetic variants of *ADAMTS13* affecting each of the ADAMTS13 protein domains, with the exception of the small signal peptide domain, have been described.

N-terminal ADAMTS13 domains (metalloprotease through spacer) are highly conserved among ADAMTS proteins[22] and are all required for ADAMTS13-

mediated cleavage of VWF.[23-25] C-terminally truncated mutations that lack the spacer domain indeed result in a protein unable to cleave VWF *in vitro*.[23] The C-terminal TSP1 domains, which are conserved among ADAMTS proteins, and the CUB domains, uniquely present in ADAMTS13 among ADAMTS proteins, are dispensable for ADAMTS13 activity under static conditions.[23] However, the cooperative activity between C-terminal TSP1 domains and CUB domains is necessary for ADAMTS13-VWF interaction in conditions of shear stress that resemble those of the small vessels in which VWF cleavage takes place.[26, 27]

The conservation of ADAMTS13 amino acid residues was evaluated through a comparison of ADAMTS13 amino acid sequence alignments in 5 species (*Homo sapiens*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, and *Gallus gallus*), using Multiple Sequence Alignment (MUSCLE). Human ADAMTS13 showed the highest sequence identity with ADAMTS13 of *Bos taurus* (~75% of amino acid sequence identity), and the lowest with ADAMTS13 of *Gallus gallus* (~34%). Reported *ADAMTS13* missense mutations determine amino acid changes in residues that are highly conserved across species (at least in 3/4 of the non-human species analysed), which confirms the importance of these residues for physiological function of ADAMTS13 (Table 3). Of 45 missense mutations, only 4 were located in less conserved residues (p.I79M, p.A606P, p.R692C and p.R1060W).

Twenty-six *ADAMTS13* mutations, representing one third of those reported, were expressed in cultured cells to evaluate *in vitro* the mechanisms by which they induce ADAMTS13 deficiency (Table 4). *In vitro* studies differed in the design and methods adopted, so that it is not easy to compare results of different reports.

However, when mutations were evaluated in more than one study, results were generally concordant.[16, 28, 29] The two main mechanisms leading to ADAMTS13 deficiency are reduced secretion and reduced catalytic activity of mutant ADAMTS13. Of 26 mutations expressed, 9 result in severe reduction of ADAMTS13 secretion in culture medium. Of the remaining 17, 6 had no catalytic activity, while 11 conserved some degree of cleaving activity towards VWF. Nonsense mediated mRNA decay has been also described as a mechanism responsible for ADAMTS13 deficiency, in addition to impaired secretion and activity.[30]

Six *ADAMTS13* polymorphisms have been expressed in cell cultures. Notably, two of them (c.1423C>T leading to p.P475S, and c.1852C>G leading to p.P618A) result in a significant reduction of *in vitro* ADAMTS13 catalytic activity compared to wild type (Table 4).[20, 31] This effect of SNPs on ADAMTS13 activity and secretion might be particularly relevant in patients carrying *ADAMTS13* mutations. During *in vitro* experiments on the *ADAMTS13* mutation p.R1336W, found in 2 patients with congenital TTP, the combined presence of such SNPs and this mutation did indeed cause ADAMTS13 deficiency *in vitro*, while the mutation or the SNPs alone did not.[31]

Clinical relevance: does *ADAMTS13* genotype influence the clinical features of the disease?

Probably owing to the rarity of patients homozygous for *ADAMTS13* mutations and to the paucity of *in vitro* expression studies, no genotype-phenotype association has been firmly established in congenital TTP. In Figure 2 the

ADAMTS13 genotypes that occurred more than once in the considered case material were plotted against the age of onset of the corresponding patients, which is the only phenotypic parameter for which enough information can be retrieved in the literature. We observed that in patients with a given *ADAMTS13* genotype, the age of disease onset was similar (Figure 2), suggesting the existence of a genotype-phenotype association. A concordance of age of onset could also be observed when patients came from different case series (patients with homozygous mutations c.4143dupA, p.R692C and p.R1060W). All 5 patients (patients 33, 67, 68, 76, 88 in Table 1) with ADAMTS13 deficiency who reached adulthood without developing symptoms of TTP were siblings of patients with adult or intermediate (15 years) disease onset. In addition, mutation p.R1060W, a frequently reported *ADAMTS13* mutation, was found in 4/15 patients with adult-onset congenital TTP and in two asymptomatic siblings of congenital TTP patients, but not in early or intermediate-onset cases. Only one of the patients was homozygous for p.R1060W.[19] In this study, we report an additional Italian patient carrying p.R1060W in homozygosis, who developed two TTP episodes at 18 and 19 years old, in occasion of her two pregnancies. These suggest the existence of a genotype-phenotype association in congenital TTP.

The mechanisms of the association between *ADAMTS1*3 mutations and disease phenotype are unknown. Since the catalytic activity of mutant recombinant ADAMTS13-R1060W is fully conserved (compared with wild type ADAMTS13), it has been postulated that a partial conservation of ADAMTS13 activity in patients' plasma, which is undetectable with current ADAMTS13 activity assays, is perhaps responsible for the milder phenotype of late onset

patients.[19, 32, 33] An association of the results of *in vitro* studies with patients' laboratory and clinical phenotype has been reported in a few families with congenital TTP and homozygous *ADAMTS13* mutations.[33] Using data available from the literature it is difficult to obtain a confirmation of these results. No clear association was found in the available data between age of disease onset, *in vitro* expression study results and conservation of mutated amino acid residues across species (Table 3).

Molecular diagnosis, animal models and disease modifiers

Congenital TTP should be suspected in patients who present with severe ADAMTS13 deficiency and absence of anti-ADAMTS13 auto-antibodies (severe congenital deficiency of ADAMTS13). In these patients, genetic analysis carried out by PCR amplification and sequencing of the exon areas and the intron-exon boundaries of *ADAMTS13*, is used to identify disease-causing genetic defects.

Although congenital severe deficiency of ADAMTS13 is a specific laboratory marker of congenital TTP, both clinical and experimental evidence indicates that the deficiency is not sufficient to determine acute TTP episodes. More than 50% of patients with a severe deficiency have their first TTP episode well after the neonatal period, during infancy or even adulthood, after years of apparent well-being and absence of disease manifestations. Consistent with these clinical observations the *ADAMTS13* knock-out mouse was viable and exhibited normal survival. Only the introduction in the animal of a genetic background associated with high levels of VWF resulted in chronic spontaneous hemolysis and thrombocytopenia. A TTP-like syndrome could be elicited in the latter animal

model only after treatment with *E coli*-derived shigatoxin.[34] Since genetic defects of *ADAMTS13* cannot entirely explain the variable clinical features of congenital TTP, an effect on clinical phenotype of genetic defects other than those that cause ADAMTS13 deficiency has been postulated. The role of possible disease modifiers has not been the object of systematic investigations. However, Noris et al. reported that the pattern of clinical presentation of congenital TTP in a family carrying mutations p.G1239V and p.V88M was influenced by a mutation in complement factor H gene, a gene the mutations of which are associated with the familial form of hemolytic uremic syndrome.[35]

Environmental modifiers may as well explain part of the variability of the phenotype of congenital TTP. The onset of acute TTP episodes in individuals with congenital ADAMTS13 deficiency is often associated with conditions and events such as pregnancy, infections, traumas, and surgical procedures.[16, 18, 21] It is possible that these conditions and events precipitate the onset of acute episodes by inducing the release of the pro-thrombotic ULVWF forms by endothelial cells. However, such triggering conditions are not always present or clinically overt.

Future perspectives

The discovery that *ADAMTS13* mutations cause congenital TTP has led to improvements in the knowledge of this rare but life threatening disease. The rare patients with homozygous *ADAMTS13* mutations are particularly suitable to study genotype-phenotype relationships. From a clinical point of view clinical studies are needed to characterize, in a uniform way, disease-related clinical features, such as age and severity of presentation and the tendency to relapse.

Finally, while fresh frozen plasma infusion is established as the treatment of choice of acute disease episodes, it is important to establish which patients are clinically so severe as to warrant plasma infusions during disease remission to prevent relapses.

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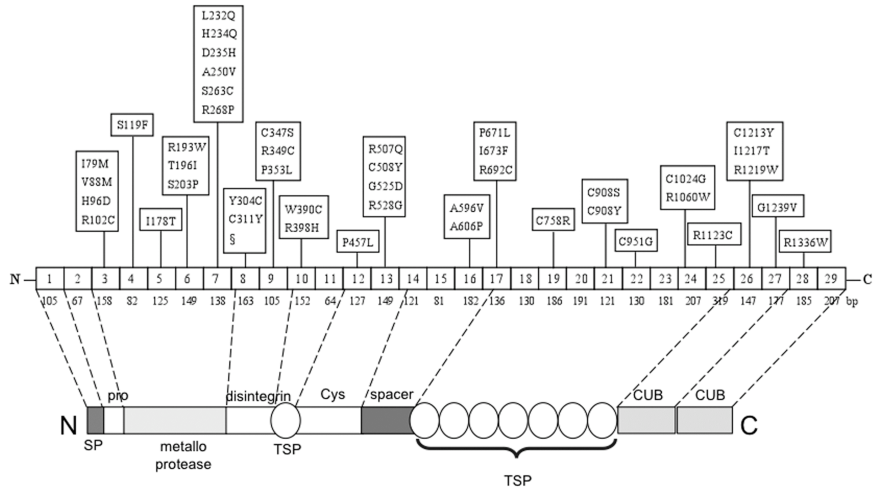
Figures

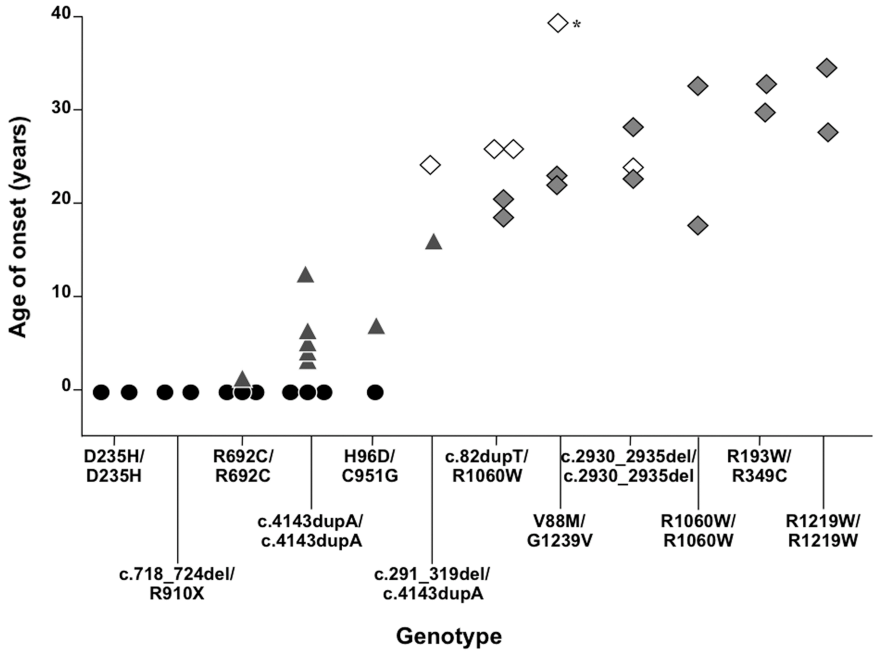
Figure 1. Linear map of the distribution of *ADAMTS13* missense mutations on the gene and on the protein. Number within boxes indicate exon number, numbers below boxes exon length expressed in base pairs.



§ p.[C322G (+) T323R (+) F324L].

SP, signal peptide domain; TSP, thrombospondin-like domain; Cys, cysteine-rich domain; CUB, complement C1r/C1s, Uegf, Bmp1 domain.

Figure 2. Relationship between *ADAMTS13* genotype and age of TTP onset. Genotypes are ordered on the basis on the mean age of onset of patients with each genotype. Circles represent patients who had neonatal TTP onset, triangles, patients with intermediate (2 months-18 years) onset, closed squares, patents with adult onset (>18 years), open squares, patients with no episodes of TTP at the time of their last follow up.



* This patient had not had any TTP episodes and had 53 years.

Tables

Table 1. Mutations of *ADAMTS13*. When available, the geographic origin of the patients carrying the mutations is indicated. The geographic origin of the parents of the patients with a given mutation is also indicated in brackets. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Location	DNA	Effect (protein or mRNA)	Domain	Ethnicity	Reference
Splice site					
Int 3	c.330+1G>A	r.[330_331ins105, 330_331ins27]		Japan/Korea	Uchida et al. [2004]
Int 4	c.414+1G>A	r.spl?		Japan	Matsumoto et al. [2004]
Int 6	c.686+1G>A			Japan	Matsumoto et al. [2004]
Int 6	c.687-2 A>G			Turkey	Studt et al. [2005]
Int 10	c.1244+2T>G	r.spl?		Japan	Matsumoto et al. [2004]
Int 11	c.1309-?G>A			France	Veyradier et al. [2004]
Int 13	c.1584+5G>A				Levy et al. [2001]
Deletion					
Ex 3	c.291_319del	p.E98PfsX31	Metalloprotease*	Turkey	Garagiola et al. [2008]
Ex 7	c.718_724del	p.S240AfsX7	Metalloprotease*		Assink et al. [2003]
Int.8_Ex.8	c.825-?_?del			France	Veyradier et al. [2004]
Ex 10	c.1095_1112del	p.W365_R370del	Disintegrin	USA	Tao et al. [2006]
Ex 15	c.1783_1784del	p.L595GfsX19	Spacer*	USA (Yemen)	Savasan et al. [2003]
Ex 19	c.2279del	p.G760AfsX18	TSP1-3*		Assink et al. [2003]
Ex 19	c.2376_2401del	p.A793PfsX43	TSP1-4*		Levy et al. [2001]
Ex 20	c.2549_2550del	p.D850GfsX7	TSP1-5*		Schneppenheim et al. [2003]
Ex 23	c.2930_2935del	p.C977_R979delinsW	TSP1-6	Iran	Palla et al. [2009]
Ex 25	c.3254_3255del	p.S1085CfsX12	TSP1-8*	France (Haiti)	Veyradier et al. [2004]
Insertion					
Ex 1	c.82dupT	p.W28LfsX111	Metalloprotease*		Donadelli et al. [2006]
Ex 4	c.372_373insGT	p.R125VfsX6	Metalloprotease*		Fujimura et al. [2009]
Ex 27	c.3770dupT	p.L1258VfsX36	CUB-1*		Levy et al. [2001]
Ex 29	c.4143dupA	p.I1382RfsX6	CUB-2*	Multiple countries[d]	Schneppenheim et al. [2003]
Nonsense					
Ex 2	c.130C>T	p.Q44X	Propeptide*		Antoine et al. [2003]
Ex 10	c.1169G>A	p.W390X	Disintegrin*		Schneppenheim et al. [2003]
Ex 12	c.1345C>T	p.Q449X	Cysteine-rich*	Japan	Kokame et al. [2002]
Ex 21	c.2728C>T	p.R910X	TSP1-5*	Germany	Schneppenheim et al. [2003]
Ex 22	c.2785C>T	p.Q929X	TSP1-5*		Fujimura et al. [2009]
Ex 24	c.3047G>A	p.W1016X	TSP1-7*		Donadelli et al. [2006]
Ex 24	c.3100A>T	p.R1034X	TSP1-7*	Germany	Schneppenheim et al. [2003]
Ex 26	c.3616C>T	p.R1206X	CUB-1*		Shibagaki et al. [2006]
Ex 27	c.3735G>A	p.W1245X	CUB-1*	Germany	Licht et al. [2004]
Ex 28	c.3904C>T	p.Q1302X	CUB-2*		Fujimura et al. [2009]
Missense					
Ex 3	c.237C>G	p.I79M	Metalloprotease	France (Haiti)	Veyradier et al. [2004]
Ex 3	c.262G>A	p.V88M	Metalloprotease	Italy	Bestetti et al. [2003]
Ex 3	c.286C>G	p.H96D	Metalloprotease		Levy et al. [2001]
Ex 3	c.304C>T	p.R102C	Metalloprotease		Levy et al. [2001]
Ex 4	c.356C>T	p.S119F	Metalloprotease	Tunisia	Meyer et al. [2008]a
Ex 5	c.533T>C	p.I178T	Metalloprotease		Fujimura et al. [2009]
Ex 6	c.577C>T	p.R193W	Metalloprotease	Japan	Matsumoto et al. [2004]
Ex 6	c.587C>T	p.T196I	Metalloprotease	Australia (Germany)	Levy et al. [2001]
Ex 6	c.607T>C	p.S203P	Metalloprotease	France (Haiti)	Veyradier et al. [2004]
Ex 7	c.695T>A	p.L232Q	Metalloprotease	Turkey	Schneppenheim et al. [2003]
Ex 7	c.702C>A	p.H234Q	Metalloprotease		Shibagaki et al. [2006]
Ex 7	c.703G>C	p.D235H	Metalloprotease		Assink et al. [2003]
Ex 7	c.749C>T	p.A250V	Metalloprotease	Japan	Uchida et al. [2004]
Ex 7	c.788C>G	p.S263C	Metalloprotease	Germany	Schneppenheim et al. [2003]
Ex 7	c.803G>C	p.R268P	Metalloprotease	Japan/France (Haiti)	Kokame et al. [2002]
Ex 8	c.911A>G	p.Y304C	Disintegrin		Fujimura et al. [2009]
Ex 8	c.932G>A	p.C311Y	Disintegrin		Assink et al. [2003]
Ex 8	‡	§	Disintegrin	Japan	Kokame et al. [2008]
Ex 9	c.1039T>A	p.C347S	Disintegrin	Croatia	Schneppenheim et al. [2006]
Ex 9	c.1045C>T	p.R349C	Disintegrin		Fujimura et al. [2009]
Ex 9	c.1058C>T	p.P353L	Disintegrin	Germany	Schneppenheim et al. [2003]
Ex 10	c.1170G>C	p.W390C	TSP1-1	Germany	Licht et al. [2004]
Ex 10	c.1193G>A	p.R398H	TSP1-1		Levy et al. [2001]
Ex 12	c.1370C>T	p.P457L	Cysteine-rich		Assink et al. [2003]
Ex 13	c.1520G>A	p.R507Q	Cysteine-rich	France/Norway	Veyradier et al. [2004]
Ex 13	c.1523G>A	p.C508Y	Cysteine-rich	Japan	Kokame et al. [2002]
Ex 13	c.1574G>A	p.G525D	Cysteine-rich		Fujimura et al. [2009]
Ex 13	c.1582A>G	p.R528G	Cysteine-rich		Levy et al. [2001]
Ex 16	c.1787C>T	p.A596V	Spacer	France/France (Haiti)	Veyradier et al. [2004]

Ex 16	c.1816G>C	p.A606P	Spacer		Fujimura et al. [2009]
Ex 17	c.2012C>T	p.P671L	Spacer	Sweeden	Schneppenheim et al. [2006]
Ex 17	c.2017A>T	p.I673F	Spacer		Matsumoto et al. [2004]
Ex 17	c.2074C>T	p.R692C	TSP1-2		Levy et al. [2001]
Ex 19	c.2272T>C	p.C758R	TSP1-3	France	Veyradier et al. [2004]
Ex 21	c.2723G>C	p.C908S	TSP1-5	France	Veyradier et al. [2004]
Ex 21	c.2723G>A	p.C908Y	TSP1-5	Japan	Matsumoto et al. [2004]
Ex 22	c.2851T>G	p.C951G	TSP1-5		Levy et al. [2001]
Ex 24	c.3070T>G	p.C1024G	TSP1-7		Levy et al. [2001]
Ex 24	c.3178C>T	p.R1060W	TSP1-7	USA/Italy/UK	Tao et al. [2006]
Ex 25	c.3367C>T	p.R1123C	TSP1-8	Japan	Matsumoto et al. [2004]
Ex 26	c.3638G>A	p.C1213Y	CUB-1		Levy et al. [2001]
Ex 26	c.3650T>C	p.I1217T	CUB-1	Korea	Park et al. [2008]
Ex 26	c.3655C>T	p.R1219W	CUB-1		Donadelli et al. [2006]
Ex 27	c.3716G>T	p.G1239V	CUB-1	Italy	Noris et al. [2005]
Ex 28	c.4006C>T	p.R1336W	CUB-2		Antoine et al. [2003]
Ex 21	c.2723G>C	p.C908S	TSP1-5	France	Veyradier et al. [2004]
Ex 21	c.2723G>A	p.C908Y	TSP1-5	Japan	Matsumoto et al. [2004]
Ex 22	c.2851T>G	p.C951G	TSP1-5		Levy et al. [2001]
Ex 24	c.3070T>G	p.C1024G	TSP1-7		Levy et al. [2001]
Ex 24	c.3178C>T	p.R1060W	TSP1-7	USA/Italy/UK	Tao et al. [2006]
Ex 25	c.3367C>T	p.R1123C	TSP1-8	Japan	Matsumoto et al. [2004]
Ex 26	c.3638G>A	p.C1213Y	CUB-1		Levy et al. [2001]
Ex 26	c.3650T>C	p.I1217T	CUB-1	Korea	Park et al. [2008]
Ex 26	c.3655C>T	p.R1219W	CUB-1		Donadelli et al. [2006]
Ex 27	c.3716G>T	p.G1239V	CUB-1	Italy	Noris et al. [2005]
Ex 28	c.4006C>T	p.R1336W	CUB-2		Antoine et al. [2003]

* The domain localization of these mutations refers to the localization of the stop codon.

‡ c.[964T>G (+) 968C>G (+) 969C>A (+) 970T>C].

§ p.[C322G (+) T323R (+) F324L].

d Germany/Poland/Czech Republic/Norway/Sweden/Croatia/Australia (Germany)/Turkey

Table 2. Non synonymous polymorphisms of the coding area of *ADAMTS13*.

Exon/intron	DNA	Protein	Domain	dbSNP ID number	Reference
Missense and nonsense					
Ex 1	c.19C>T	p.R7W	Signal peptide	rs34024143	Levy et al. [2001]
Ex 9	c.1016C>G	p.T339R	Disintegrin	/	Fujimura et al. [2009]
Ex 12	c.1342C>G	p.Q448E	Cysteine-rich	rs2301612	Levy et al. [2001]
Ex 12	c.1368G>T	p.Q456H	Cysteine-rich	rs36220239	/
Ex 12	c.1370C>T	p.P457L	Cysteine-rich	rs36220240	/
Ex 12	c.1423C>T	p.P475S	Cysteine-rich	rs11575933	Kokame et al. [2002]
Ex 13	c.1451G>A	p.R484K	Cysteine-rich	rs28375042	/
Ex 16	c.1810G>A	p.V604I	Spacer	rs34256013	/
Ex 16	c.1852C>G	p.P618A	Spacer	rs28647808	Levy et al. [2001]
Ex 16	c.1874G>A	p.R625H	Spacer	rs36090624	Levy et al. [2001]
Ex 16	c.1879G>T	p.E627X	Spacer*	rs60398774	/
Ex 16	c.1900G>A	p.E634K	Spacer	rs34569244	/
Ex 18	c.2195C>T	p.A732V	TSP1-2	rs41314453	Levy et al. [2001]
Ex 19	c.2218G>A	p.E740K	TSP1-2	rs36221451	/
Ex 20	c.2494G>A	p.V832M	TSP1-4	rs34104386	/
Ex 21	c.2699C>T	p.A900V	TSP1-5	rs685523	Levy et al. [2001]
Ex 21	c.2708C>T	p.S903L	TSP1-5	/	Kokame et al. [2007]
Ex 23	c.2944G>A	p.G982R	TSP1-6	rs36222275	/
Ex 24	c.3097G>A	p.A1033T	TSP1-7	rs28503257	Levy et al. [2001]
Ex 25	c.3287G>A	p.R1096H	TSP1-8	rs61751476	/
Ex 26	c.3677C>T	p.T1226I	CUB-1	rs36222894	/
Indels					
Ex 17	c.2059_2060insG	p.V687GfsX158	TSP1-4*	rs34245610	/
Ex 29	c.4190del	p.A1397VfsX35	CUB-2*	rs35876612	/

* The localization of these polymorphisms refers to the localization of the stop codon.

Table 3. Localization, results of in vitro studies, associated age of disease onset and aminoacid residue conservation across species of *ADAMTS13* missense mutations.

Protein	Domain	Secretion compared with WT*	Activity compared with WT*	Age of onset#	Conservation A_B_C_D_E
p.I79M	Metalloprotease				I_V_I_I_V
p.V88M	Metalloprotease	++	++		V_V_V_V_A
p.H96D	Metalloprotease				H_H_H_H_H
p.R102C	Metalloprotease				R_R_R_R_R
p.S119F	Metalloprotease			Intermediate	S_S_S_S_S
p.I178T	Metalloprotease				I_I_I_I_V
p.R193W	Metalloprotease	++	-		R_R_R_R_R
p.T196I	Metalloprotease				T_T_T_T_T
p.S203P	Metalloprotease	++	-		S_S_S_S_S
p.L232Q	Metalloprotease			Neonatal	L_L_L_L_I
p.H234Q	Metalloprotease				H_H_H_H_H
p.D235H	Metalloprotease			Neonatal	D_D_D_D_D
p.A250V	Metalloprotease	++	-		A_A_A_A_G
p.S263C	Metalloprotease				S_S_S_S_S
p.R268P	Metalloprotease	++	-		R_R_R_R_E
p.Y304C	Disintegrin				Y_Y_Y_F_Y
p.C311Y	Disintegrin			Intermediate	C_C_C_C_C
‡	Disintegrin				§
p.C347S	Disintegrin				C_C_C_C_C
p.R349C	Disintegrin				R_R_R_R_R
p.P353L	Disintegrin	+++	+		P_P_P_P_P
p.W390C	TSP1-1				W_W_W_W_W
p.R398H	TSP1-1				R_R_R_R_R
p.P457L	Cysteine-rich	+++	+		P_P_P_P_P
p.R507Q	Cysteine-rich	-	-		R_R_R_R_R
p.C508Y	Cysteine-rich				C_C_C_C_C
p.G525D	Cysteine-rich				G_G_G_G_G
p.R528G	Cysteine-rich				R_R_R_R_R
p.A596V	Spacer	++	+	Neonatal	A_A_A_A_/
p.A606P	Spacer				A_A_A_S_/
p.P671L	Spacer				P_P_P_P_/
p.I673F	Spacer	-	-		I_I_I_I_/
p.R692C	TSP1-2			Neonatal	R_Q_R_R_/
p.C758R	TSP1-3				C_C_C_C_C
p.C908S	TSP1-5				C_C_C_C_/
p.C908Y	TSP1-5	-	-		C_C_C_C_/
p.C951G	TSP1-5				C_C_C_C_/
p.C1024G	TSP1-7				C_C_C_C_C
p.R1060W	TSP1-7	+	++++	Adult	R_Q_R_R_K
p.R1123C	TSP1-8	-	+++	Neonatal	R_R_R_R_R
p.C1213Y	CUB-1	++	+++		C_C_C_C_C
p.I1217T	CUB-1				I_I_I_I_I
p.R1219W	CUB-1	-	+++	Adult	R_R_R_R_R
p.G1239V	CUB-1	-	+++		G_G_G_G_G
p.R1336W	CUB-2	++	++		R_R_R_R_H

***** Secretion and activity of the recombinant mutant proteins in comparison to wild type (WT) ADAMTS13 are presented by means of semi quantitative symbols:**-** indicates not detectable; + severely reduced; ++ reduced; +++ slightly reduced; ++++ similar to WT.

\# The age of disease onset associated with a certain mutation was reported when the mutation was reported in the homozygous state.

‡ p.[C322G (+) T323R (+) F324L].

§ C322G: C_C_C_C_C; T323R: T_T_T_T_T; F234L: F_F_F_F_F.

A, *Homo sapiens*; **B**, *Bos Taurus*; **C**, *Mus musculus*; **D**, *Rattus norvegicus*; **E,** *Gallus gallus*.

Table 4. Results of in vitro expression studies of *ADAMTS13* mutations and single nucleotide polymorphisms (SNPs).

Position	DNA	Protein	System	Secretion	Activity	Reference
Missense						
Ex 3	c.262 G>A	p.V88M	HEK293	40%	18%	Peyvandi et al.
			HEK293/Drosophila	30%	40%*#	Donadelli et al.
Ex 6	c.577C>T	p.R193W	HeLa	Reduced	Not	Matsumoto et al.
Ex 6	c.607T>C	p.S203P	HeLa	21%	/	Hommais et al.
			COS-7	18%	Not	Hommais et al.
Ex 7	c.749 C>T	p.A250V	HEK293	Reduced	Not	Uchida et al. [2004]
Ex 7	c.803G>C	p.R268P	HeLa	Not detectable	Not	Kokame et al.
			HeLa	38%	/	Hommais et al.
			COS-7	25%	Not	Hommais et al.
Ex 9	c.1058C>T	p.P353L	COS-7	69%	3%	Manea et al. [2007]
Ex 12	c.1370C>T	p.P457L	COS-7	70%	4%	Manea et al. [2007]
Ex 13	c.1520 G>A	p.R507Q	HeLa	Not detectable	/	Hommais et al.
			COS-7	1%	Not	Hommais et al.
Ex 13	c.1523G>A	p.C508Y	HeLa	Not detectable	Not	Kokame et al.
Ex 16	c.1787 C>T	p.A596V	HeLa	17%	/	Hommais et al.
			COS-7	20%	25%*	Hommais et al.
Ex 17	c.2017A>T	p.I673F	HeLa	Not detectable	Not	Matsumoto et al.
Ex 21	c.2723 G>A	p.C908Y	HeLa	Not detectable	Not	Matsumoto et al.
Ex 24	c.3178 C>T	p.R1060W	HeLa	11%	35%*	Tao et al. [2006]
			HEK293	Severely	100%*	Camilleri et al.
Ex 25	c.3367 C>T	p.R1123C	HeLa	Not detectable	Not	Matsumoto et al.
			HEK293/Drosophila	Not detectable	64%*#	Donadelli et al.
Ex 26	c.3638G>A	p.C1213Y	HEK293	29%	80%*	Zhou et al. [2009]
Ex 26	c.3655C>T	p.R1219W	HEK293/Drosophila	Not detectable	62%*#	Donadelli et al.
Ex 27	c.3716G>T	p.G1239V	HEK293	Not detectable	6%	Peyvandi et al.
			HEK293/Drosophila	Not detectable	66%*#	Donadelli et al.
Ex 28	c.4006C>T	p.R1336W	HEK293	23%	12%*	Plaimauer et al.
Other						
Ex 2	c.130C>T	p.Q44X	HEK293	Not detectable	/	Plaimauer et al.
Ex 12	c.1345C>T	p.Q449X	HeLa	Conserved	Not	Kokame et al.
Ex 27	c.3735 G>A	p.W1245X	HeLa	24%	100%*	Zhou et al. [2009]
Ex 29	c.4143dupA	p.E1382RfsX6	COS-7	14%	85%*	Pimanda et al.
			HEK293	4%	10%	Garagiola et al.
Ex 27	c.3770dupT	p.L1258VfsX36	HeLa	8%	100%*	Zhou et al. [2009]
Ex 3	c.291_319del	p.E98PfsX31	HEK293	Not detectable	Not	Garagiola et al.
Ex 10	c.1096_1113d	p.C365-C370del	HeLa	7%	Not	Tao et al. [2006]
Ex 23	c.2930_2935d	p.C977_R979delin	HEK293	5%	6%	Palla et al. [2009]
SNPs						
Ex 1	c.19C>T	p.R7W	HEK293	99%	86%*	Plaimauer et al.
Ex 12	c.1342C>G	p.Q448E	HeLa	Conserved	Conserved	Kokame et al.
			HEK293	95%	75%*	Plaimauer et al.
Ex 12	c.1423C>T	p.P475S	HeLa	Conserved	Reduced	Kokame et al.
Ex 16	c.1852C>G	p.P618A	HEK293	27%	14%*	Plaimauer et al.
Ex 18	c.2195C>T	p.A732V	HEK293	60%	71%*	Plaimauer et al.
Ex 24	c.3097G>A	p.A1033T	HeLa	Conserved	80%	Tao et al. [2006]

* Activity expressed as 'specific activity', normalized for equal amounts of wild type protein.

° The localization of these mutations refers to the localization of the stop codon.

\# Donadelli et al, 2006 used a BiP promoter and *Drosophila* cell lines to obtain secretion of mutant ADAMTS13 that were not secreted in eukaryotic cell lines. The activity of mutant proteins was then measured.

System indicates the type of cells used for the experiments.

Supplementary Material S1. Patients with inherited ADAMTS13 deficiency reported in the literature. In all patients mutations have been identified on both alleles of *ADAMTS13*. Only one mutation is reported in the table for patients with homozygous mutations, whereas both mutations are reported for compound heterozygotes. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Patients	Mutation	Mutation effect	Age of onset	Reference
Individuals with two mutations				
1	c.2074C>T	p.R692C	Neonatal	
2	c.2074C>T	p.R692C	Neonatal	
3	c.2074C>T	p.R692C	Neonatal	
4	c.286C>G	p.H96D	Neonatal	
	c.2851T>G	p.C951G		
5	c.286C>G	p.H96D	8 years	
	c.2851T>G	p.C951G		
6	c.1582A>G	p.R528G	Neonatal	
	c.3770dupT	p.L1258VfsX36		***Levy 2001***
7	c.1193 G>A	p.R398H	Neonatal	
	c.3070T>G	p.C1024G		
8	c.304C>T	p.R102C	Neonatal	
	c.587C>T	p.T196I		
9	c.2376_2401del	p.A793PfsX43	Neonatal	
	c.3638G>A	p.C1213Y		
10	c.1584+5G>A		4 years	
11	c.803G>C	p.R268P	Neonatal	
	c.1523G>A	p.C508Y		***Kokame 2002***
12	c.1345C>T	p.Q449X	Neonatal	
13	c.1169G>A	p.W390X	Neonatal	
	c.2549_2550del	p.D850GfsX7		
14	c.2728C>T	p.R910X	3 years	
	c.4143dupA	p.E1382RfsX6		
15	c.1058C>T	p.P353L	4 years	
	c.4143dupA	p.E1382RfsX6		
16	c.695T>A	p.L232Q	Neonatal	***Schneppenheim 2003***
17	c.3100A>T	p.R1034X	Neonatal	
	c.4143dupA	p.E1382RfsX6		
18	c.1058C>T	p.P353L	2 years	
	c.2728C>T	p.R910X		
19	c.788C>G	p.S263C	Neonatal	
	c.4143dupA	p.E1382RfsX6		
20	c.130C>T	p.Q44X	21 years	
	c.4006C>T	p.R1336W		***Antoine 2003***
21	c.130C>T	p.Q44X	N.A.	
	c.4006C>T	p.R1336W		
22	c.1783_1784del	p.L595GfsX19	Neonatal	***Savasan 2003***
23	c.932G>A	p.C311Y	23 months	
24	c.703G>C	p.D235H	Neonatal	
25	c.703G>C	p.D235H	Neonatal	
26	c.1058C>T	p.P353L	20 months	
	c.1370C>T	p.P457L		
27	c.718_724del	p.S240AfsX7	Neonatal	
	c.2728C>T	p.R910X		***Assink 2003***
28	c.718_724del	p.S240AfsX7	Neonatal	
	c.2728C>T	p.R910X		
29	c.2279del	p.G760AfsX18	7 months	
30	c.4143dupA	p.E1382RfsX6	Neonatal	
31	c.4143dupA	p.E1382RfsX6	64 months	
32	c.262G>A	p.V88M	22 years	
	c.3716G>T	p.G1239V		
33	c.262 G>A	p.V88M	No episodes at 53 years	***Bestetti 2003***
	c.3717 G>T	p.G1239V		
34	c.262 G>A	p.V88M	23 years	
	c.3717 G>T	p.G1239V		
35	c.414+1G>A	r.spl?	4 years	***Matsumoto 2004***
36	c.414+1G>A	r.spl?	Neonatal	
	c.2017A>T	p.I673F		

37	c.577C>T	p.R193W	Neonatal	
	c.1244+2T>G	r.spl?		
38	c.2017A>T	p.I673F	Neonatal	
	c.2723G>A	p.C908Y		
39	c.686+1G>A		Neonatal	
40	c.1309-?G>A		Neonatal	***Veyradier 2004***
	c.2723G>C	p.C908S		
41	c.1520G>A	p.R507Q	Neonatal	
	c.1787C>T	p.A596V		
42	c.825-? ?del		Neonatal	
	c.2272T>C	p.C758R		
43	c.237C>G	p.I79M	Neonatal	
	c.803G>C	p.R268P		
44	c.1787C>T	p.A596V	Neonatal	
45	c.607T>C	p.S203P	Neonatal	
	c.3254_3255del	p.S1085CfsX12		
46	c.1170G>C	p.W390C	3 years	***Licht 2004***
	c.3735G>A	p.W1245X		
47	c.749C>T	p.A250V	N.A.	***Uchida 2004***
	c.330+1 G>A	r.[330_331ins1055, 330_331ins127]		
48	c.2074C>T	p.R692C	2 months	***Snider 2004***
49	c.687-2A>G		Neonatal	***Studt 2005***
50	c.702C>A	p.H234Q	3 months	***Shibagaki 2006***
	c.3616C>T	p.R1206X		
51	c.1095_1112del	p.W365-R370del	N.A.	***Tao 2006***
	c.3178C>T	p.R1060W		
52	c.3178C>T	p.R1060W	N.A.	***Schneppenheim 2006***
	c.4143dupA	p.E1382RfsX6		
53	c.4143dupA	p.E1382RfsX6	14 years	
54	c.587C>T	p.T196I	1.5 years	
	c.4143dupA	p.E1382RfsX6		
55	c.2012C>T	p.P671L	6 years	
	c.4143dupA	p.E1382RfsX6		
56	c.4143dupA	p.E1382RfsX6	N.A.	
57	c.1520G>A	p.R507Q	N.A.	
	c.4143dupA	p.E1382RfsX6		
58	c.1039T>A	p.C347S	N.A.	
	c.4143dupA	p.E1382RfsX6		
59	c.4143dupA	p.E1382RfsX6	4 years	
60	c.4143dupA	p.E1382RfsX6	4.5 years	
61	c.4143dupA	p.E1382RfsX6	Neonatal	
62	c.3367C>T	p.R1123C	Neonatal	***Donadelli 2006***
63	c.3655C>T	p.R1219W	35 years	
64	c.3655C>T	p.R1219W	28 years	
65	c.82dupT	p.W28LfsX111	19 years	
	c.3178C>T	p.R1060W		
66	c.82dupT	p.W28LfsX111	21 years	
	c.3178C>T	p.R1060W		
67	c.82dupT	p.W28LfsX111	No episodes at 25 years	
	c.3178C>T	p.R1060W		
68	c.82dupT	p.W28LfsX111	No episodes at 25 years	
	c.3178C>T	p.R1060W		
69	c.4143dupA	p.E1382RfsX6	Neonatal	***Manea 2007a***
70	c.4143dupA	p.E1382RfsX6	3 years	***Manea 2007b***
71	‡	§	3 years	***Kokame 2008***
	c.2723G>A	p.C908Y		
72	c.3178C>T	p.R1060W	33 years	***Camilleri 2008***
73	c.330+1G>A	r.[330_331ins1055, 330_331ins127]	15 months	***Park 2008***
	c.3650T>C	p.I1217T		
74	c.356C>T	p.S119F	17 years	***Meyer 2008a***
75	c.291_319del	p.Q97fsX31	15 years	***Garagiola 2008***
	c.4143dupA	p.E1382RfsX6		
76	c.291_319del	p.Q97fsX31	No episodes at 22 years	
	c.4143dupA	p.E1382RfsX6		
77	c.911A>G	p.Y304C	N.A.	***Fujimura 2009***
	c.1574G>A	p.G525D		
78	c.911 A>G	p.Y304C	Neonatal	
	c.1574G>A	p.G525D		
79	c.372_373insGT	p.R125VfsX6	27 years	
	c.3904C>T	p.Q1302X		
80	c.372_373insGT	p.R125VfsX6	N.A.	
	c.3904C>T	p.Q1302X		

81	c.577C>T	p.R193W	33 years	
	c.1045 C>T	p.R349C		
82	c.577C>T	p.R193W	30 years	
	c.1045 C>T	p.R349C		
83	c. 533 T>C	p.I178T	26 years	
	c.2785 C>T	p.Q929X		
84	c.577C>T	p.R193W	N.A.	
	c.1816G>C	p.A606P		
85	c.577C>T	p.R193W	N.A.	
86	c.2930_2935del	p.C977_R979delinsW	23 years	
87	c.2930_2935del	p.C977_R979delinsW	29 years	***Palla 2009***
88	c.2930_2935del	p.C977_R979delinsW	No episodes at 24 years	
89	c.3178C>T	p.R1060W	18 years	***This study***
Individuals with one mutation				
90	c.3047G>A	p.W1016X	5 years	***Donadelli 2006***
	Not found			
91	c.3178C>T	p.R1060W	21 years	***Camilleri 2008***
	Not found			
92	c.3178C>T	p.R1060W	31 years	
	Not found			

‡ c.[964T>G (+) 968C>G (+) 969C>A (+) 970T>C].

§ p.[C322G (+) T323R (+) F324L].

N.A., not available.

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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.

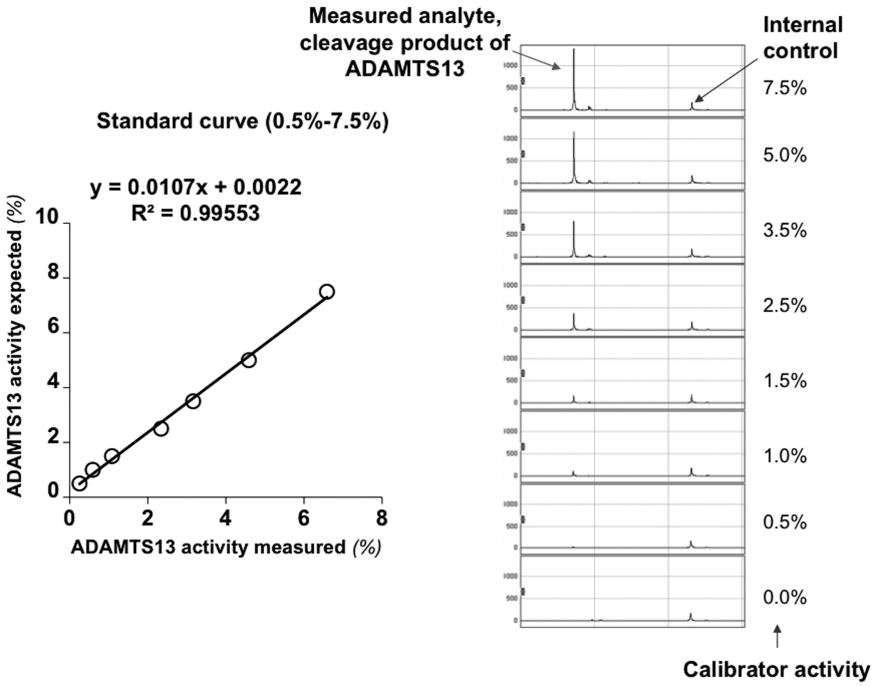


Figure 2. Relationship between residual ADAMTS13 activity and clinical features of congenital TTP. Panel A: association between residual ADAMTS13 deficiency and age of disease onset in 29 patients with congenital TTP at linear regression analysis. Only a few outliers were detected. A patient from the 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.

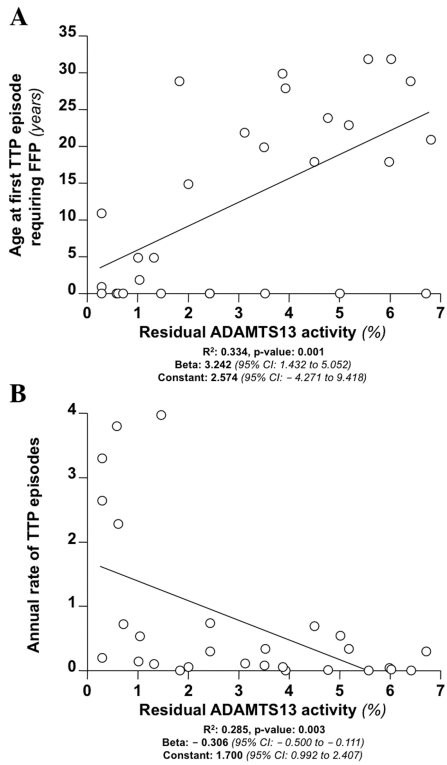


Figure 3. Relationship between *ADAMTS13* genotype, residual activity and age of disease onset. Age of disease onset and residual ADAMTS13 activity in multiple patients carrying the same mutations of *ADAMTS13*. A clustering of both the age of onset and the plasmatic activity of ADAMTS13 can be observed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

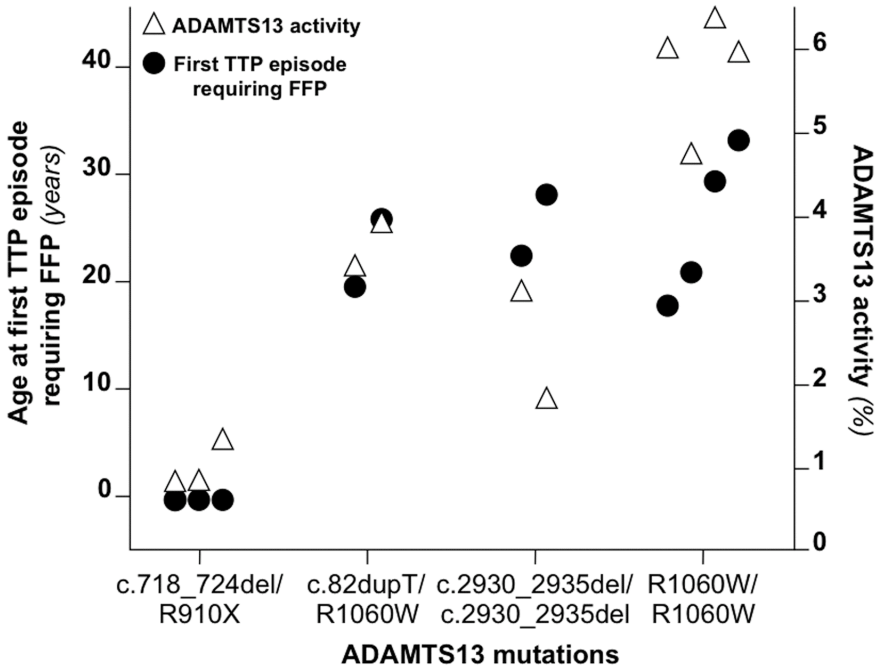


Figure 4. Evolutionary conservation, associated ADAMTS13 plasmatic activity and age of disease onset of N- and C-terminal mutations of ADAMTS13. Panel A: evolutionary conservation of amino acid residues affected by missense mutations of *ADAMTS13* associated with congenital TTP in all cases reported 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.

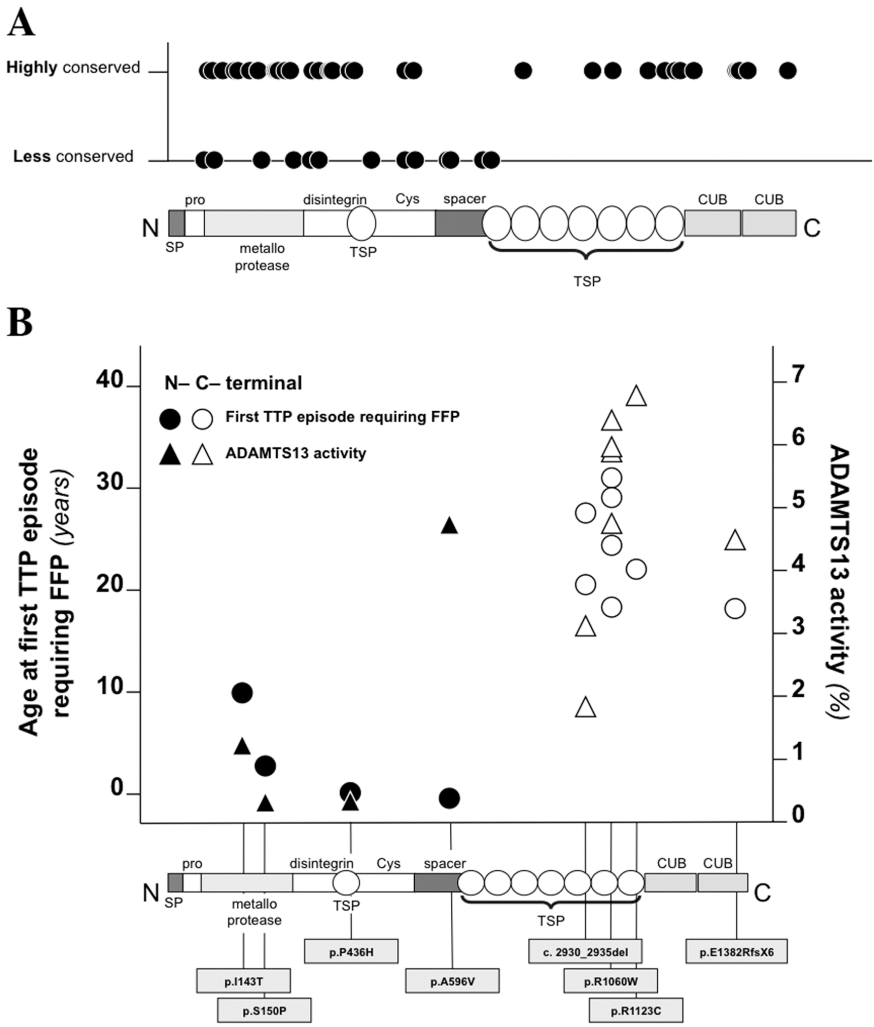
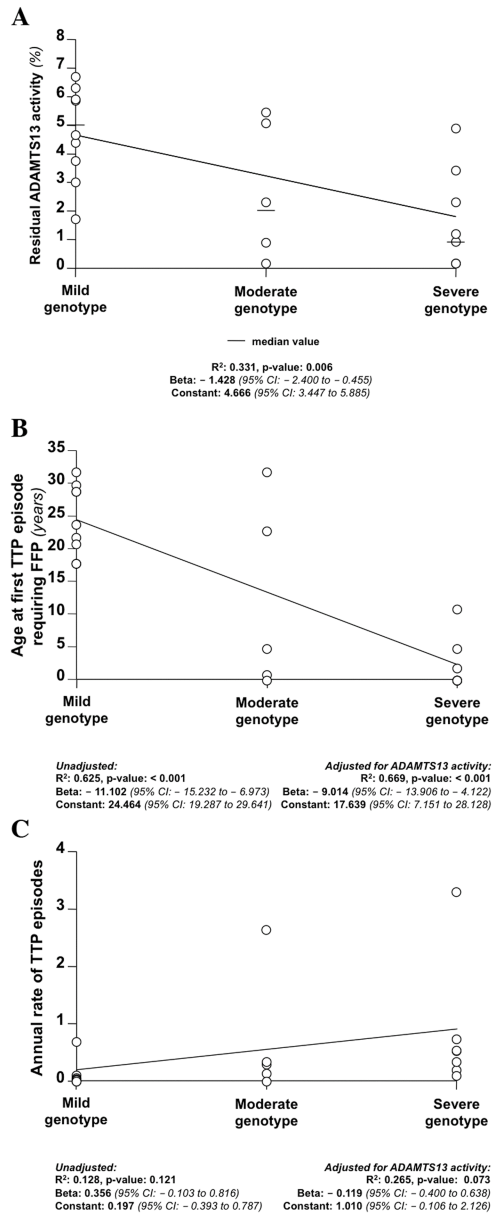


Figure 5. Association between genotype severity score, plasmatic activity of ADAMTS13, age of disease onset and annual rate of TTP episodes. Increasing severity in the genotype score (i.e. an increasing number of N-terminal mutations; 'mild genotype' = 2 C-terminal mutations, 'moderate genotype' = 1 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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CHAPTER 3

Clinical case: drop of residual plasmatic activity of ADAMTS13 to undetectable levels during acute disease in a patient with adult-onset congenital thrombotic thrombocytopenic purpura

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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 a congenital severe deficiency of ADAMTS13 plasmatic activity caused by mutations in the *ADAMTS13* gene.[1-4] Congenital TTP displays a heterogeneous clinical severity with respect to age of disease onset, frequency of acute disease episodes and need for fresh frozen plasma (FFP) prophylaxis.[1-4] We recently found that residual ADAMTS13 activity, measured by highly-sensitive surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry,[5] is inversely associated with the severity of the disease. Low residual activity measured during disease remission was associated with a history of early disease onset, a high annual rate of TTP episodes and prescription of FFP prophylaxis.[5] These results suggest that congenital TTP patients who have some residual plasmatic activity of ADAMTS13 (we found 2-3% to be the critical level) are protected from early disease and frequent recurrences, developing the disease only when the fragile balance between residual ADAMTS13 and its cleavage substrate, the pro-thrombotic ultralarge forms of von Willebrand factor (VWF), is shifted towards ultralarge VWF release by environmental perturbations (e.g. infections, traumas, pregnancy, etc.). According to this "two-hit" model, one would expect to see ADAMTS13 activity drop during acute disease even in patients who during remission have some degree of residual plasmatic activity. However, in the aforementioned study residual ADAMTS13 activity was measured only during disease remission.[5] In this paper, we report the case of an Italian patient with congenital TTP who developed one single acute episode of

the disease in her adult life. The patient had been included in the study of residual ADAMTS13 during remission, but the clinical history of the patient is hereby presented in full and we also report the results of SELDI-TOF-based ADAMTS13 activity measurement during and after the acute disease episode. The patient provided informed consent and the study was approved by the institutional review board of the Fondazione IRCCS Ca' Granda – Ospedale Maggiore Policlinico.

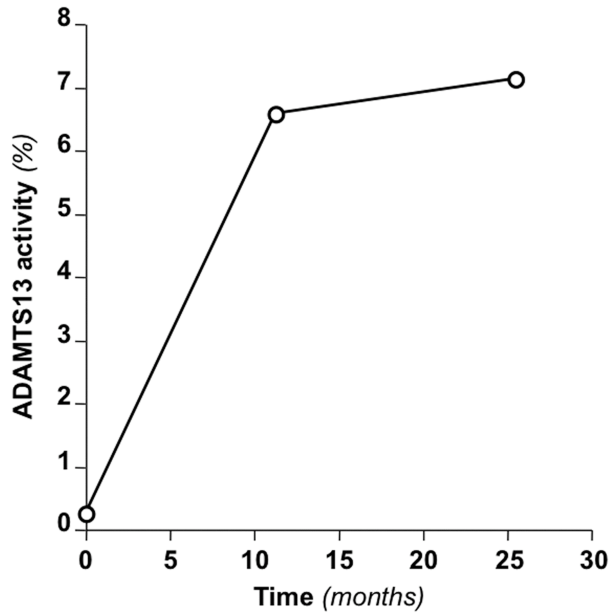
At the age of 32, during the 19th week of her first pregnancy, the patient was admitted to the gynecology department of her local hospital with epigastric pain, fatigue and headache. Complete blood count showed anemia (hemoglobin: 8.7 g/L) and thrombocytopenia (platelet count: 42 x 10^9/L) with increased reticulocyte count and presence of schistocytes at the blood smear. Lactate dehidrogenase and indirect bilirubin levels were also above the upper normal value, direct Coombs tests was negative. There was no sign of renal involvement (creatinine: 79 μmol/L). Both alanine and aspartate transaminases were elevated. While elevation of liver enzymes suggested a diagnosis of HELLP, the presence of schistocytes supported a diagnosis of TTP. Plasma exchange (PEX) was implemented, with rapid increase of platelet counts. A total of 5 daily PEX procedures were performed. After the 5th procedure the patient developed an allergic reaction with bronchospasm, which led to the administration of corticosteroids and to the temporary suspension of PEX. At this time, fetal distress developed and the patient agreed to the therapeutic termination of pregnancy. Platelet counts dropped to 59 x 10^9/L and PEX was resumed. A total of 7 procedures were carried out, with resolution of the disease symptoms and

correction of laboratory abnormalities. After remission was achieved, the patient was referred to the outpatient clinic for thrombotic microangiopathies of the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center. ADAMTS13 assays hereby mentioned have been described elsewhere.[6, 7] Measurement of ADAMTS13 plasmatic activity by collagen binding assay on plasma samples collected during the first and second visits to the clinic, as well as on a sample that had been collected at the time of acute disease showed severely deficient plasmatic activity of ADAMTS13 (i.e. activity below 10% of normal). ADAMTS13 antigen levels were severely reduced. No anti-ADAMTS13 autoantibody was detectable by western blotting or ELISA in any of the samples, indicating congenital ADAMTS13 deficiency. *ADAMTS13* gene analysis by PCR and Sanger sequencing identified two novel missense mutations, not present in the dbSNP database. The two mutations were a c.578G>A substitution in exon 6 of *ADAMTS13* leading to a p.R193Q protein change in the metalloprotease domain and a c.3283C>T substitution in exon 25 leading to a p.R1095W protein change in the eighth thrombospondin-1-like domain of ADAMTS13. Both changes were predicted to be potentially damaging for protein function by Polyphen 2 (URL: http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) software and were not present in 198 alleles of thrombosis free individuals sequenced in the frame of the DVT-Milan study. A diagnosis of congenital TTP was made. The patient remained asymptomatic during follow-up, with no need for FFP prophylactic infusion. In order to assess whether or not the patient had measurable ADAMTS13 activity during acute disease and remission, the residual plasmatic activity of ADAMTS13 was quantified by SELDI-TOF

mass spectrometry.[5] A detectable ADAMTS13 cleavage activity was found in the two samples collected during disease remission months after the last PEX procedure, whereas it was undetectable during acute disease before the implementation of PEX (Figure). Herein, we have reported the measurement of residual plasmatic activity of ADAMTS13 by SELDI-TOF mass spectrometry in a patient with congenital TTP during both remission and acute disease. During remission, ADAMTS13 activity was detectable, with values above 5%. Consistent with the patient's clinical history, similar residual activity levels were shown to be associated with adult-onset disease and low-tendency towards recurrence. By contrast, activity during acute disease was completely abolished, consistent with the hypothesis that onset of acute TTP is associated with a drop in ADAMTS13 levels. Pregnancy, a recognized TTP-triggering condition that is physiologically associated with reduction in ADAMTS13 and raise in VWF plasmatic levels, is likely to have precipitated the onset of acute disease in the patient. In conclusion, this case report of a patient with congenital TTP highlighted the reduction of ADAMTS13 activity to undetectable levels in a patient with otherwise detectable residual ADAMTS13. This case is consistent with the paradigm of a 'two-hit' model in TTP, whereby a triggering agent can cause the onset of acute disease by abrogating residual activity in patients with already severely-reduced plasmatic ADAMTS13.

Figures

Figure 1. Residual ADAMTS13 activity measured by SELDI-TOF mass spectrometry at different moments of the clinical course.



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CHAPTER 4

Residual ADAMTS13 activity in ADAMTS13-deficient thrombotic thrombocytopenic purpura: an emerging concept

Luca A Lotta, Haifeng M Wu, Khaled M Musallam, Flora Peyvandi.

Abstract

Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening thrombotic microangiopathy characterized by acute episodes of widespread microvascular thrombosis. The discovery that the plasmatic activity of the von Willebrand factor (VWF) cleaving protease, ADAMTS13, is severely deficient in a great proportion of individuals with TTP partially clarified the pathophysiology of the disease. However, the finding of severe deficiency of ADAMTS13 alone is unable to fully explain the clinical heterogeneity of patients with TTP. The recent development of methods that measure ADAMTS13 activity with great analytical sensitivity and precision offers the opportunity to study the spectrum of ADAMTS13 activity below 10% (herein defined as "residual ADAMTS13 activity"). Recent exploratory studies on residual ADAMTS13 activity suggest that the amount of residual activity of ADAMTS13 might be a major determinant of the clinical heterogeneity of TTP in patients with severe ADAMTS13 deficiency. In this article, we review the recent findings on residual ADAMTS13 activity and their implications for research and clinical practice in the field.

Introduction and scope of this review

Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening thrombotic microangiopathy characterized by acute episodes of widespread microvascular thrombosis.[1] The discovery that the plasmatic activity of the von Willebrand factor (VWF) cleaving protease, ADAMTS13, is severely deficient in a great proportion of individuals with TTP partially clarified the pathophysiology of the disease.[2] Severe ADAMTS13 deficiency is defined as an activity of the protease below 10% of normal, with normal values usually ranging form 50% to 150%. Severe ADAMTS13 deficiency is either due to circulating anti-ADAMTS13 autoantibodies (i.e. acquired deficiency)[3] or, less frequently, to recessively inherited mutations of *ADAMTS13* (i.e. congenital deficiency).[4] Although not all of the patients with a clinically defined TTP present with severely reduced ADAMTS13, the finding of severe ADAMTS13 deficiency has been consistently shown to define a distinct subgroup of the disease, with peculiar behavior and clinical features.[5-7] In these patients, the pathologic presence in the circulation of ultralarge VWF multimers that remain uncleaved is regarded as the mechanism responsible for VWF-mediated platelet aggregation and thrombosis.[8] The peculiar clinical characteristics of TTP with severe ADAMTS13 deficiency include a relatively mild acute-disease prognosis, with better response to plasma exchange (PEX) and lower episode-fatality rate, and high tendency to recur.[6, 9] In spite of the recognized importance of measuring ADAMTS13 activity at acute disease presentation, the finding of severe deficiency of ADAMTS13 alone is unable to fully explain the clinical heterogeneity of patients with TTP. For example, patients with auto-antibody mediated severe deficiency of ADAMTS13 may

remain in remission for years, in spite of unmeasurable or very low ADAMTS13 activity and persistence of anti-ADAMTS13 autoantibodies.[9] Also, some patients with congenital severe deficiency of ADAMTS13 develop their first disease episode in their adult age, whereas others have early-onset disease and frequent recurrences.[10, 11] The recent development of methods that measure ADAMTS13 activity with great analytical sensitivity and precision offers the opportunity to study the spectrum of ADAMTS13 activity below 10%.[11, 12] Residual ADAMTS13 activity is herein used to define the amount of activity below 10% that is detectable in patients with severe ADAMTS13 deficiency. Recent exploratory studies found that patients with severe deficiency of ADAMTS13 show a range of residual plasmatic activity and that residual activity is associated with clinically relevant endpoints.[13-16] This suggests that the amount of residual activity of ADAMTS13 might be a major determinant of the clinical heterogeneity of TTP in patients with severe ADAMTS13 deficiency. In this article, we review the recent findings on residual ADAMTS13 activity and their implications for research and clinical practice in the field. The review will focus only on TTP with severely deficient ADAMTS13 activity.

TTP with severe deficiency of plasmatic ADAMTS13 activity as a distinct subtype of the disease

TTP was originally described by Eli Moschcowitz in 1924 as a syndrome with a pentad of clinical manifestations: thrombocytopenia, microangiopathic hemolytic anemia, neurologic symptoms, renal involvement and fever.[17] It was later recognized that fever, neurologic symptoms or renal involvement are not always

present during TTP, so that TTP is currently defined by the presence of thrombocytopenia and microangiopathic hemolytic anemia and by the exclusion of an alternative diagnosis.[18] The pathophysiology of TTP remained unexplained for many decades until studies in the early 1980s demonstrated that patients with chronic relapsing TTP had a defect in their ability to cleave unusually large VWF multimers.[19] Subsequently, in the late 1990s it was found that the plasma of patients with the disease was severely deficient of von Willebrand factor (VWF) cleaving protease activity.[8] Further studies identified anti-ADAMTS13 antibodies[3] and mutations in ADAMTS13[4] as causes of severely reduced ADAMTS13 activity. The discovery of the role of ADAMTS13 sparked investigations on laboratory measurements related to the protease. A number of observational studies demonstrated that ADAMTS13 deficiency is not invariably present in patients with a diagnosis of TTP, but also that patients with severe deficiency of ADAMTS13 have distinct clinical characteristics and prognosis.[5-7, 9, 20-22] Severe ADAMTS13 deficiency in TTP patients is associated with idiopathic rather than secondary disease (i.e., disease associated with clinical conditions such as cancer, bone marrow transplantation or use of certain drugs), lower platelet count at presentation and lower prevalence of renal involvement.[5, 6, 9, 22] Patients with severely reduced ADAMTS13 activity at acute disease presentation have milder acute disease prognosis with better response to PEX and lower episode fatality rate than those with ADAMTS13 above 10%.[5, 6, 9] This association is caused by the poor prognosis of secondary TTP forms in which severe ADAMTS13 deficiency has low prevalence (e.g., bone-marrow transplantation associated TTP or cancer-associated TTP). Patients with severe

deficiency are also more prone to recurrence.[9, 14, 20, 23] A recent study found a 41% percent recurrence risk at 7.5 years in TTP survivors who presented with severe ADAMTS13 deficiency, whereas survivors with activity above 10% had a recurrence rate of only 4%.[9] Studies on ADAMTS13-related measurements as markers for recurrence are summarized in Table 1. Also ADAMTS13-related laboratory measurements other than ADAMTS13 activity were shown to be associated with disease outcomes. These markers include ADAMTS13 antigen levels and IgG titre, inhibitor titre, Ig subclass and specificity of anti-ADAMTS13 antibodies.[6, 9, 20, 23-26] However, the value of these markers as predictors after accounting for ADAMTS13 activity is uncertain. This prompted researchers to look for additional prognostic markers that could help predict the risk of acute disease morbidity and mortality and the imminence of disease recurrence.[27]

Residual ADAMTS13 activity in congenital and acquired ADAMTS13 deficiency

Until recently the quantification of ADAMTS13 plasmatic activity relied on assays that were developed in order to distinguish patients with severely reduced from patients with normal or slightly reduced protease activity. Assays standardization, and limitations in sensitivity, linear behavior and reproducibility were well recognized issues affecting early-developed methods.[28] The performance of ADAMTS13 assays improved through the years, with the development of new methods and improvement of existing ones.[29] Recently, assays able to measure residual ADAMTS13 activity with great analytical

sensitivity (limit of detection [LOD] of up to 0.5%) have been developed.[11, 12] In particular, a method based on SELDI-TOF mass spectrometry for the detection of cleavage product showed linear behavior for the measurement of activities below 10% and great reproducibility.[13] The assay was employed in a number of exploratory studies that sought for associations between residual ADAMTS13 plasmatic activity and disease outcomes.[13-16]

Residual ADAMTS13 activity in congenital TTP

Congenital TTP (also known as Upshaw-Schulman syndrome, OMIM #274150) is caused by mutations in *ADAMTS13* and accounts for less than 10% of all cases of TTP. As noted, the phenotype of congenital TTP is variable in its clinical severity.[10, 11] A study was recently conducted in which the residual activity of ADAMTS13 was measured by SELDI-TOF mass spectrometry in a cohort of 29 patients with congenital TTP.[15] The study was designed to test the hypothesis that genetically-determined patterns of residual ADAMTS13 activity were responsible for the variable clinical phenotype. The study found that a residual plasmatic activity of ADAMTS13 was measurable in 90% of the study participants. It also showed that the amount of residual plasmatic activity of ADAMTS13 measured by SELDI-TOF mass spectrometry is negatively associated with the clinical severity of the phenotype (as assessed by the age of disease onset, the annual rate of TTP episodes and the prescription of FFP prophylaxis). Residual plasmatic activity levels below 2-3% pinpointed patients with adverse clinical outcomes (i.e. age of onset below 18 years, annual rate of TTP episodes greater than 1 and need for FFP prophylaxis). Although these discriminative levels should be

validated in an independent cohort before being introduced in clinical practice, the results of the study suggest that the use of a sensitive ADAMTS13 activity measurement could be used to tailor individual management, in particular concerning the decision of whether or not to implement long-term FFP prophylaxis. The results also provide pathophysiological insights. A model of congenital TTP is suggested whereby 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, infection, surgery). In contrast, patients with a low or with no residual activity are more vulnerable to environmental challenges, developing frequent disease episodes. Consistent with this model is the relationship between residual ADAMTS13 activity and the age of first disease episode of congenital TTP patients. Patients with higher residual activity had an older age of onset (Figure 1A), although a few patients had early age of onset in spite of relatively high residual activity. This may be due to the presence of genetic modifiers or to a varying impact of environmental triggering conditions (Figure 1B); most importantly, none of the patients with low residual activity had adult age of onset (Figure 1C). Additional support for this model came from the measurement of residual activity during acute TTP and remission in a patient with congenital TTP.[16] While activity was detectable in multiple remission samples, a drop to undetectable levels was observed during an acute disease episode. Overall, these studies highlight an important contribution of residual ADAMTS13 as a determinant of disease severity in congenital TTP.

Residual ADAMTS13 activity in acquired TTP

In patients with acquired TTP, the amount of residual ADAMTS13 activity in patients with severely deficient ADAMTS13 (activity below 10%) was associated with both subsequent TTP exacerbation (i.e. reappearance of disease within 30 days of the remission of a previous event)[14] and recurrence (i.e. reappearance of disease after 30 days).[13] In the former study, mean ADAMTS13 activity at presentation was significantly lower in patients who suffered exacerbations than in those who did not (mean residual ADAMTS13 of 1.4% vs 2.9%). Receiver operating characteristic curve analysis of all study subjects indicated that the discriminative level of residual ADAMTS13 activity for the risk of exacerbation was 1.15%. In the latter study, the association between residual ADAMTS13 activity and disease recurrence was obtained within a group of patients who already had a diagnosis of severely reduced ADAMTS13. This indicates that the measurement of ADAMTS13 in TTP patients is informative of the risk of recurrence also for activities below 10%. The relationship between ADAMTS13 activity and risk for recurrence is characterized by an exponential curve (Figure 2). This pattern confirms the importance of being able to measure low ADAMTS13 activity, as the risk of recurrence witnesses a steep increase for decreasing activity below levels of 20% and especially below 10%. Anecdotic reports on the risk of pregnancy-associated TTP support these results, showing that women who develop TTP associated with pregnancy have very low levels of ADAMTS13 activity (below 2.5%) at the beginning of their pregnancy.

Future directions

The concept that the residual activity of ADAMTS13 in patients with ADAMTS13-deficient TTP is an important determinant of disease severity and of clinically relevant outcomes has implications for future research in the field. The development of a new generation of assays able to measure in a fast, cost effective and reproducible way ADAMTS13 activity at low concentrations is warranted in order to apply residual activity measurement to large-scale research and in clinical practice. Similarly sensitive and precise assays of ADAMTS13 antigen levels might help calculating the specific activity of mutated ADAMTS13 in congenital patients in whom residual plasmatic activity has been quantified. In this group of patients the benefit of measuring residual ADAMTS13 repeatedly and that of using the recently identified discriminative levels for clinical decisions will be the focus of future research. In the field of acquired TTP, the exponential increase of TTP recurrence risk for decreasing values of ADAMTS13 activity implies that much of predictive power of measuring ADAMTS13 activity may stand in a tail of the ADAMTS13 activity spectrum (which is poorly ascertained by most of the currently available assays). The hypothesis merits further and thorough testing, as researchers in the field of TTP might happen to already have in hand the clinical marker they were looking for.

Figures

Figure 1. Relationships between residual ADAMTS13 activity and age of disease onset in congenital TTP. Increasing residual activity is associated with older age of onset (A). A few patients with high residual activity have early age of onset (B). None of the patients with low residual activity have late disease onset (C).

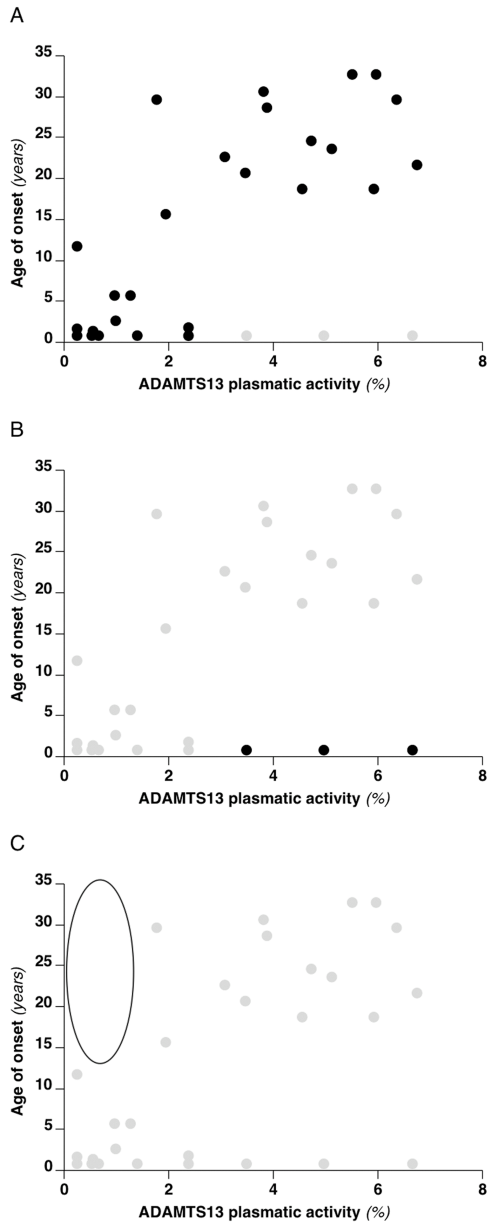
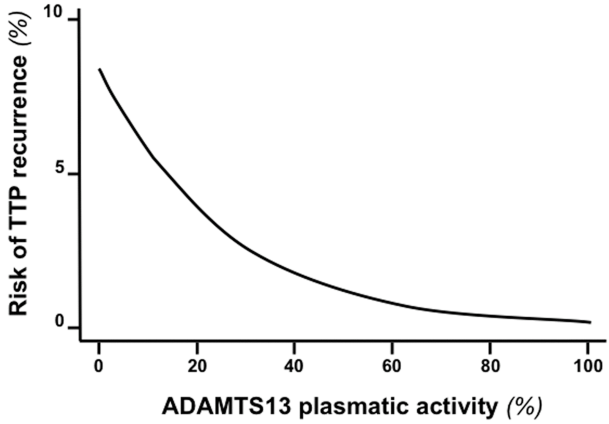


Figure 2. Exponential decay of the risk of thrombotic thrombocytopenic purpura recurrence for increasing levels of ADAMTS13 activity.



Tables

Table 1. Studies that investigated ADAMTS13-related measurements in thrombotic thrombocytopenic purpura.

Reference	Desing	Timing of measurement	Marker	Endpoint	Result
Ferrari et al. Blood 2007	Cohort	Acute TTP	Anti-ADAMTS13 inhibitor levels	Acute disease mortality	Inhibitor at presentation not associated with death
		Acute TTP	Anti-ADAMTS13 Ig class	Acute disease mortality	High-IgA titres associated with death
		Remission	ADAMTS13 activity	Recurrence	Severe ADAMTS13 deficiency increases risk
Peyvandi et al. Haematologica 2008	Case-control	Remission	ADAMTS13 activity	Recurrence	Severe ADAMTS13 deficiency increases risk
		Remission	ADAMTS13 antigen	Recurrence	Severe antigen deficiency not associated with increased risk
		Remission	Anti-ADAMTS13 autoantibodies	Recurrence	Presence of antibodies increases risk
Jin et al. BJH 2008	Cohort	Remission	ADAMTS13 activity	Recurrence	Decresing ADAMTS13 levels associated with incresed risk
Ferrari et al. JTH 2009	Case-control	Acute TTP	Anti-ADAMTS13 IgG subclasses	Recurrence	High IgG4 associated with recurrence
Kremer-Hovinga et al. 2010	Inception cohort	Acute TTP	ADAMTS13 activity	Acute disease mortality	Severely deficient patients had higher survival but no statistical significance
		Acute TTP	ADAMTS13 activity	Recurrence	Severe ADAMTS13 deficiency increases risk (RR=10)
		Acute TTP	Anti-ADAMTS13 inhibitor levels	Acute disease mortality	High inhibitor associated with increased mortality in patients with severe ADAMTS13 deficiency
		Acute TTP	Anti-ADAMTS13 Ig class	Acute disease mortality	High-IgA titres associated with death

TTP indicates thrombotic thrombocytopenic purpura; RR, relative risk.

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SECTION II

CHAPTER 5

Pathogenesis and treatment of acquired idiopathic thrombotic thrombocytopenic purpura

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Adapted from Haematologica 2010;95:1444-7.

Thrombotic thrombocytopenic purpura (TTP) is a rare thrombotic disease characterized by episodes of thrombocytopenia and microangiopathic haemolytic anemia due to disseminated microvascular thrombosis. TTP was first described in 1924 by Moschowitz as a disease presenting with a pentad of signs and symptoms (anaemia, thrombocytopenia, fever, hemiparesis and haematuria).[1] Post-mortem examination showed widespread thrombi, mainly composed of platelets, in the terminal circulation of several organs. The description of von Willebrand factor (VWF) multimers of unusually large size in the plasma of patients with TTP represented a turning point for the understanding of the disease pathophysiology.[2, 3] The presence in plasma of the highly platelet-adhesive ultralarge multimers of VWF provided a plausible explanation for the platelet- and VWF-rich thrombi observed in the small vessels of TTP patients. Then, studies in the late 1990s independently demonstrated the severe deficiency of a specific VWF cleaving-protease in the plasma of patients with recurrent TTP.[4] This protease was identified as the thirteenth member of the ADAMTS (a disintegrin and metalloprotease with thrombospondins 1 repeats) family of metalloproteases, ADAMTS13.[5-7] Severe ADAMTS13 deficiency can be due to mutations in the ADAMTS13 gene (congenital TTP, see review[8]) or to anti-ADAMTS13 autoantibodies (autoimmune TTP).[9-11] The antibody-mediated severe deficiency of ADAMTS13 can be detected in most patients with idiopathic TTP (i.e. TTP occurring without associated clinical conditions/events), whereas its prevalence is much lower in the secondary forms of TTP (i.e. TTP associated with pregnancy, infections, autoimmune diseases and the use of drugs such as ticlopidine and clopidogrel).[12] It should also be mentioned that there are

idiopathic TTP cases with only slightly deficient or even normal ADAMTS13 levels at presentation, but these cases are not object of the present article and we will use idiopathic and autoantibody-mediated TTP as synonyms.

Epidemiology and clinical course of idiopathic TTP

The incidence of idiopathic TTP is estimated to be 4.5/1 million person/years, higher in blacks than in whites and Asians and with a male to female ratio of 1:2, similarly to other autoimmune diseases.[13] Idiopathic TTP tends to have a less severe acute-disease prognosis, but much higher risk of recurrent disease in comparison to secondary forms.[14, 15] The overall mortality of TTP was higher than 90%, but has decreased to 8-30% after the introduction of plasma exchange (PEX), which is the treatment of choice of acute TTP episodes.[16, 17]

The lower mortality of idiopathic TTP (21% vs 39% in the frame of the Oklahoma TTP registry)[15] is probably due to the higher response to PEX of patients with auto-antibodies and to the mortality related to associated conditions in secondary cases.[18] Up to 40% of patients **w**ith TTP develop recurrent episodes of the disease, with higher risk for recurrences in patients with severe ADAMTS13 deficiency and anti-ADAMTS13 autoantibodies during acute episodes. The cumulative risk for recurrence at 7.5 years from the first episode in patients with ADAMTS13 activity below 10% at presentation was estimated to be 41%, 10 times that of patients with activity above 10% (4% risk at 7.5 years).[16] Persistence of ADAMTS13 deficiency and autoantibodies during disease remission is also associated with increased risk for recurrence.[19, 20]

Anti-ADAMTS13 antibody characterization

Anti-ADAMTS13 autoantibodies have been the focus of several research efforts trying to characterize their immunoglobulin (Ig) subclass, specificity and mechanisms of action. Early studies distinguished two classes of anti-ADAMTS13 autoantibodies: inihibitory and non-inihibitory antibodies. Inihibitory antibodies are present in 50-90% and their mechanism of action is the inihibition of ADAMTS13-mediated proteolyisis of VWF.[21, 22] When non-inhibitory antibodies are also measured, the anti-ADAMTS13 autoantibodies are detectable in the majority of patients.[10] The core binding site for VWF, located in the spacer domain of ADAMTS13 and consisting of aminoacid residues Tyr572-Asn579 and Arg657-Gly666, represents the target site of the autoantibodies found in the plasma of several TTP patients.[23, 24] The mechanism of action of non-inhibitory antibodies has been proposed to be the opsonisation and enhanced clearance of ADAMTS13, but this mechanism has never been proven.[21] Studies on the class of anti-ADAMTS13 autoantibodies showed they are usually of IgG type, particularly IgG1 and IgG4 subtypes,[10, 25] but in a few cases autoantibodies of IgA and/or IgM isotype were also found.[19] Most anti-ADAMTS13 IgG found in TTP patients were demonstrated to be directed against the spacer domain, but additional antibodies against other ADAMTS13 domains were also detected.[26, 27] However, these studies were conducted in small patient cohorts. Zheng et al. in this issue of Haematologica report the first study of antibody specificity on a relatively large group of patients with TTP.[28] They found that, although almost all patients with IgG had antibodies directed against the N-terminal ADAMTS13 domains (Cys-rich through spacer), up to 46% of TTP patients also had

antibodies towards the C-terminal ADAMTS13 domains (TSP1-5 through CUB). Moreover, two patients had antibodies against the C-terminal domains of ADAMTS13, but not against the N-terminal domains. These findings suggest a functional role of C-terminal domains of ADAMTS13 in vivo, also in light of the importance of these domains in the VWF-ADAMTS13 interaction under fluid shear stress. Importantly, Zheng et al.[29] combined antibody specificity with clinical data, showing that patients with antibodies against ADAMTS13 C-terminal domains had lower platelet counts at presentation. This is not the first time anti-ADAMTS13 antibody features are found to correlate with clinical outcomes in TTP. Patients with IgG subclasses 4 were found to be more likely to have disease recurrence than patients with IgG subclass 1.[28] Patients with high inhibitor titers were found to have worse acute-disease prognosis.[29] Consistently, patients with high levels of IgG (both inhibitory and non-inhibitory) were found to have higher likelihood of developing cardiac involvement and, hence, poorer prognosis in comparison to patients with low IgG levels.[30] All these findings indicate that different antibody features might be associated with clinical outcomes in TTP, but more comprehensive studies should be carried out before antibody characterization can be introduced in routine clinical practice of TTP. Moreover there remain other questions to be addressed. Acquired TTP is an autoimmune disorder, at least in those patients with an autoantibody-mediated severe ADAMTS13 deficiency, but the mechanisms involved in the loss of tolerance of the immune system against ADAMTS13 remain unknown. The higher incidence of autoimmune idiopathic TTP in specific ethnical groups such as Afro-Caribbeans, as well as the report of idiopathic TTP in two monozygotic

twins both developing anti-ADAMTS13 antibodies,[31] strongly argues in favor of a genetic predisposition even in the acquired form of the disease. In the last year, two groups independently demonstrated an association between human leukocyte antigen (HLA) alleles and idiopathic TTP: HLA-DR and HLA-DQ typing suggests an underlying genetic risk for the development of TTP in Europeans.[32, 33] As for the antibody characterization, confirmation of these results in larger groups of patients and in other ethnic groups are required prior of the introduction of HLA typing in the control of the disease.

Treatment and clinical trials in idiopathic TTP

PEX remains the treatment of choice of acute episodes of TTP.[34] As mentioned, its introduction greatly reduced the disease mortality and it has been proven superior to plasma infusion.[17] Several different immunosuppressive drugs (corticosteroids, cyclosporine, azathioprine and, recently, rituximab) are added by many centers to PEX, with the rationale that they help stopping antibody production in autoimmune cases, but their efficacy has never been confirmed by large clinical trials. In addition to these treatments, novel drugs have been developed or are undergoing pre-clinical development that could potentially be used in idiopathic TTP along with PEX. These could tackle different aspects of TTP pathophysiology (Table 1). First, it is possible to reduce or abolish the production of anti-ADAMTS13 autoantibodies with anti-CD20 monoclonal antibodies that target B-lymphocytes (e.g. rituximab, but other more potent compound are being developed).[35] Second, it could be in principle possible to restore VWF cleavage in patients with severe ADAMTS13 deficiency with the

use of recombinant ADAMTS13. Third, novel compounds that inhibit VWF binding to platelet glycoprotein Ib-alpha have been developed that could block VWF-mediated platelet activation. There is hope in the TTP community that these novel therapeutic strategies be able to reduce the persistently high disease mortality. However, the availability of these new options generates new challenges for clinicians who have to deal with TTP patients. These include uncertainties on the safety of the drugs in this delicate clinical setting and on the subgroup(s) of patients (idiopathic, secondary, TTP with prominent renal impairment, etc.) that could benefit from the treatment. The efficacy and safety of these novel therapeutic strategies will need to be assessed in the frame of large clinical trials, a challenge for clinical scientists who work on this rare disease. Idiopathic TTP incidence is such that anyone willing to carry out a 3-year clinical trial involving roughly 100 patients would need to be able to cover a population of approximately 7 million people (assuming an incidence of 4.5/1 million person/years). The choice of the clinical end-point will similarly be a challenge: mortality is ~10-20% which makes it a hardly targetable end-point with small sample sizes. Such surrogate endpoints as the incidence of stroke, renal failure, myocardial infarction, time to platelet recovery or clinical remission may be adequate for the definition of therapeutic efficacy but there are few data available from cohort studies that could inform the design of clinical trials employing these endpoints. The picture is made even more complicated by the heterogeneity in the pathophysiologic background of TTP. The inclusion in a TTP trial of secondary cases, patients with atypical hemolytic uremic syndrome, patients at first TTP episodes or recurrence may in principle conceal the effect of a treatment which is

highly effective in a subgroup of TTP patients (e.g. only those with anti-ADAMTS13 autoantibodies). Recently, a phase 2 double-blind, placebo-controlled, clinical trial of intravenous ARC1779, an inhibitor of VWF binding to platelet glycoprotein Ib-alpha, was stopped due to slow recruitment (clinicaltrials.gov identification number NCT00726544). New trials are nonetheless being designed and carried out. These will be critical to the efforts of translating preclinical achievements in improvements in the care of this rare, but still life-threatening thrombotic disease.

Table. Novel drugs developed or undergoing pre-clinical development that could potentially be used in idiopathic TTP along with plasma exchange.

Pathogenic Mechanism	**Drug class**	**Drug(s)**	**Mechanism of action**	**Stage of development**
Production of anti-ADAMTS13 auto antibodies	Anti-CD20 antibodies	Rituximab. Other drugs under development	B-lymphocyte depletion	Available
Impaired VWF cleavage	Recombinant replacement products	recombinant ADAMTS13	Replacement of deficient ADAMTS13	Pre-clinical development
VWF binding to platelets and VWF-mediated thrombosis	VWF-platelet interaction inhibitors	ARC1770 and ALX-0681	Blockage of VWF-mediated platelet aggregation	Phase 2 of clinical development

TTP, thrombotic thrombocytopenic purpura; VWF, von Willebrand factor.

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CHAPTER 6

Different clinical severity of first episodes and recurrences of thrombotic thrombocytopenic purpura

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Abstract

The clinical course of thrombotic thrombocytopenic purpura (TTP) is characterized by recurrent disease episodes in up to 50% of the cases. The clinical presentation and severity of different TTP episodes have not been systematically compared. Laboratory and clinical information from 51 patients with recurrent disease, stemming from 136 patients with TTP included in the Milan TTP registry (URL: http://www.ttpdatabase.org), were used to compare episode fatality, symptoms and disease-related laboratory measurements in different disease episodes. The prevalence of severe neurological symptoms (coma, seizures, and focal neurological defects) was significantly lower in recurrences than in the first episode. Platelet counts and haemoglobin levels at presentation were higher in recurrences than in the first disease episode, and lactate dehydrogenase levels were lower. Also episode fatality tended to be lower in the second and third disease episodes than in the first. Recurrences of TTP are generally milder than first episodes. These differences in severity should be taken into account in clinical research on TTP and in patient management.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare, life-threatening disease characterized by acute episodes of thrombocytopenia and microangiopathic hemolytic anemia due to disseminated microvascular thrombosis.[1] The clinical presentation of TTP is variable in terms of type and severity of clinical symptoms.[2-5] Before the introduction of plasma exchange (PEX) for the treatment of acute TTP episodes mortality reached 90%, but decreased to 8-30% following the adoption of PEX.[3, 6, 7] Patients who survive the first acute episode of the disease may remain asymptomatic for the rest of their life or develop one or more recurrence. It has been estimated that approximately one third of TTP patients recur, with a higher risk of recurrence in those with severe deficiency of the von Willebrand factor cleaving-protease ADAMTS13 during acute episodes[4, 8, 9] and remission.[10-12] Few data are available on the clinical presentation and severity of recurrences. It is not uncommon in clinical practice to observe that TTP patients, aware of their condition and under close medical monitoring after the first episode, are diagnosed with recurrence before the development of such severe symptoms as coma or other signs of severe neurological involvement. Accordingly, Scully et al. observed that patients from the UK TTP registry needed less PEX procedures to reach remission during recurrences than during the first episode.[3] However, the different severity of recurrent TTP episodes has never been the object of a systematic investigation. In addition, it is possible that a number of patients who die from a recurrence are not referred for recurrence to expert clinical centers which are the major source of studies on this disease, biasing the perception of the real severity of recurrent episodes. If confirmed, the

knowledge that recurrences are clinically milder than first disease episodes could be useful for those who design and carry out observational studies or clinical trials in TTP, in order to avoid lumping together patients with first episodes and recurrences. It would also help in clinical practice to better balance the risk of recurrent disease with that of side effects from potentially harmful preventive therapies such as the use of immunosuppressive agents.

Patients and Methods

Patients and definition of clinical categories

Between 1999 and 2009 physicians from Italy and 10 additional countries (Hungary, Serbia, Canada, Germany, Iran, Lebanon, Romania, Russia, Slovenia and Turkey) referred patients who had a suspected diagnosis of TTP to the registry established at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre and provided detailed clinical information and, when available, plasma samples. Information was collected using a standardized clinical questionnaire (available upon request). When necessary to fill gaps of information, clinicians were asked to provide additional clinical documentation and samples. Physicians in Milan (L.A.L. or F.P.) reviewed the cases and confirmed or excluded the diagnosis of TTP. Criteria for TTP diagnosis were the documentation of at least one episode of: (a) thrombocytopenia, (b) microangiopathic haemolytic anemia (c) exclusion of alternative explanations for thrombocytopenia (such as the enterohemorrhagic form of hemolytic uremic syndrome, catastrophic anti-phospholipid antibodies syndrome, pre-eclampsia and related syndromes, sepsis, systemic inflammatory response syndrome, disseminated intravascular

coagulation, disseminated malignancy or bone-marrow transplantation associated TTP-like syndrome). Remission was defined as persistence of normal platelet counts and hemoglobin levels for at least 30 days after the most recent acute episode and freedom from new disease symptoms. The study was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda – Ospedale Maggiore Policlinico and all patients or their tutors gave informed consent. The first TTP episode was classified as secondary when associated to one or more of the following conditions: (1) pregnancy or post-partum, (2) use of drugs reported to be associated with TTP, (3) additional disease associated with TTP (autoimmune diseases or HIV infection). Patients who did not fit these categories were classified as having idiopathic disease. Presence of symptoms in each TTP episode was defined as prevalence of symptoms at presentation or during the acute phase of the disease before remission or death. Symptoms related to thrombocytopenia included petechiae (purpura), ecchymoses and superficial hematomas. Renal involvement included an increase in the serum level of creatinine (serum levels above the normal reference value of each laboratory) or presence of alterations of urinalysis such as proteinuria or hematuria. Severe neurological symptoms were coma, seizure and focal neurological signs (motor/sensory deficit or aphasia). Less severe symptoms (headache, amaurosis fugax, mild gait alterations) were included in the category "other neurological symptoms". Cardiovascular involvement included acute myocardial infarction, other acute coronary syndromes, increase in cardiac troponin-T, T wave electrocardiographic alterations and severe hypertension. Gastrointestinal symptoms included abdominal pain, vomiting and diarrhea, whereas jaundice was

excluded from this category owing to the confounding effect of hemolysis. The category "other symptoms" included fatigue, dyspnea and other symptoms not included in the aforementioned categories. PEX procedures for each episode were calculated as the number of 1 volume PEX sessions carried out since diagnosis until remission or death; double volume exchanges were considered equivalent to 2 single volume PEX sessions. According to Vesely et al.,[4] laboratory data recorded as "at presentation" were the most abnormal findings recorded on the day of diagnosis ±7 days. To compare data from different laboratories, LDH values were normalized to an upper normal limit of 480 IU/L. The plasmatic activity of ADAMTS13 was measured centrally at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre using collagen binding assay on citrated plasma as previously described.[12] Activity of ADAMTS13 below 10% was considered severely deficient. The normal range for ADAMTS13 activity was 46-160%. Data of all patients are included in a digital registry (URL: www.ttpdatabase.org).

Study design

Figure 1 provides a flow-chart of the study design. To assess the severity of different episodes mortality rates were compared. In addition, each disease episode was compared with the others in patients who had more than one episode. Indicators of disease severity used for comparison were the differences in the prevalence of symptoms, in laboratory values (platelet counts, hemoglobin, LDH, and creatinine), and in the number of PEX procedures carried out in the time span between diagnosis and remission or death. In this matched-pair analysis each individual served as a control for him or herself in different episodes. Patients

referred for only one episode were followed-up for vital status to assess how many of them had died of recurrent disease without notification to the caregiver physician and to our registry, because these events might bias the selection of patients with recurrent disease towards non-fatal, less severe cases. This check was carried out only for patients referred from Italian centres. Information on their vital status and (when applicable) cause of death was searched for by different methods: (a) direct contact of the patient, (b) check against mortality registries, (c) postal follow-up through the vital statistic offices of the city of birth or last residence of the patient.

Statistical analysis

Wilcoxon signed-rank test was used to compare continuous variables in the two disease episodes. Categorical variables were compared by McNemar test. P values <0.05 were considered significant. STATA 11 software was used for the analysis (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP)

Results

After exclusion of patients who did not match the diagnostic criteria and those with insufficient information to establish the diagnosis (n=167), 136 patients with TTP were included in the cohort (number updated at July 2009). The general features of the cohort and clinical information on the first disease episode are in Table 1. Twelve patients died of the disease (9% of all patients), 10 during the first disease episode (7.3% of 136 patients with a first disease episode), 2 during the second episode (3.5% of 57 patients referred for more than one episode).

None of the 33 patients who had 3 or more disease episodes died of the disease. Of the 12 patients who died, 9 had severe deficiency of ADAMTS13 (activity below 10%). Plasma samples for the centralized measurement of ADAMTS13 activity were available for 135 patients (99%): for 36 (27%) patients only acute disease samples were available, for 53 (39%) only remission samples and for 46 (34%) both acute and remission samples. The prevalence of severe ADAMTS13 deficiency was higher in the group of patients with availability of acute disease samples (67%, 55/82) than in those without, whereas it was lower in patients with only remission samples available (36%, 19/53). Among the symptom categories analyzed, cardiovascular symptoms had the lowest prevalence (Table 1): an acute coronary syndrome was diagnosed in only 5 patients, 3 of whom had an acute myocardial infarction. Eight of the 136 patients had an acute renal failure according to the RIFLE (Risk of renal dysfunction, Injury to the kidney, Failure of kidney function, Loss of kidney function, End-stage renal disease) criteria for the definition of renal failure in critically ill patients.[13] Two of these 8 patients had severe ADAMTS13 activity, three (one with severe ADAMTS13 deficiency) died of the disease at the first episode and only one, who had relapsing episodes, was included in the paired analysis of disease severity. Of 57 patients who had two or more disease episodes, clinical information on the first two episodes of the disease was available for 51 (90%). Their general features and clinical information pertaining to the two episodes are summarized in Table 2. The general characteristics and the clinical features at first episode in patients with recurrence were not different from those in patients without recurrence (not shown), but a severe deficiency of ADAMTS13 activity was more prevalent

among patients who had recurrences (65%, 37/57) than those without (43%, 29/68). In patients with multiple disease episodes, the prevalence of severe neurological symptoms and fever was lower during the second than the first episode (Table 2). Among disease-related laboratory measurements, platelet counts and hemoglobin levels were higher in the second episode, whereas serum LDH was lower (Table 2). Creatinine levels did not significantly differ between the two episodes. Finally, the number of PEX procedures carried out between diagnosis and hospital discharge was smaller for the second episode. Subgroup analyses were performed in groups of recurrent patients with (n=36) or without (n=15) severe deficiency of ADAMTS13. In the group of patients with severe deficiency of ADAMTS13 statistically significant differences were found for prevalence of severe neurological symptoms, fever, hemoglobin levels, platelet counts, LDH values and number of PEX until remission/death. In the group of patients in whom severe deficiency of ADAMTS13 was not found in the available samples (n=15) significant differences in the two episodes were found for platelet counts, hemoglobin and LDH levels. All statistically significant differences found in subgroup analyses were in the same direction of those observed in the primary analysis. Thirty-three (65%) of the 51 patients included in the paired analysis of disease severity comparing the first and second episode in patients with recurrent disease had at least a third disease episode that developed at a median time of 1.3 years (range: 0.3-8 years) after the second episode. The third episode generally displayed milder clinical and laboratory features in comparison to the first and even to the second episode (Figures 2 and 3). The prevalence of purpura (p=0.004, McNemar's test, prevalence at first vs

third episode), of severe (p=0.001) and other (p=0.007) neurological symptoms, and of fever (p=0.002) were lower in the third than in the first episode (Figure 2). Hemoglobin was largely influenced by episode number (p<0.001, Wilcoxon signed rank test, levels at first vs third episode) and smaller but statistically significant effects were also observed for platelet number (p=0.004) and LDH (p=0.03), whereas creatinine levels were not influenced by recurrence (p=0.85) (Figure 3). Vital status information were requested for Italian patients reported to have survived the first TTP episode and not known to have had additional disease episodes (n=52, 75% of the 69 survivors of the first TTP episode not referred for recurrence) (Figure 1). Vital status information through October 2009 was collected for all 52 patients: by phone calls or e-mail contact with the patients (n=13, 25%), through mortality registries (n= 16, 31%), or from the vital statistic office of the birth or residence city (n=23, 44%). Only one of the 52 patients had died, from a cause that was deemed unrelated to TTP (rupture of mechanic aortic valve prosthesis).

Discussion

The results of this study show that TTP recurrences usually have a milder clinical course than first episodes of the disease. Neurological involvement, the degree of anemia and thrombocytopenia, which were previously shown to be predictors of 6-month mortality in TTP patients,[14] were less severe in the second and third disease episodes than in the first. PEX sessions employed during second episodes were significantly less than those of the first, corroborating the other study

findings. The third disease episode displayed even milder clinical course than the first two episodes.

There are several plausible explanations for TTP recurrences to be less severe than first episodes. A first argument is that recurrences are more rapidly diagnosed and treated. Indeed, many recurrences were diagnosed on the occasion of complete blood counts, in the presence of the laboratory features of TTP (microangiopathic hemolytic anemia and thrombocytopenia), but with mild or no clinical manifestations. A low prevalence of neurological symptoms, relatively high platelet counts and low number of PEX needed to achieve remission in TTP recurrences were previously reported by Bohm et al. in a study evaluating the course of ADAMTS13 activity during acute TTP, including 11 patients with relapsing TTP and 14 with a first disease episode.[15] However, only two of the relapsing patients of that study were also evaluated during the first episode rendering a direct comparison impossible. Secondly, patients who tend to experience the most severe episodes have the highest risk of death during the first episode, so that survivors as a group tend to have less severe disease. Thirdly, selection of survivors of recurrence might have occurred; this, however, was ruled out by follow-up. An advantage of our study is that 51 patients with 2 or more TTP episodes could be directly compared during their different episodes. This paired-comparison design minimizes the influence of individual determinants of disease severity, which might arise when different individuals are compared in different episodes. The design of this study also allowed minimizing heterogeneity in the ascertainment of clinical characteristics of different episodes in the same patient, because patients were usually referred to the same centre for

first episode and recurrences. Limitations of this study were missing information on some patients and the small absolute number of patients studied. However, given the rarity of TTP and the difficulties of gathering detailed clinical information on clinical episodes we believe that the number and quality of data available for analysis were sufficient to enable the comparison of disease severity described in the study. The overall prevalence of fatal TTP in the TTP cohort of this study was 9%, with lower mortality in relapsing episodes (unmatched analysis). This proportion of fatal disease is consistent with the rate of mortality reported for other cohorts. However, because ours is a tertiary care centre for TTP patients offering free ADAMTS13 testing and medical assistance and counseling, it is possible that recruitment of our patients is biased towards survivors of TTP episodes. Patients who died during the first episode without being referred to our registry do not affect the findings of this study, which is focused on survivors of the first episode who developed recurrences, but may have attenuated the contrast in severity between first and second events. Instead, we reasoned that deaths for TTP occurring during recurrences, not reported to our registry, might have biased this study towards the recruitment of survivors of TTP recurrence, i. e., a less severe subpopulation of recurrent patients. To exclude the presence of this potential distortion of the results, patients referred for a first TTP episode were followed-up to assess whether or not they had died of the disease without notice to the Hemophilia and Thrombosis Centre. The result of this evaluation showed no evidence of this bias, with the only death that occurred not being causally related to TTP. The knowledge that recurrences are generally milder than first disease episodes should be considered in the design and analysis of observational

studies and clinical trials in TTP. An unbalance in the prevalence of patients with recurrent episodes in groups with a risk factor under evaluation or in two different treatment groups might indeed confound the ascertainment of the effect of an exposure or a treatment on acute disease clinical outcomes. The results of this study also have clinical relevance. Although recurrent episodes of TTP should always be regarded as a potentially fatal condition, the relatively less severe clinical course of recurrences should be taken into account when potentially harmful preventive therapies, like long-term immunosuppression, are being planned in patients considered at risk for TTP recurrence.

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Figures

Figure 1. Study flow-chart.

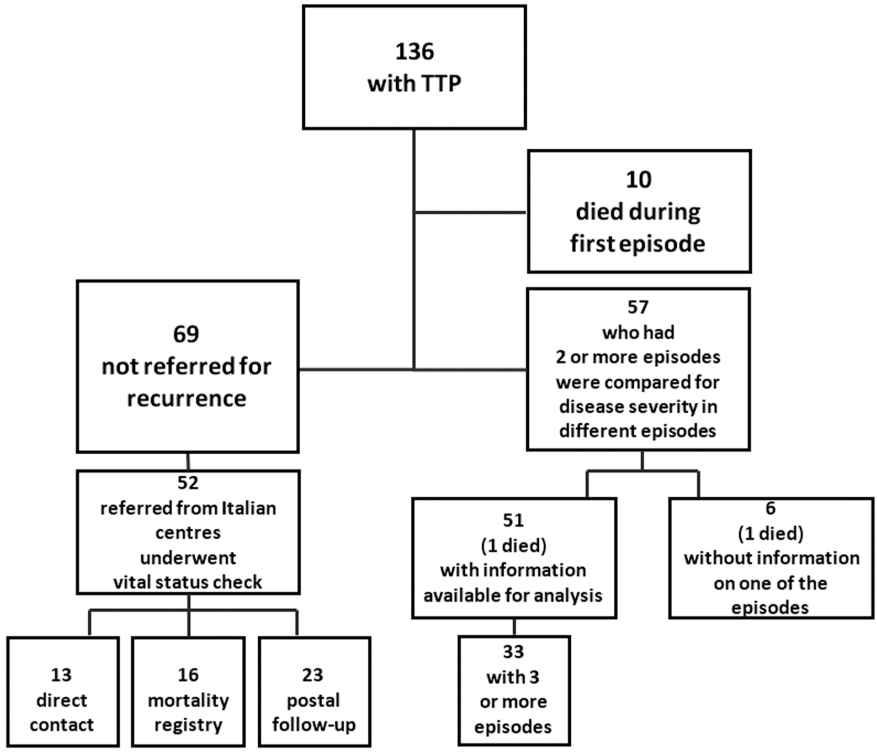


Figure 2. Prevalence of clinical symptoms in different episodes for patients with at least 3 episodes.

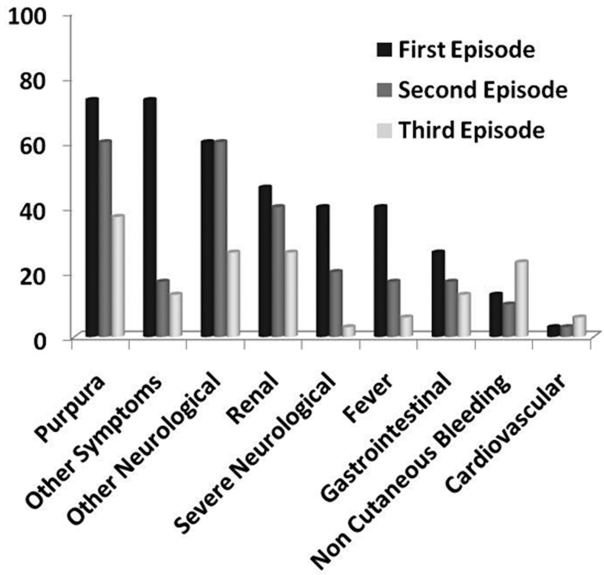
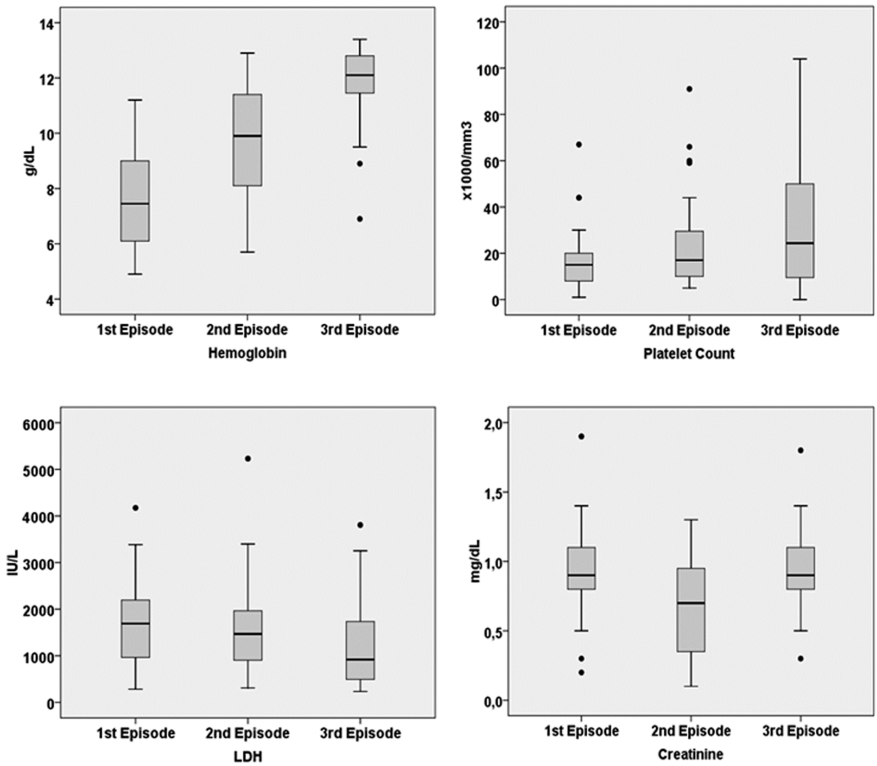


Figure 3. Disease-related laboratory measurements in different episodes for patients with at least 3 episodes.



Tables

Table 1. General, clinical and laboratory features at first episode of the patients included in the study.

Variable	Value
n=	136
GEOGRAPHIC ORIGIN	
Italy	107 (79%)
Other country[a]	29 (21%)
REFERRED DURING:	
Acute disease	56 (41%)
Remission	80 (59%)
MALE SEX	29 (21%)
AGE AT FIRST EPISODE, years	37 (28-50)
DIED OF THE DISEASE	12 (9%)
IDIOPATHIC DISEASE	107 (79%)
SECONDARY DISEASE	29 (21%)
Pregnancy	13 (9%)
Autoimmune disease[b]	9 (7%)
Drug induced[c]	5 (4%)
More than one condition[d]	2 (1%)
SEVERE DEFICIENCY OF ADAMTS13 ACTIVITY[e]	74 (54%)
REFERRED FOR MORE THAN ONE EPISODE	57 (42%)
CLINICAL SYMPTOMS AT FIRST EPISODE (n=130)	
Severe neurological	64 (49%)
Other neurological	69 (53%)
Cutaneous manifestations of thrombocytopenia	71 (55%)
Bleeding	15 (12%)
Renal involvement	47 (36%)
Cardiovascular symptoms	15 (12%)
Gastrointestinal symptoms	49 (38%)
Fever	47 (36%)
Other symptoms	50 (38%)
LABORATORY MEASUREMENTS AT FIRST EPISODE (n=117)	
Platelet counts ($x10^9$/l)(n=117)	14 (7-22)
Hemoglobin (g/l) (n=117)[f]	80 (65-93)
Lactate dehydrogenase (IU/l) (n=113)	1571 (953-2330)
Creatinine (µmol/l) (n=107)	79 (70-106)
TREATMENT AT FIRST EPISODE (n=129)	
Plasma exchange	118 (92%)
Number of plasma exchanges (n=105)	11 (7-19)
Other transfusional therapy	81 (63%)
Immunosuppressive treatment	118 (92%)

Continuous variables were expressed as median (interquartile range).

a Hungary (n=11), Serbia (n=10), Canada, Germany, Iran, Lebanon, Romania, Russia, Slovenia and Turkey (n=1 each).

b Autoimmune thyroid disease (n=5), systemic lupus eritematosus (n=3), myasthenia gravis (n=1).

c Ticlopidine (n=4), clopidogrel (n=1).

d Pregnancy and autoimmune thyroid disease (n=1), pregnancy and systemic lupus eritematosus (n=1).

e Measured during acute disease or during remission.

Table 2. General and clinical features at their first two episodes of the 51 recurrent patients included in paired analysis of disease severity.

Variable	Value	
n=	51	
GEOGRAPHIC ORIGIN		
Italy	41 (80%)	
Other country	10 (20%)	
MALE SEX	11 (21%)	
AGE AT FIRST EPISODE, years	35 (26-44)	
AGE AT SECOND EPISODE, years	38 (30-48)	
TIME TO FIRST RELAPSE	1.7 (0.5-3.7)	
IDIOPATHIC DISEASE	37 (73%)	
SEVERE DEFICIENCY OF ADAMTS13 ACTIVITY[a]	36 (70%)	
REFERRED FOR MORE THAN TWO EPISODES	33 (64%)	
	FIRST EPISODE	*SECOND EPISODE*
CLINICAL SYMPTOMS (n=51)		
Severe neurological	25 (49%)	9 (18%)
Other neurological	35 (68%)	25 (49%)
Cutaneous manifestations of thrombocytopenia	33 (65%)	29 (56%)
Bleeding	9 (18%)	3 (6%)
Renal involvement	15 (41%)	18 (35%)
Cardiovascular symptoms	2 (4%)	2 (4%)
Gastrointestinal symptoms	18 (35%)	10 (20%)
Fever	21 (41%)	8 (16%)
Other symptoms	17 (33%)	11 (22%)
LABORATORY FEATURES (n=47)		
Platelet counts ($x10^9$/l)	15 (8-20)	20 (10-34)
Hemoglobin (g/l)	77 (62-97)	10 (81-117)
Lactate dehydrogenase (IU/l)	1691 (940-2379)	1110 (563-1900)
Creatinine (µmol/l)	70 (70-97)	79 (53-114)
NUMBER OF PLASMA EXCHANGES (n=37)[b]	12 (7-19)	7 (5-12)

Continuous variables were expressed as median (interquartile range).

a Measured during acute disease or during remission.

b Information available on n=37 patients for both episodes; information was not available for the first episode in n=6 patients, for the second in n=2 patients, n=6 patients were not treated with PEX.

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CHAPTER 7

Platelet-reactive conformation and multimeric pattern of von Willebrand factor in acquired thrombotic thrombocytopenic purpura during acute disease and remission

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Abstract

Binding of von Willebrand factor (VWF) multimers of ultralarge size to platelets is considered the triggering mechanism of microvascular thrombosis in thrombotic thrombocytopenic purpura (TTP). We assessed the potential of VWF-related measurements as markers of disease activity and severity in TTP. VWF antigen (VWF:Ag), platelet glycoprotein-Ib-α binding-conformation (GPIb-α/BC) and multimeric pattern were investigated in 74 patients with acquired TTP and 73 healthy controls. VWF ristocetin co-factor activity (VWF:RCo) and collagen binding (VWF:CB) were also measured in a subgroup of patients. VWF:Ag and VWF-GPIb-α/BC were higher in TTP patients than controls. However, there was no apreciable difference in VWF-GPIb-α/BC between samples obtained during acute TTP and remission. Larger VWF multimers were frequently lacking in acute TTP patients, who displayed ultralarge multimers at remission. The degree of loss of larger VWF multimers was associated with the degree of abnormality of hemoglobin, platelet counts and serum LDH and was also associated with low levels of both VWF:RCo/Ag and VWF:CB/Ag ratios. In TTP, the platelet-binding conformation of VWF is not exclusively present in acute disease, nor is it associated with its clinical and laboratory severity. The loss of larger VWF multimers, accompanied by low VWF:RCo/Ag and VWF:CB/Ag ratio values, represents an index of disease activity and severity of acute TTP in patients with severe ADAMTS13 deficiency.

Introduction

Thrombotic thrombocytopenic purpura (TTP), a rare disease characterized by single or multiple episodes of disseminated microvascular thrombosis, is associated with the severe plasmatic deficiency of the von Willebrand factor (VWF)-cleaving metalloprotease ADAMTS13.[1] In patients with TTP the presence in plasma of highly platelet-reactive VWF forms of ultralarge (UL) molecular weight (ULVWF), which in physiological conditions are cleaved by ADAMTS13 into regular-sized multimers,[2] is the main mechanism of intravascular platelet adhesion/aggregation and disseminated thrombosis in the microcirculation.[3-6] A nanobody (i.e., a single domain antibody) with specificity towards the platelet glycoprotein Ib-α binding conformation of VWF (GPIb-α/BC) enables to measure by immunoassay the proportion of plasma VWF which is highly reactive with this platelet glycoprotein.[7] In TTP, VWF-GPIb-α/BC (measured as the ratio of VWF in its platelet-binding conformation to the whole circulating mass of the protein) was 2-fold (acquired TTP) to 8-fold (congenital TTP) higher than normal in 17 patients with acute disease,[7] but only 1.5-fold higher in 22 patients in clinical remission.[8] The higher amounts of VWF-GPIb-α/BC suggest that a change in the conformation of circulating VWF is responsible for the heightened platelet reactivity that leads to disseminated microvascular thrombosis.

In this study, we sought to replicate these findings and to evaluate in a large cohort of patients the relationship between the amount of platelet-reactive VWF and different clinical and laboratory presentations of acquired TTP (acute disease/remission, with and without ADAMTS13 deficiency). Antigen

(VWF:Ag) levels, ristocetin cofactor activity (VWF:RCo), collagen binding (VWF:CB) and the multimeric pattern of VWF were also investigated.

Patients and Methods

Patient and sample selection

The 74 patients investigated in the study were selected from a larger group of 136 patients with TTP included in the Milan TTP registry (URL: www.ttpdatabase.org). The features of the registry and the clinical definitions adopted have been described elsewhere.[9, 10] A patient selection flow-chart is in Figure 1. Selected patients with acute TTP were those with available plasma samples, after excluding those transfused with blood products during the 10 days preceding sampling. Plasma samples were also available from patients during disease remission. Patients included in the study with acute phase samples were excluded from the choice of remission samples in order to respect the independence of observations. A summary of the study design is in Figure 2. Study measurements were investigated in four primary subgroups (a-d) chosen according to the following criteria (see below "Rationale for subgroup selection"): (a) 18 patients with a first episode of acute TTP and severe ADAMTS13 deficiency (<6%) at presentation; (b) 16 patients with a recurrent episode of acute TTP (second through eighth episode) and severe ADAMTS13 deficiency at the time of recurrence; (c) 20 patients with previous acute TTP currently in remission, with severe ADAMTS13 deficiency at the time of remission; (d) 20 patients with previous acute TTP, currently in remission with normal plasma levels of ADAMTS13 (46-160%). Each patient was included in

only one of the four primary subgroups. Additional analyses of the study results were carried out in subgroups formed by combining in various ways primary subgroups a-d: (e) 34 patients with acute TTP (group a + b, i. e., patients with first acute episodes plus patients during acute recurrence, all with severe ADAMTS13 deficiency); (f) 40 patients with previous acute TTP currently in remission (group c + d, i. e., those with severe deficiency (<6%) plus those with normal ADAMTS13 (>46%); (g) 54 patients with severe ADAMTS13 deficiency (group a + b + c, i. e., those with first acute episodes plus those with recurrences plus those in remission, all with ADAMTS13 levels <6%). For 17 of the 34 patients with acute TTP (group e) samples collected both during acute disease and remission were also available and used for paired comparisons of the study measurements at different times of the clinical course in the same individuals. In this subgroup, VWF:RCo and VWF:CB were also measured and the VWF:RCo/Ag and VWF:BC/Ag ratios calculated.

Rationale for subgroup selection

Patients with first acute episodes and those with acute recurrences (n=34) were included in two distinct primary subgroups (a and b) because recurrences are usually diagnosed earlier and are milder than first acute episodes of TTP in terms of clinical and laboratory abnormalities, creating a possible source of confounding.[10] We chose to analyze separately 54 patients with ADAMTS13 <6%, whether in the acute phase or during disease remission, in order to investigate patients with a similar disease mechanism, i.e., the severe deficiency of the VWF-cleaving protease. The subgroup of 20 patients with normal ADAMTS13 during remission was chosen in order to allow comparison of the

VWF-related measurements in patients with and without severe ADAMTS13 deficiency.

Control group selection

Controls for VWF:Ag and VWF-GPIb-α/BC measurements were 73 blood donors at the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, similar in age and sex with the whole group of TTP patients and with each study subgroup.

Study measurements

The multimeric pattern of plasma VWF was analyzed by sodium dodecyl sulfate agarose gel electrophoresis followed by luminographic visualization of multimers. EDTA plasma samples were diluted in order to obtain final VWF:Ag concentrations of 10%. Gel systems of 1% high gelling temperature agarose (HGT-Seakem) were chosen for low-resolution multimeric analysis. After electrophoresis, proteins were electrotransferred to an immobilon polyvinylidene fluoride membrane (Millipore, Billerica, MA) overnight and multimers visualized using a rabbit polyclonal anti-human VWF antibody (Dako Cytomation, Glostrup, Denmark) and a goat anti-rabbit IgG peroxidase conjugated antibody (BioRad Laboratories, Hercules, CA). The filters were stained with a luminol-iodophenol solution, covered with transparent film, and densitometry of multimers was then carried out with Typhoon 8600 Variable Mode Imager (Image Quant TM Software; Amersham Biosciences, Uppsala, Sweden). The multimeric patterns of patient and control samples were compared with that of a reference pooled plasma run on the same gel. Densitometric analysis was performed on the blotted membrane using the graph line obtained by the single

lane of the gel and plasma samples with similar VWF concentrations in order to obtain comparable area values. According to Budde and Scheneppenheim,[11] the reference plasma lane was divided into small (1-5), intermediate (6-10) and larger (>10) multimers of regular size. The proportion of larger multimers in the sample was calculated by dividing the area corresponding to larger multimers by the total area of the lane. The ultralarge VWF (ULVWF) ratio was calculated as the ratio of (i) the proportion of large multimers in the test sample to (ii) the corresponding area in the reference plasma within the same gel. A normal range was established by calculating the ULVWF ratio in 36 healthy subjects. VWF-GPIb-α/BC was measured using an immunoadsorbent assay,[7] based upon the nanobody AU/VWF-a11 that specifically recognizes the VWF-GPIb-α/BC. Antibodies were provided by P. G. de Groot and P. J. Lenting. Before incubation, serial dilutions of VWF:Ag were obtained (4 dilutions with concentrations ranging from 250 ng/ml to 31 ng/ml). The same pooled normal plasma used for the VWF:Ag assay was used as reference in each experiment. The VWF-GPIb-α/BC was calculated as the ratio of the slope of the close response curve of plasma samples to the slope of normal plasma pool (set to a value of 1). Plasma from a patient with type 2B von Willebrand disease was included in all the experiments as a positive control, because these patients have higher amounts of VWF-GPIb-α/BC in their plasma.[12] ADAMTS13 activity was measured using the collagen binding assay, anti-ADAMTS13 antibodies were searched for by western blotting.[9] The lower laboratory limit of sensitivity of the collagen binding assay is 6%, so that ADAMTS13 deficiency was arbitrarily defined as severe when plasma levels were lower than 6%. VWF:Ag was quantified using the ACL TOP Analyzer and

pooled normal plasma as reference. VWF:RCo was measured by the Siemens Healthcare Diagnostics BC von Willebrand Reagent assay using lyophilized platelets in the presence of ristocetin. The test was performed in an automatic coagulometer (BCS, Siemens Healthcare, Milano, Italy). VWF:CB was measured on plasma using a solution of 95% type I and 5% type III collagens (Horm®, Nycomed Austria GmbH, Linz, Austria) as described previously.[13]

Statistical analysis

The chi-square or Fisher's exact tests and Mann-Whitney U- or Student t-tests were used when appropriate to compare categorical and continuous variables in different primary and secondary study groups. The Wilcoxon signed-ranks test was used for paired comparisons of continuous variables. Pearson's correlation was used to assess the relationships between the VWF-related measurements and the clinical and laboratory features of TTP patients at the time of acute disease presentation.

Results

The general features and results of VWF and ADAMTS13 measurements in the whole group of TTP patients and controls are shown in Table 1. Median values of VWF:Ag and VWF-GPIb-α/BC were higher in TTP patients than controls (Mann-Whitney U test, $p<0.001$ and $p=0.004$), whereas there was no statistically significant difference pertaining to ULVWF multimers, which, however, showed a much wider range of ratio values in cases than in controls.

Among study subgroups, VWF:Ag was higher during acute TTP (first and recurrent episodes combined) than during remission (median value in 34 acute

cases 202% vs 130% in 40 remission cases, p=0.002). VWF-GPIb-α/BC values were not significantly different in 34 cases with acute TTP (first and recurrent episode combined) compared with 40 cases in remission (median values 1.20 vs 1.31, p=0.28). There was also no difference in the paired analysis of the 17 cases with both acute and remission samples available (Table 2). In patients with first acute disease episode and remission with severe ADAMTS13 deficiency, VWF-GPIbα/BC values were higher than in those with recurrent episodes, those with normal protease levels and controls (see Table 1). Owing to the lack of large VWF multimers, the ULVWF multimer ratio was markedly decreased in patients with first acute episodes, whereas it was higher than normal (due to the presence in plasma of ultralarge multimers) during remission in patients with severe ADAMTS13 deficiency (Table 1 and Figure 3A). The marked changes of the multimeric pattern of VWF from defective to ultralarge are shown in Figure 3B, which compares the ratio values during acute episodes and remission obtained in paired samples from 17 TTP cases. These changes occurred particularly in patients who had low ULVWF ratio at the time of their acute episode and severe ADAMTS13 deficiency at remission (Figure 3B). The multimeric structure was not different from normal in patients during acute recurrences and during remission in patients with normal ADAMTS13 activity (Table 1 and Figure 3A). Figure 4 shows representative examples of VWF multimer patterns in the aforementioned patient subgroups. In the 17 patients with both acute and remission samples available, antigen-normalized values of VWF:RCo and, particularly, VWF:CB correlated with ULVWF ratio (VWF:RCo/Ag: r=0.71, p=0.01; VWF:CB/Ag: r=0.88, p=0.0006). Similarly to what observed for

ULVWF ratio, the VWF:CB/Ag ratio was reduced in acute TTP compared to remission (Table 2). The VWF:RCo/Ag ratio was reduced in the first acute episodes of TTP compared to the corresponding remissions (0.47 vs 0.78; n=10; p=0.01), but not in recurrent episodes compared to their remissions (1.03 vs 0.75; n=7; p=0.13). ABO blood groups influenced both VWF:Ag and VWF-GPIb-α/BC, because both in patient and controls plasma levels were higher in carriers of non-O blood groups (Table 3). However, the associations of high levels of VWF:Ag and VWF-GPIb-α/BC with TTP remained statistically significant after stratification for O/non-O blood group (Table 3). The ULVWF multimer ratio was independent from blood groups (not shown). In patients, VWF:Ag and VWF-GPIb-α/BC levels did not correlate with the ULVWF ratio. Finally, we chose to evaluate whether or not during acute TTP there was a correlation between the aforementioned VWF-related measurements, the degree of abnormality of laboratory markers of disease activity and severity (platelet count, hemoglobin, serum lactate dehydrogenase) and the prevalence of clinical symptoms (neurological, renal, cardiovascular, hemorrhagic). While there was no correlation between these laboratory measurements of TTP activity and VWF:Ag, VWF:RCo and VWF-GPIb-α/BC levels (data not shown), the ULVWF multimer ratio was positively correlated with the degree of anemia ($r=0.48$; $p=0.007$), thrombocytopenia ($r=0.59$; $p<0.001$) and negatively correlated with serum LDH levels ($r=-0.54$; $p=0.003$). VWF:CB/Ag and VWF:RCo/Ag ratio were similarly correlated with TTP-related laboratory measurements at presentation. VWF:CB/Ag displayed statistically significant correlations with platelet counts ($r=0.65$, $p=0.01$) and LDH (negative correlation with $r=-0.57$ and $p=0.05$) and a

trend towards correlation with hemoglobin (r=0.47, p=0.09). VWF:RCo/Ag showed a statistically significant correlation with platelet counts (r=0.74; p=0.003) and a trend towards negative correlation with LDH (r=-0.53; p=0.07). There was no statistically significant association with hemoglobin (r=0.37; p=0.19), perhaps due to the smaller sample size and lower correlation of VWF:RCo/Ag with ULVWF ratios compared with VWF:CB/Ag (see above). None of the study measurements was associated with the prevalence and type of clinical symptoms (data not shown).

Discussion

This study aimed to investigate the relationships between VWF-related properties and different clinical and laboratory presentations of acquired TTP (acute disease or remission with or without ADAMTS13 deficiency). In previous studies,[7, 8] plasma levels of VWF in its platelet-binding conformation (VWF-GPIb-α/BC) were high during acute TTP,[7] suggesting that this rise might be associated with, or even be responsible of, the formation of platelet-rich thrombi in the microcirculation, and hence of disease activity. In our cohort, platelet-reactive VWF was indeed increased in the acute phase of the first disease episode (subgroup a) in comparison to controls, but during remission the plasma levels of this conformation of VWF were not different from those measured during the first acute episode. Moreover, even in patients during the acute phase there was a large overlap of values between them and controls. No correlation was found with the degree of abnormalities of such laboratory markers of TTP activity and severity as platelet count, hemoglobin and LDH levels. Hence, platelet-reactive

VWF appears to be a weak index of disease activity in these patients. Its presence in plasma was closely associated with the presence of severe ADAMTS13 deficiency, indicating that the rise in the platelet-reactive conformation of VWF is among the prothrombotic changes induced by the deficiency of the VWF-cleaving protease.

There were striking differences in the multimeric structure of VWF between patients in the acute phase of the first disease and those in remission. The largest multimeric fraction of VWF was markedly reduced on electrophoresis in patients with a first acute episode of TTP, and this reduction was correlated with the degree of impairment of laboratory markers of disease activity and severity. The defect of larger VWF multimers was pronounced in patients during their first acute episode but not in those with recurrences, consistent with the views that recurrences are generally less severe than first disease episodes in terms of clinical and laboratory measurements, perhaps because they are diagnosed and managed earlier.[4] During remission, the multimeric pattern changed dramatically, with the fresh appearance in plasma of ultralarge VWF multimers similar to those present in vascular endothelial cells and platelets, in agreement with the pioneering observations made by Moake et al in 1982.[14] The lack of larger VWF multimers in patients with acute TTP was previously described in the frame of case reports and small case series.[15-17] In this study the correlation between the degree of defect of larger multimers of regular size and laboratory markers of TTP activity and severity suggests that the electrophoretic measurement of the ULVWF ratio is an index of disease activity and severity at presentation, at least in patients with ADAMTS13 deficiency. Lack of larger VWF multimers strongly

correlated with low VWF:CB/Ag and VWF:RCo/Ag ratios, suggesting that VWF:CB/Ag and VWF:RCo/Ag ratios, which are more easily measurable than the ULVWF ratio, may be candidate markers of acute disease severity in TTP. Pertaining to the striking differences in the multimeric structure of VWF observed during the first acute episode of TTP in comparison with the same patients during remission, we offer the following mechanistic explanations. During acute disease the plasmatic deficiency of the metalloprotease impairs the cleavage of the ultralarge multimeric forms of VWF secreted in plasma from markedly activated endothelial cells. However, these forms and the largest regular sized multimers are not seen in patient plasma because they avidly bind to platelets, thereby causing a multimeric defect that resembles that seen in acquired and type 2 von Willebrand disease. Perhaps this VWF defect adds to that of thrombocytopenia in causing the bleeding tendency sometimes observed in the acute phase of TTP. Indeed no association was found in this study between hemorrhagic symptoms and degree of multimeric effect, perhaps owing to the low prevalence in our patients of these symptoms, that tend to be overlooked and reported less frequently in TTP cases than the more striking ischemic symptoms. In contrast, during TTP remission, the degree of endothelial cell activation and the associated secretion of ultralarge multimers are likely to be much smaller, so that the balance between secretion of ultralarge VWF and platelet uptake is less turned towards the latter than during acute disease. The same situation of relative balance does perhaps take place at the time of recurrence, because recurrent episodes are diagnosed earlier, before massive platelet uptake of VWF is large enough to cause the loss of larger multimers in plasma. In TTP cases

characterized by normal ADAMTS13 levels during remission, the normal multimeric pattern uniformly observed in this study is likely to be explained by the restoration of a physiological degree of secretion of ultralarge VWF from endothelial cells that are no longer abnormally activated, as well as by the capacity of plasma ADAMTS13 to process ultralarge multimers into smaller regular sized multimers. This study has a number of limitations. The selection of the case material is based upon a registry for information on clinical data, symptoms and routine laboratory parameters such as platelet count, hemoglobin and serum LDH. However, the ADAMTS13 and VWF-related measurements, i.e., the core of this study, were centralized in one laboratory. The criteria for the diagnosis of TTP in the acute phase and remission are those based upon the exclusion of other thrombotic microangiopathies usually adopted in the medical literature. The criteria chosen for the analysis of primary and secondary subgroups are arbitrary, but the rationale for their choice is explained.

In conclusion, this study confirms that the platelet-reactive form of VWF as measured with the nanobody-based immunoassay is sometime present at increased concentrations during TTP. However, it also demonstrates that this measurement is not an accurate and sensitive index of disease activity and severity, being detectable also in several patients at the time of disease remission. The most striking and consistent finding was a defect of the large VWF multimers during the acute phase of TTP, accompanied by low VWF:CB/Ag and VWF:RCo/Ag ratios. The defect of VWF multimers was associated with disease activity and severity, as indicated by the degree of thrombocytopenia, anemia and organ damage measured by serum LDH.

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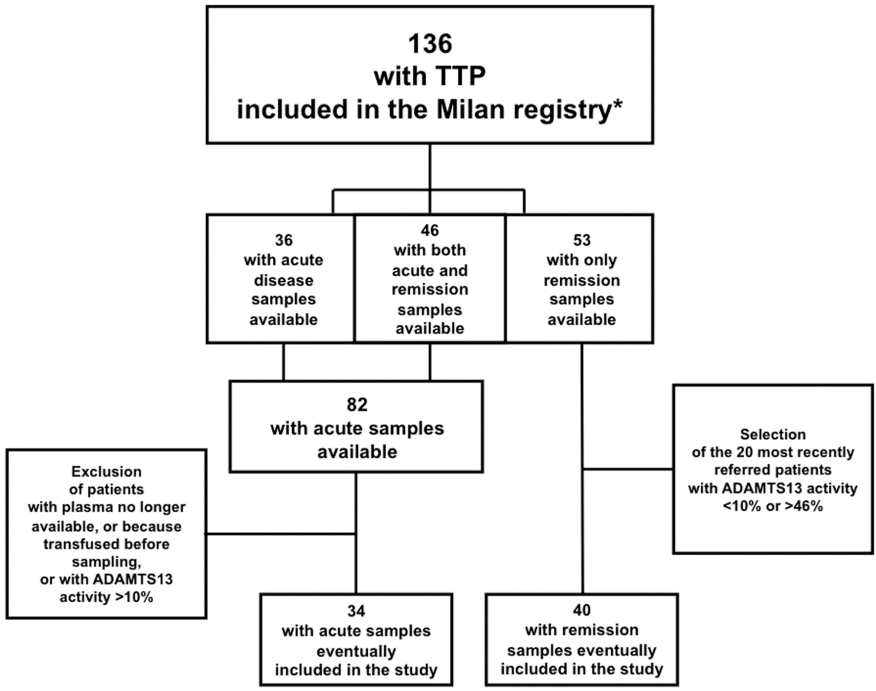
Figures

Figure 1. This flow-chart depicts the criteria for patient inclusion in the study.



* Number updated at the beginning of the study (July 2009). One patient had no available samples.

Figure 2. This flow chart shows the criteria followed in the selection of the primary and secondary subgroups from the 74 patients selected from the Milan TTP registry (see Figure 1).

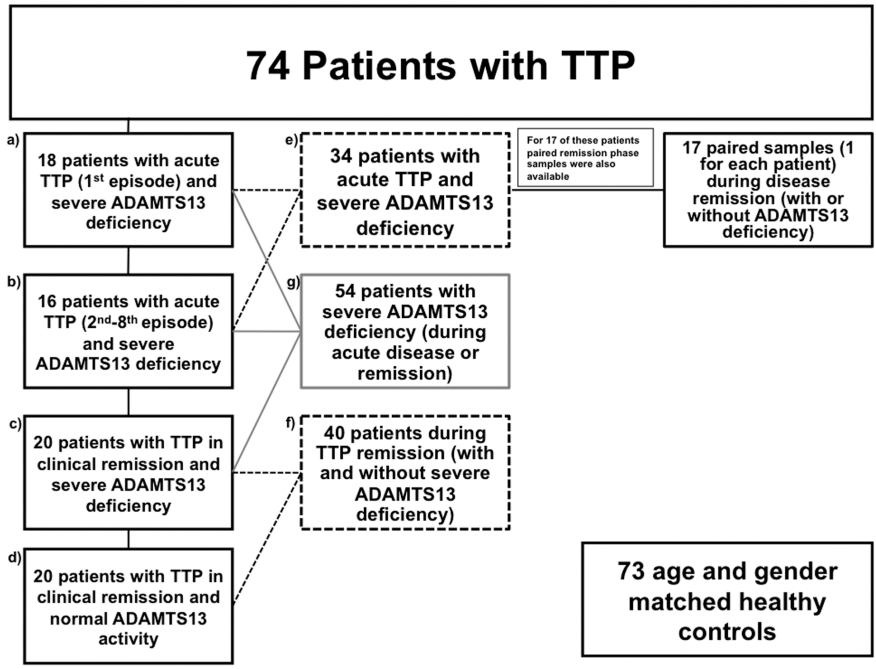


Figure 3. ULVWF multimer ratio in study subgroups (A) and according to paired samples analysis (B). In (A) box plots were used to describe the distribution of ULVWF ratio across study groups. Each box plot represents median, quartiles and range of ULVWF ratio. Panel (B) represents paired comparisons of ULVWF during acute disease (left) and remission (right). In (B) closed circles denote patients investigated at the time of their first TTP episode, open circles those investigated at the time of recurrences. All patients represented in (B) had severely deficient ADAMTS13 activity at the time of acute disease. During remission, only four of the patients represented in (B) had severe ADAMTS13 deficiency, displaying ultralarge VWF multimers in plasma with an ULVWF ratio above 1.20. The other patients investigated during remission in paired comparisons had normal ADAMTS13 activity.

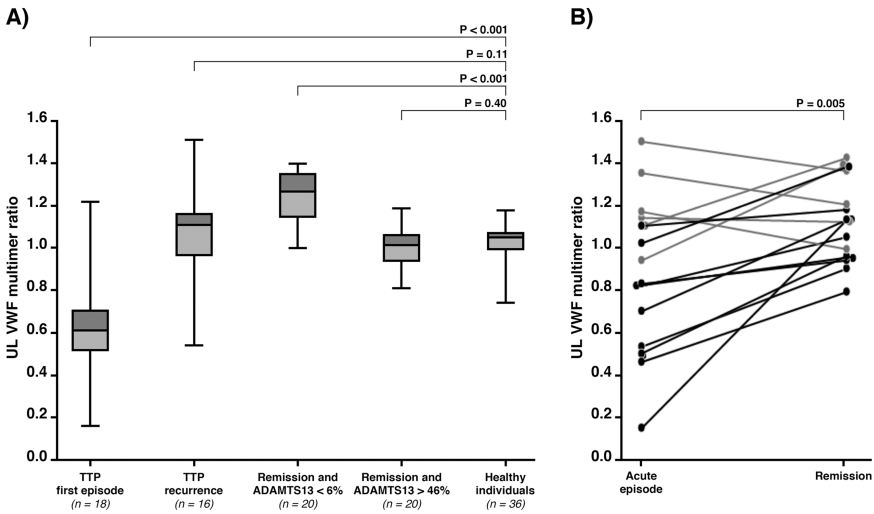
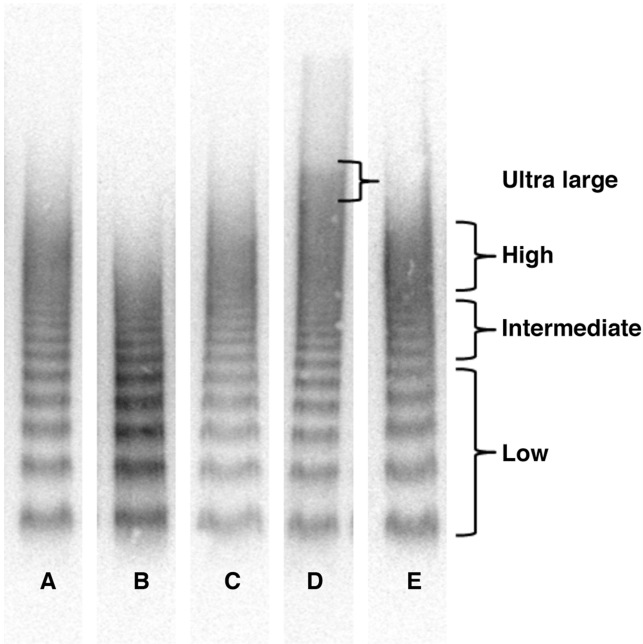


Figure 4. Multimeric analysis in pooled normal plasma (A), patient at first TTP episode (B), patient at recurrent (third) episode (C), patient during remission with severe ADAMTS13 deficiency (D), patient during remission with normal ADAMTS13 activity (E). Low-, intermediate-, high and ultralarge molecular weight VWF multimers are highlighted by parentheses at the right side of the figure.



Tables

Table 1. General features and study results in TTP (all patients and primary subgroups a-d) and controls.

Groups and subgroups	Controls	All TTP	Group (a) first acute episode, ADAMTS13 <6%	Group (b): recurrent acute episode, ADAMTS13 <6%	Group (c): remission, ADAMTS13 <6%	Group (d): remission ADAMTS13, normal
Number of cases (males/females)	73 (18/55)	74 (18/56)	18 (5/13)	16 (3/13)	20 (7/13)	20 (3/17)
Age at first episode, years	/	40 (14-71)	45 (18-64)	36 (14-69)	38 (19-54)	36 (23-71)
Age at sampling, years	46 (18-72)	46 (18-72)	45 (18-64)	43 (14-70)	47 (33-63)	44 (27-72)
O blood group, n (%)	30 (41)	32 (43)	6 (33)	7 (43)	8 (40)	11 (55)
Hemoglobin at first episode, g/dL	/	8.3 (4.1-13.5)	8.7 (4.1-11.4)	7.2 (5.7-13.5)	7.7 (5.5-11.2)	8.3 (4.7-12)
Platelets at first episode, x1000/mm^3	/	15 (1-89)	15 (5-24)	17 (3-44)	12 (1-67)	17 (2-89)
Platelets at sampling, x1000/mm^3	/	145 (2-410)	15 (5-24)	36 (2-117)	240 (150-410)	250 (152-366)
VWF antigen[a], %	105 (84-124)	147 (104-234)	200 (125-268)	202 (124-290)	134 (99-194)	110 (89-152)
VWF-gpIb-α/BC ratio[a]	0.92 (0.74-1.3)	1.30 (0.83-1.73)	1.32 (0.98-1.96)	1.07 (0.85-1.89)	1.41 (0.75-1.55)	1.17 (0.69-1.51)
ULVWF multimer ratio[a]	1.05 (0.97-1.07)	1.04 (0.85-1.17)	0.61 (0.50-0.74)	1.11 (0.95-1.17)	1.26 (1.14-1.35)	1.01 (0.94-1.06)
ADAMTS13 activity %	93 (46-160)	<6	<6	<6	<6	89 (55-170)

All continuous variables were expressed as median (with ranges between parentheses) unless specified.

a Expressed as medians (interquartile ranges).

Table 2. Study measurements in 17 patients with available acute disease and remission samples (paired comparison).

	Acute disease	**Remission**	**P value**
VWF antigen, %	234 (144-311)	184 (150-263)	0.35
ULVWF multimer ratio	0.85 (0.54-1.14)	1.14 (0.98-1.29)	0.005
VWF-GPIb-α/BC ratio	1.05 (0.98-168)	1.46 (0.81-1.94)	0.30
VWF:Rco, %	109 (77-180)	129 (92-119)	0.20
VWF:Rco/Ag ratio	0.65 (0.38-0.93)	0.77 (0.60-0.92)	0.37
VWF:CB, IU/dL	112 (74-250)	166 (136-220)	0.15
VWF:CB/Ag ratio	0.75 (0.40-0.90)	1.01 (0.78-1.12)	0.005

Acute vs remission values were compared by Wilcoxon signed ranks test. All values expressed as median (interquartile range).

Table 3. Results of VWF:Ag and VWF-GPIb-α/BC according to O/non-O blood groups.

Blood group	Variable	VWF:Ag, %		P=
		Cases	Controls	
O	N=	32	30	<0.001
	Median	118	85	
non-O	N=	42	43	<0.001
	Median	194	115	

Blood group	Variable	VWF-GPIb-α/BC ratio		P=
		Cases	Controls	
O	N=	32	30	0.06
	Median	1.01	0.79	
non-O	N=	42	43	0.03
	Median	1.41	1.15	

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CHAPTER 8

ADAMTS13 activity and autoantibodies classes and subclasses as prognostic predictors in acquired thrombotic thrombocytopenic purpura

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Abstract

Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening disease. Of surviving patients, 45% develops an exacerbation or a late recurrence. Severe ADAMTS13 deficiency is a well-established predictor of recurrence. The predictive value of anti-ADAMTS13 antibodies, their inhibitory activity and Ig class subtype is still to be established. We sought to analyze ADAMTS13 related biomarkers (ADAMTS13 and anti-ADAMTS13 immunoglobulins, classes and subclasses) and their potential relationship with prognosis. In 115 patients with TTP, we assessed the association between levels of these biomarkers, the severity of acute episodes and the risk of recurrence. During acute TTP, higher IgA, IgG1 and IgG3 titres showed the strongest association with acute episode severity. In the survival analyses, the presence of IgG at acute disease, the presence of ADAMTS13 inhibitor or IgG during remission were all associated with a higher hazard of recurrence. Both the Ig class and subclass are of predictive value for acute episode severity in patients with TTP. Although markers that could predict the risk of recurrence in the acute phase are limited, a thorough assessment of ADAMTS13 related parameters during remission is warranted.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare disease characterized by widespread hyaline thrombi, composed primarily of platelets and rich in von Willebrand factor (VWF). TTP manifests in the terminal arterioles and capillaries of multiple organs, most extensively in the heart, brain, kidney, pancreas, spleen, mesentery and adrenal glands.[1, 2] The disorder is associated with a severe deficiency of the VWF-cleaving protease ADAMTS13 causing persistence of highly adhesive ultralarge VWF (ULVWF).[3-6] Within the microcirculation, unfolded ULVWF multimers spontaneously seize platelets and promote platelet thrombi formation, eventually leading to life-threatening microvascular thrombosis. In patients with acquired TTP, a deficiency of ADAMTS13 is caused by auto-antibodies against ADAMTS13.[6] Neutralizing antibodies that inhibit proteolytic activity but also non-neutralizing antibodies that may enhance clearance or disturb interaction with physiologic binding partners have been described.[7] In most cases, anti-ADAMTS13 autoantibodies are of the IgG isotype, with IgG4 as the most prevalent subclass followed by IgG1.[8-10] IgM and IgA autoantibodies have also been identified.[7] Exacerbation (within 30 days of remission) and late recurrences (after 30 days) are frequent complications, reported in up to 40% of TTP patients.[11-13] Most recurrences occur during the first year, but they have also been documented to occur years after the first acute event.[12, 14] Several studies assessed the potential utility of ADAMTS13-related laboratory measurements at different stages of the disease clinical course for the prediction of clinical outcomes in TTP, obtaining discordant results.[9, 10, 12, 15-19] In this multicenter study, we analyzed the association between ADAMTS13-related

biomarkers and acute episode severity as well as risk of recurrence in patients with TTP.

Patients and methods

Patients and definition of clinical categories

Clinical centers from seven countries (Italy, Hungary, Lebanon, Romania, Slovenia, Serbia and Russia) participated in the study. Data collection methods and clinical definitions were extensively described elsewhere.[20] Data of all patients were included in a digital database (URL: http://www.ttpdatabase.org). Criteria for TTP diagnosis were the documentation of at least one episode of: (a) thrombocytopenia, (b) microangiopathic haemolytic anemia (c) exclusion of alternative explanations for thrombocytopenia (such as the enterohemorrhagic form of hemolytic uremic syndrome, catastrophic anti-phospholipid antibodies syndrome, pre-eclampsia and related syndromes, sepsis, systemic inflammatory response syndrome, disseminated intravascular coagulation, disseminated malignancy or bone-marrow transplantation associated TTP-like syndrome). Patients were classified as in remission when they became free of symptoms and had normal laboratory values (except for ADAMTS13 levels) for at least 30 days from the conclusion of plasma therapy. Recurrence was defined as the re-emergence of clinical symptoms or laboratory criteria compatible with a diagnosis of an acute TTP episode (as described above) occurring at least 30 days or later after remission of the preceeding acute episode.

Laboratory measurements

ADAMTS13 activity was measured using the residual VWF collagen binding activity (CBA) assay, as previously reported.[16] ADAMTS13 antigen levels were measured by ELISA, as previously described by Feys and colleagues.[21] The anti-ADAMTS13 antibodies were quantified using an ELISA method. Microtiter plate wells were coated with mouse anti-V5 antibody (Invitrogen, Grand Island, NY, USA) at 4 μg/mL final concentration. The plate was incubated successively with: 1) cell-culture conditioned media containing ADAMTS13 protein at a final concentration of 1μg/mL; 2) plasma samples at various dilutions (1/50, 1/100 and 1/200); and, 3) HRP-labelled anti-human IgG/IgA/IgM/IgG1,2,3,4. Between each step, the plates were washed with phosphate-buffered saline containing 0.1% (v/v) Tween-20 (pH 7.4). Bound IgG/IgA/IgM/IgG1,2,3,4 was detected adding OPD substrate. The optical density (OD) values were read at 492 nm using a reference filter at 620 nm. For IgG, the concentration of autoantibody was calculated as a percentage of a curve of a patient's plasma with a high inhibitor titre (arbitrarily assigned a value of 100%). Normal range was calculated on 40 normal plasmas and values higher than 2 standard deviations were considered positive (IgG>1.18%; IgA>0.014; IgM>0.034; IgG1>0.006; IgG2>0.013; IgG3>0.019; IgG4>0.003). To normalize the OD values in patients with more than one IgG subclass of anti-ADAMTS13 autoantibodies, a normalization procedure was applied as previously described by Ferrari and colleagues with minor modifications.[10] Purified human IgG 1-4 (lambda chain, Sigma-Aldrich, St Louis, MO, USA) antibodies were coated on an ELISA plate in a concentration range of 0.125–1 μg/ml and the OD values generated from the addition of HRP-

anti-human IgG 1-4 were recorded every 5 min up to 75 min. For each IgG subclass and concentration, five independent experiments were performed and the means of the corresponding OD values were plotted against time and analyzed by linear regression for each IgG subclass. The regression coefficients obtained at a coating concentration of 0.5µg/ml (linear range) were used to calculate the OD value of each subclass. Setting the OD value of IgG4 to 1, the resulting normalization factors were 1.1, 0.94, 1.49 for IgG1, IgG2 and IgG3, respectively. OD values of TTP patients positive for anti-ADAMTS13 IgG subclasses were normalized by multiplying them by the corresponding normalization factor. To obtain the IgG subclass percentage distribution in a single patient, positive normalized OD values of each subclass were summed and the relative proportion of each individual subclass was calculated as a percentage of the total absorbance (set at 100%).

Statistical analysis

To take into account the intra-individual correlation of repeated measurements over time, linear regression random intercept models were fitted to assess the association between levels of ADAMTS13 and anti-ADAMTS13 immunoglobulins and the severity of acute episodes, defined on the basis of platelets counts and on the number of plasma exchanges performed. Values below the limit of detection (LOD) were assigned a value equal to 50% of the LOD. When necessary, the dependent variables were transformed (logarithm or square-root) to get a normal distribution. Using Cox regression models we analysed the hazard ratio (HR) and 95% confidence interval (95% CI) of recurrence in association with levels of ADAMTS13 and anti-ADAMTS13

immunoglobulins retrieved at the previous acute episode or during remission. In these analyses, we categorized CBA levels and other biomarkers and immunoglobulins in two (low, high) according to standard thresholds. To take into account within-subject correlation, frailty models were fitted. Two separate analyses were performed, for measurements during acute episodes and remission, respectively. All the analyses were performed with Stata, version 11 (StataCorp LP, College Station, TX, USA).

Results

We identified 166 patients with a confirmed diagnosis of TTP over the study period (1994-2010). We excluded 11 patients with congenital TTP and 40 patients with plasma samples that were inadequate for analysis (gathered during plasma therapy), leaving 115 patients for this analysis. The general and clinical characteristics of the patients are summarized in Table 1. Of the 115 patients, 43 entered the study (first plasma sample collection) with a first acute TTP event and 7 of them developed at least one recurrent event during follow-up. Eleven patients entered the study with a recurrent event and 3 of them developed at least one subsequent recurrent event. The remaining patients (n=61) had their first samples retrieved during remission and 20 of them had at least one subsequent acute episode of TTP. Moreover, 9 patients (8%, 7 women and 2 men, aged 21-66 years, mean age 46 years) died during the study period. Of these, 7 died during an acute episode of TTP. The remaining two were a 34-year-old female patient who died of a cerebral tumor; and a 54-year-old female patient who died of an unknown cause.

Acute phase analysis

Samples from 70 acute TTP episodes in 62 patients were analyzed. 43 samples were collected during the first acute episode, 27 during a recurrence (eleven 2nd episodes, four 3rd episodes, five 4th episodes, three 5th episodes, one 6th episode, one 9th episode, two 10th episodes). During the acute phase, higher IgA titres were associated with lower platelet counts, while higher IgG titres were associated with an increased number of plasma exchanges required to obtain remission. Among the IgG subclasses, IgG1 had the strongest association with platelet count, while IgG3 was strongly associated with both platelet count and the number of plasma exchanges (Table 2). There was no association between severity of the acute phase and ADAMTS13 antigen, activity, or inhibitor levels.

Survival analyses

Patients with low levels of ADAMTS13 (<10%) during the acute episode did not have a significantly higher hazard of recurrence, but none of 5 patients with normal levels (≥46%) developed recurrences. Patients with presence of IgG during the acute episode had a high risk of recurrence (15/50=30%) although hazard ratio was not estimable (none of 10 patients with absence of IgG developed recurrence) (Table 3). Analyses considering only 'first' acute episodes led to the same results. Patients with low ADAMTS13 activity (<10%) or presence of IgG during remission had a five-fold increased hazard of recurrence (respectively HR=4.89, 95% CI 2.00 to 11.99 and HR=4.99, 95% CI 2.08 to 12.00) (Table 4). Moreover, low ADAMTS13 antigen (<10%) and presence of inhibitor in blood drawn during remission were associated with a high hazard of

recurrence (respectively HR=5.66, 95% CI 2.10 to 15.24 and HR=4.30, 95% CI 2.00 to 9.21) (Table 4).

Discussion

In recent years, several studies have investigated the role of biomarkers in predicting recurrence of episodic autoimmune disease. Particularly meaningful in this respect are studies about the distribution of the specific Ig subclasses in relation to a number of autoimmune diseases such as rheumatoid arthritis, celiac disease, type I diabetes and others.[22-24] These studies showed how IgG subclasses have a distinct biologic properties with different actions on complement activation and immune functions. Published data suggest that patients with a reduced ADAMTS13 activity during the acute episode have a higher risk of recurrence.[12] In the present study, conducted on a large number of TTP patients, we did not identify a higher hazard of recurrence for patients with severe ADAMTS13 deficiency (<10% of activity) during the acute phase compared with patients with intermediate or normal levels (≥10%), but none of 5 patients with normal levels (≥46%) developed recurrences. Conversely, patients with severe ADAMTS13 deficiency during remission had a 5-times higher hazard of recurrence (Table 5).

In patients with acquired idiopathic TTP and in some secondary forms, the deficiency of ADAMTS13 depends on the presence of anti-ADAMTS13 autoantibodies. The predictive value of disease recurrence offered by the presence of anti-ADAMTS13 antibodies, their inhibitory activity, Ig classes and subclasses is still contraversial.[15, 16] Our data add to previous findings by showing that the

presence of anti-ADAMTS13 inhbitors during remission also predicts the risk of recurrence. They also confirm that presence of anti-ADAMTS13 IgG has a strong predictive value for recurrence both during acute phase and remission. However, we did not find any association between IgG subclasses and recurrence risk (Table 5). We did not find an association between ADAMTS13 activity or antigen level and acute disease severity in acquired TTP. Moreover, we did not observe an association between anti-ADAMTS13 inhibitor levels and acute episode severity which is in disagreement with previous reports.[19] Our study also showed that during the acute TTP phase, IgA represented the Ig class which is most strongly associated with the clinical severity of the acute episode (estimated by the number of platelets at presentation). IgA could contribute to the severity of the clinical manifestations by activating the complement system through the mannose-binding lectin pathway, thus increasing complement-mediated inflammation.[25] In fact IgA levels have been associated with increase mortality from acute phase TTP.[9] In this study, we identified that the IgG class and subclasses are also predictive of the severity of acute TTP. High IgG titres were associated with a higher number of plasma exchanges; and of the IgG subclasses, IgG1 and IgG3 were associated with the clinical severity of the acute phase of disease. In fact high IgG1 levels were previously suggested to increase mortality risk in patients with TTP.[10] Detailed knowledge about the pathogenicity of the different isotypes may be useful to develop subclass specific immunoaphereses and immunotherapies capable of redirecting the isotopic switch towards less pathogenic isotypes and of blocking complement and inflammatory cell activation by acting on specific IgG subclasses. The main limitation of our study

is related to the nature of data retrieval. This is not a formal cohort study, since we have identified patients from current local registries and patients records, and there is no inception cohort, leading to potential referral bias. The patients, referred to a tertiary center from many other regional centers, could be representing those with highest severity, or highest recurrence rate. Those with a first event long ago who never had a recurrence were less likely to be included than those with a recent recurrence, so we may have overestimated recurrence risk. Those who would have had a recurrence but died, were also not included. Survival analyses comparing groups by laboratory parameters and acute phase analyses, however, were not affected by these limitations. So, while our estimates of the proportion of patients with severe disease and the recurrence risk per se may be inaccurate, we believe the analyses that showed a preponderance of specific biomarkers in patients with severe events or recurrence is reliable. The large number of patients, the long observation time available for most patients and the centralized measurement of ADAMTS13-related biomarkers collocate ours among the most precise estimates available in the literature.

In conclusion, we undertook a comprehensive assessment of several biomarkers, retrieved both at acute phase and remission, to predict outcomes in patients with TTP. Disease recurrence seems to be only associated with the presence of IgG antibodies during the acute phase, while alterations in several ADAMTS13-related biomarkers could predict recurrence risk when measured during disease remission. Hence, adequate laboratory workup in this setting could help identify patients at risk that require closer follow up. Although the study of IgG

subclasses does not seem helpful in predicting disease recurrence, its value in identifying the severity of the acute episodes warrants further study.

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Tables

Table 1. Characteristic of patients.

	N	%
Total	115	100
Geographic Origin		
Italy	92	80
Other Country	23	20
Sex		
Male	25	22
Female	90	78
Age at first acute episode, years		
<20	9	8
20-30	26	23
30-40	33	28
40-50	17	15
50-60	19	16
60-70	10	8
>70	2	2
Died from the disease	7	6
Idiopathic disease	92	80
Secondary disease	23	20
other autoimmune disease	4	3
Pregnancy	9	8
Ticlopidine use	4	3
Surgery	3	3
Herpes Virus Infection	2	2
Malignant disease	1	1
ADAMTS13 at acute episode (N=62)		
<10	43	69
≥10,<46	14	23
≥46	5	8

Table 2. Relationship between ADAMTS13 related biomarkers and clinical severity during the acute phase.

Variable	Log Platelet count		
	Slope	**95%CI**	**P value**
ADAMTS13 activity	0.005	-0.001 to 0.011	0.12
ADAMTS13 antigen	0.003	-0.002 to 0.008	0.27
Inhibitor	0.003	-0.0001 to 0.005	0.06
IgG	-0.0005	-0.002 to 0.001	0.42
IgM	-0.188	-0.657 to 0.280	0.43
IgA	-0.262	-0.480 to -0.045	0.018
IgG1	-0.708	-1.197 to -0.218	0.005
IgG2	-0.096	-1.266 to 1.075	0.87
IgG3	-0.478	-0.817 to -0.139	0.006
IgG4	0.069	-0.073 to 0.212	0.34
	√Plasma exchange		
ADAMTS13 activity	0.005	-0.021 to 0.031	0.73
ADAMTS13 antigen	0.007	-0.010 to 0.024	0.43
Inhibitor	0.006	-0.003 to 0.017	0.17
IgG	0.005	0.0002 to 0.010	0.04
IgM	0.71	-0.796 to 2.218	0.36
IgA	0.59	-0.501 to 1.675	0.29
IgG1	1.47	-1.021 to 3.964	0.25
IgG2	0.63	-3.034 to 4.301	0.74
IgG3	2.55	0.005 to 5.095	0.05
IgG4	0.21	-0.565 to 1.740	0.38

CI, confidence interval.

Table 3. Cox regression analysis of hazard of recurrence in relation to biomarkers levels at previous acute episode.

	Number of patients	Patients with recurrences	Hazard Ratio	95% CI	P value
ADAMTS13 activity (%)					
≥10	18	3	1.00	Reference	Reference
<10	22	4	1.68	0.43-6.59	0.45
ADAMTS13 antigen (%)					
≥10	18	4	1.00	Reference	Reference
<10	16	2	0.86	0.23-3.23	0.83
Inhibitor (U/ml)					
Absent	20	4	1.00	Reference	Reference
Present	15	3	0.70	0.19-2.63	0.60
IgG (%)					
≤1.18	5	0	1.00	Reference	Reference
>1.18	27	6	∞	-	-
IgM					
≤0.034	25	4	1.00	Reference	Reference
>0.034	6	1	1.37	0.24-7.97	0.72
IgA					
≤0.014	17	3	1.00	Reference	Reference
>0.014	14	2	ne		
IgG1					
≤0.006	5	1	1.00	Reference	Reference
>0.006	24	4	1.24	0.21-7.23	0.81
IgG2					
≤0.013	6	1	1.00	Reference	Reference
>0.013	23	4	1.09	0.22-5.46	0.92
IgG3					
≤0.019	10	2	1.00	Reference	Reference
>0.019	19	3	0.65	0.20-20.6	0.46
IgG4					
≤0.003	7	2	1.00	Reference	Reference
>0.003	22	3	0.49	0.12-2.12	0.34

CI, confidence interval.

Table 4. Cox regression analysis of hazard of recurrence in relation to biomarkers levels at previous remission observation.

	Number of patients	Patients with recurrences	Hazard Ratio	95% CI	P value
ADAMTS13 activity (%)					
≥10	71	18	1.00	Reference	Reference
<10	19	9	4.89	2.00-11.99	0.001
ADAMTS13 antigen (%)					
≥10	77	19	1.00	Reference	Reference
<10	5	3	5.66	2.10-15.24	0.001
Inhibitor (U/mL)					
Absent	43	9	1.00	Reference	Reference
Present	13	9	4.30	2.00-9.21	<0.001
IgG (%)					
≤1.18	50	7	1.00	Reference	Reference
>1.18	26	11	4.99	2.08-12.00	<0.001
IgM					
≤0.034	74	16	1.00	Reference	Reference
>0.034	2	2	2.67	0.39-18.44	0.32
IgA					
≤0.014	59	16	1.00	Reference	Reference
>0.014	16	1	0.30	0.07-1.27	0.10
IgG1					
≤0.006	25	6	1.00	Reference	Reference
>0.006	17	6	1.29	0.61-2.71	0.50
IgG2					
≤0.013	30	6	1.00	Reference	Reference
>0.013	12	6	1.85	0.86-3.98	0.12
IgG3					
≤0.019	29	7	1.00	Reference	Reference
>0.019	13	5	1.45	0.59-3.58	0.42
IgG4					
≤0.003	11	2	1.00	Reference	Reference
>0.003	31	10	1.49	0.49-4.49	0.48

CI, confidence interval.

Table 5. Studies that investigated ADAMTS13-related measurements in thrombotic thrombocytopenic purpura.

Reference	Design	Sample size*	Timing of measurement	Marker	Endpoint	Result
This study	Cohort	62	Acute TTP	ADAMTS13 activity	Acute episode severity	No association
		59	Acute TTP	ADAMTS13 antigen	Acute episode severity	No association
		56	Acute TTP	Anti-ADAMTS13 inhibitor level	Acute episode severity	No association
		52	Acute TTP	Anti-ADAMTS13 Ig class	Acute episode severity	Higher IgG and IgA titres associated with more severe acute episode
		47	Acute TTP	Anti-ADAMTS13 IgG subclasses	Acute episode severity	Higher IgG1 and IgG3 titres associated with more severe acute episode
		62	Acute TTP	ADAMTS13 activity	Recurrence	No recurrence in patients with ADAMTS13 ≥46%
		62	Acute TTP	ADAMTS13 antigen	Recurrence	No association
		62	Acute TTP	Anti-ADAMTS13 inhibitor	Recurrence	No association
		52	Acute TTP	Anti-ADAMTS13 Ig class	Recurrence	Presence of IgG associated with recurrence
		47	Acute TTP	Anti-ADAMTS13 IgG subclasses	Recurrence	No association
		106	Remission	ADAMTS13 activity	Recurrence	ADAMTS13 activity <10% associated with recurrence
		101	Remission	ADAMTS13 antigen	Recurrence	ADAMTS13 antigen<10% associated with recurrence
		68	Remission	Anti-ADAMTS13 inhibitor	Recurrence	Presence of inhibitor associated with recurrence
		74	Remission	Anti-ADAMTS13 Ig class	Recurrence	Presence of IgG associated with recurrence
		61	Remission	Anti-ADAMTS13 IgG subclasses	Recurrence	No association
Coppo et al. 2005	Cohort	33	Acute TTP	Anti-ADAMTS13 inhibitor	Acute episode severity	Patients with inhibitor took longer to achieve remission and required more plasma volume to obtain remission han patients without inhibitor
Ferrari et al. 2007	Cohort	35	Acute TTP	Anti-ADAMTS13 inhibitor level	Acute disease mortality	No association
		35	Acute TTP	Anti-ADAMTS13 Ig class	Acute disease mortality	High IgA titres associated with death
		32	Remission	ADAMTS13 activity	Recurrence	ADAMTS13 activity <5% deficiency associated with recurrence

Peyvandi et al. 2008	Case-control	109	Remission	ADAMTS13 activity	Recurrence	ADAMTS13 activity <10% deficiency associated with recurrence
		77	Remission	ADAMTS13 Ag	Recurrence	No association
		97	Remission	Anti-ADAMTS13 autoantibodies	Recurrence	Presence of antibodies associated with recurrence
Jin et al. 2008	Cohort	24	Remission	ADAMTS13 activity	Recurrence	Lower ADAMTS13 activity associated with higher risk or recurrence
		24	Remission	Anti-ADAMTS13 Ig G	Recurrence	No association
Ferrari et al. 2009	Case-control	48	Acute TTP	Anti-ADAMTS13 IgG subclasses	Acute disease mortality	High IgG1 titres associated with mortality
		48	Acute TTP	Anti-ADAMTS13 IgG subclasses	Recurrence	High IgG4 titres associated with recurrence
Kremer-Hovinga et al. 2010	Inception cohort	261	Acute TTP	ADAMTS13 activity	Acute disease mortality	ADAMTS13 activity <10% associated with better survival (p=0.11)
		183	Acute TTP	ADAMTS13 activity	Recurrence	ADAMTS13 activity <10% associated with recurrence
		60	Acute TTP	Anti-ADAMTS13 inhibitor level	Acute disease mortality	High inhibitor (≥2 Bethesda units) associated with mortality
		37	Remission	ADAMTS13 activity	Recurrence	No association
Zheng et al. 2010	Cohort	67	Not specified	Anti-ADAMTS13 autoantibody antigen specificity	Clinical symptoms and disease-related laboratory measurements at hospital admission	Antibodies against C-terminal domains associated with higher platelet counts at admission
Yang et al. 2011	Cohort	40	Acute TTP	ADAMTS13 antigen	Acute disease mortality	Lower antigen levels associated with mortality
		40	Upon response to PEX	ADAMTS13 antigen	Sustained remission	Higher antigen levels associated with sustained remission

* Different analyses had different sample size.

TTP, thrombotic thrombocytopenic purpura; Ig, immunoglobulin; PEX, plasma exchange.

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CHAPTER 9

Clinical case: use of thienopyridines in a patient with acquired idiopathic thrombotic thrombocytopenic purpura

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Abstract

Thienopyridines are commonly used anti-platelet drugs that may be associated with the development of secondary, drug-induced thrombotic thrombocytopenic purpura (TTP), a rare but potentially life-threatening condition. We report the case of a 70 year-old man with a history of recurrent idiopathic TTP episodes who was treated with clopidogrel and then ticlopidine for thromboprophylaxis after percutaneous coronary intervention. Treatment was successful with no signs of TTP recurrence. Platelet counts and ADAMTS13 activity levels remained normal for months after the initiation of anti-platelet therapy, with no reappearance of anti-ADAMTS13 autoantibodies. This report suggests that thienopyridines can be used in patients with a history of TTP who are in disease remission.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare, life-threatening disease characterized by single or multiple episodes of disseminated microvascular thrombosis, with thrombocytopenia, microangiopathic hemolytic anemia and damage of multiple organs.[1-4] TTP can be idiopathic or secondary. Secondary TTP is defined when the disease develops in association with medical conditions (i.e. HIV infection, bone-marrow transplantation and disseminated malignancy) or with the use of certain drugs.[1] Drug-induced TTP is very rare, accounting for a tiny proportion of all TTP cases.[2] Drugs that have been reported in association with TTP include quinine and thienopyridines (ticlopidine and clopidogrel), a class of inhibitors of platelet ADP-receptor commonly used for anti-platelet therapy in patients with cardiovascular disease.[5] Idiopathic TTP is associated with severe deficiency of the von Willebrand factor cleaving protease, ADAMTS13, due to the presence of circulating anti-ADAMTS13 autoantibodies. By contrast, secondary TTP is rarely associated with the presence of anti-ADAMTS13 autoantibodies.[1] However, case-series and reviews of reported cases of thienopyridine-induced TTP showed a high prevalence of anti-ADAMTS13 autoantibodies, suggesting that the use of these drugs in genetically susceptible patients may elicit an anti-ADAMTS13 autoimmune response similar to that of idiopathic TTP patients.[6, 7] There are no data on the use of thienopyridines in patients with a history of idiopathic TTP.

Case description

A 70 year-old Italian man was admitted to the Cardiology unit for elective coronary angiography, after a recent stress-test had shown evidence of stress-induced angina with reversible ischemia of the inferior surface. The patient had a history of idiopathic thrombotic thrombocytopenic purpura with severe deficiency of ADAMTS13 due to anti-ADAMTS13 autoantibodies. At the age of 69 he was admitted to another hospital for the sudden appearance of fatigue, diarrhea and focal neurological signs (aphasia and right upper limb weakness and paresthesia). Laboratory tests showed severe thrombocytopenia (platelet counts: 20 10^9/L), and Coombs-negative mechanical hemolytic anemia (hemoglobin: 7.6 g/dL), increased lactate dehydrogenase (LDH: 1968 IU/L) and hemoglobinuria. A diagnosis of TTP was made and the patient was treated with corticosteroids (prednisone, 75 mg daily) and plasma exchange (PEX), achieving remission with normalization of the laboratory parameters after 4 PEX procedures. Two months later, a new TTP episode developed. Measurement of ADAMTS13 activity by collagen binding assay[8, 9] on plasma sampled before the initiation of PEX revealed severe deficiency of ADAMTS13 (ADAMTS13 activity: <6%; normal values, n.v.: 46-160%) with presence of anti-ADAMTS13 IgG auto-antibodies at western blotting analysis and ELISA measurement (antibody titre: 18%; n.v.: <1%).[8, 9] The second episode resolved after 12 plasma exchanges. Measurement of ADAMTS13 at 6 months after remission, when corticosteroid maintenance treatment was discontinued, showed normal ADAMTS13 activity (ADAMTS13 activity: 85%; n.v.:46-160%) with absence of anti-ADAMTS13 autoantibodies. Table 1 summarizes ADAMTS13-related laboratory measurements at acute

disease and remission. After remission, the patient started regular follow-up at the out-patient clinic for thrombotic microangiopathies of the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy), with regular measurement of ADAMTS13 activity and follow-up clinical visits every 3-4 months. At coronary angiography, the patient showed coronary artery bivascular disease with 80% stenosis of the anterior descending and circumflex arteries. Bare metal stents were successfully placed after pre-dilatation. During the procedure, the patient was treated with clopidogrel (75 mg daily) and ASA (100 mg daily). After the procedure, a cutaneous rash developed, which was treated with corticosteroids. Clopidogrel was precautionarily suspended. Dual anti-platelet therapy with ticlopidine (250 mg twice a day) and ASA (100 mg daily) was prescribed for the duration of one year. Complete blood counts before, during and after the intervention consistently showed normal platelet counts. ADAMTS13 activity was normal in 4 measurements 2-weeks after the procedure, at 3, 6 and 12 months, with no appearance of anti-ADAMTS13 autoantibodies (Figure).

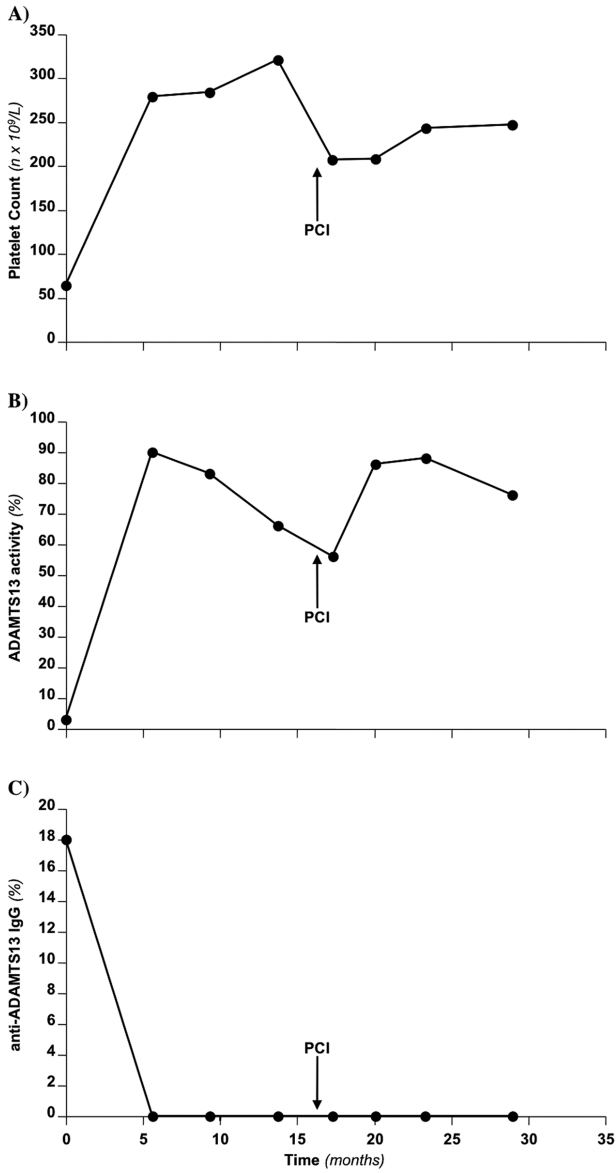
Discussion

We reported the case of a 70 year-old patient with a history of autoimmune idiopathic TTP treated with thienopyridines after percutaneous coronary intervention. The development of drug-induced TTP is a rare side effect of treatment with thienopyridines with an incidence rate of 1/5000 treated patients for ticlopidine and 1/1 million treated patients for clopidogrel.[6] Thienopyridine-induced TTP has been reported to be associated with the presence of anti-

ADAMTS13 autoantibodies in a large proportion of cases, suggesting the assumption of these drugs can elicit anti-ADAMTS13 autoimmune responses.[6] The still unexplained mechanisms leading to anti-ADAMTS13 autoimmune response in patients with thienopyridine-induced TTP could share pathophysiological pathways with autoimmune idiopathic TTP. There are no data on the use of thienopyridines in patients with a history of autoimmune TTP. In this case, thienopyridines were administered in a patient with a history of recurrent autoimmune TTP with severe ADAMTS13 deficiency and anti-ADAMTS13 IgG autoantibodies. At the time of treatment the patient was in remission and had no evidence of anti-ADAMTS13 autoantibodies. Autoantibodies did not reappear and TTP did not develop after treatment with thienopyridine. This indicates that thienopyridines do not necessarily induce TTP when used in patients with a history of TTP who are in clinical remission and suggests that the mechanisms that lead to anti-ADAMTS13 autoimmunity in patients with idiopathic and those with drug-induced TTP are likely distinct.

Figure

Figure 1. Platelet count and ADAMTS13 activity and antibody levels after the initiation of anti-platelet therapy. PCI indicates percutaneous coronary intervention.



Table

Table 1. ADAMTS13-related laboratory profile of the patient.

Variable	Normal values	Acute disease - second episode	Remission - 6 months after the second episode
A13:Activity, %[a]	46-160%	<6	90
A13:Antigen, %[b]	40-155%	40	85
WB[c]	Absent	Present	Absent
ADAMTS13 inhibitor, bethesda units[d]	Absent	2	Absent
IgG, %[e]	<1.18%	18	0
IgG1, %[e]	<0.006%	0.012	0
IgG2, %[e]	<0.019%	0.109	0
IgG3, %[e]	<0.019%	0	0
IgG4, %[e]	<0.003%	1.161	0
IgM, %[e]	<0.034%	0	0
IgA, %[e]	<0.014	0	0

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SUMMARY

In this thesis, we reported original studies on the pathophysiology of TTP and on severe ADAMTS13 deficiency. The findings of these studies are accompanied by a review of previously published data which collocates the research in the context of available literature. Illustrative clinical cases of the disease are also presented. The work herein reported enables the elaboration of a revised model of the pathophysiology of this rare disease. The manuscript is organized in two Sections. Section I is on congenital TTP, Section II is on acquired TTP. Below we report a summary of the Sections and Chapters of the manuscript.

Section I: congenital thrombotic thrombocytopenic purpura.

Chapter 1: this chapter reports a systematic analysis of *ADAMTS13* gene mutations and polymorphisms. This includes original analyses on the allele frequency and gene localization of variants occurring in the general population and in patients with congenital TTP. This was the first study to report a clustering of age of disease onset in patients with congenital TTP carrying the same disease-causing mutations.

Chapter 2: this chapter reports a study on the residual activity of ADAMTS13 in congenital TTP and its relationships with disease phenotype. The study is the first to (a) describe the presence of some residual activity in the majority of congenital TTP patients; (b) find that the residual activity is associated with the severity of the disease; (c) identify residual ADAMTS13 levels that discriminate patients with unfavorable prognosis; (d) describe a genotype-phenotype correlation in the disease, with mutations in the evolutionary conserved N-terminal domains of

ADAMTS13 being associated with severe disease in an allelic-dose dependent manner.

Chapter 3: a clinical case is presented. Residual ADAMTS13 activity was measured in a patient with congenital TTP during acute disease and compared with levels at remission. Activity was reduced to undetectable levels (i.e. below 0.5%) during the acute phase, whereas it was detectable and above 6% during multiple remission samples. This illustrate how onset of acute disease is characterized by a drop of residual activity, indicating that a "second condition/event" intervened to trigger acute disease in a patient with severe ADAMTS13 deficiency.

Chapter 4: this chapter summarizes emerging evidence on residual ADAMTS13 activity in ADAMTS13-deficient thrombotic thrombocytopenic purpura and discusses its implication for clinical practice and future research.

Section II: acquired thrombotic thrombocytopenic purpura

Chapter 5: the knowledge on the pathophysiology of acquired TTP is reviewed, with particular emphasis on the role of anti-ADAMTS13 autoantibodies. The feasibility of clinical trials in TTP is also discussed.

Chapter 6: this chapter describes the prevalence of clinical symptoms at TTP presentation, the disease-related laboratory measurement and the episode fatality rate of first episodes and recurrences of TTP. A milder clinical course with reduced fatality rate is observed for recurrences of TTP, suggesting that these are diagnosed and managed earlier than first episodes. The study also implies that

there is a time lag between the onset of thrombosis in TTP, the appearance of symptoms and the diagnosis.

Chapter 7: this chapter describes the study of ADAMTS13 and VWF-related laboratory measurement in acute versus remission in TTP. The goal of the study was to discover whether changes in VWF conformation, amount or multimeric pattern were associated with the onset of thrombosis in TTP. The consumption of multimers of ultralarge size of VWF was found to be a marker of acute disease severity. This Chapter shows that changes in the amount and multimeric patterns of VWF may trigger acute thrombosis in ADAMTS13 deficient individuals.

Chapter 8: this chapter describes the study of ADAMTS13- and anti-ADAMTS13 autoantibody-related measurements and their association with acute disease severity and recurrence risk in patients with acquired TTP. The study shows that both the Ig class and subclass of anti-ADAMTS13 autoantibodies are of predictive value for acute episode severity in patients with TTP.

Chapter 9: a clinical case of acquired TTP is presented. Two different thienopyridines, a class of drugs associated with the development of TTP, were safely used in a patient with a history of acquired idiopathic thrombotic thrombocytopenic purpura. This clinical case shows that drug-induced TTP develops with mechanisms that are likely distinct from those of idiopathic acquired TTP, in spite of the presence of anti-ADAMTS13 autoantibodies in both conditions.

Below we discuss the implications of the results of these studies in the understanding of the pathophysiology of TTP.

The emerging concept of residual ADAMTS13 activity and the "two-hit" paradigm for the pathophysiology of thrombotic thrombocytopenic purpura

The discovery that ADAMTS13 plasmatic activity is severely reduced in patients with TTP partially clarified the pathophysiology of the disease. Severe ADAMTS13 activity is associated with the appearance in the circulation of platelet-reactive ultralarge multimers of VWF (ULVWF).[1] These are in turn responsible for platelet aggregation and disseminated microvascular thrombosis that characterize the acute episode of TTP. However, the finding of severe deficiency alone is not sufficient to explain the clinical heterogeneity of TTP.[2, 3] TTP patients with severe deficiency of ADAMTS13 may indeed remain asymptomatic for years, without active thrombosis in spite of the presence of ULVWF in the circulation. Studies on a mouse model of severe ADAMTS13 suggested that a second event is probably required to trigger TTP in the presence of severe ADAMTS13 deficiency. *Adamts13* knock-out mice do not spontaneously develop thrombotic microangiopathy, but they do develop a TTP-like syndrome upon the injection of shiga toxin.[4] This behavior resembles that of human congenital ADAMTS13 deficiency, in which environmental triggering events such as surgery or pregnancy are often associated with the development of acute episodes.[3, 5] These conditions might precipitate the onset of acute thrombosis by triggering the release of ULVWF by activated endothelial cells. Consistently, TTP triggering conditions/events are all accompanied by endothelial activation and VWF release.

However, also the postulation of this second event needed to trigger TTP in predisposed patients incompletely explains the clinical heterogeneity of the disease. First, triggering conditions/events are not always apparent. Second, in spite of similarly reduced ADAMTS13 activity (activity below 10%) and of similarly distributed environmental exposures (as one may assume in a sufficiently large population) some patients with congenital or acquired TTP develop frequent recurrences, whereas others may remain thrombosis-free for years.[2, 3, 5] The discovery of the importance of residual ADAMTS13 activity reconciles this clinical heterogeneity with the "two-hit" paradigm. Below we present a model that incorporates recent evidence.

Environmental exposures (circadian variation, inflammation, infections, traumas, chemicals, etc.) cause ADAMTS13 activity to fluctuate around a genetically determined level. In healthy individuals, these fluctuations never reach the TTP-triggering threshold (Figure 1A). This can be defined as the ADAMTS13 activity level below which ULVWF multimers accumulate to the point that disseminated microvascular thrombosis is eventually triggered. It is unclear which is the actual TTP-triggering threshold, but it is conceivable that this is close to 0% of activity and that it is individually variable. In patients with either congenital or acquired severe deficiency of ADAMTS13, the fluctuations of ADAMTS13 activity occur at a level that is much closer to the TTP-triggering threshold. How close this is depends on the residual activity of ADAMTS13. The type and location of causal *ADAMTS13* mutations are a major determinant of residual plasmatic ADAMTS13 activity in congenital TTP patients, with mutations affecting the evolutionary conserved N-terminal domains of the protein being associated with lower

plasmatic activity. Similarly, in patients with acquired TTP the titre and affinity of anti-ADAMTS13 autoantibodies may determine the residual activity of ADAMTS13. As a result, patients with some degree of conserved activity are more resistant to ADAMTS13 activity fluctuations even under mild challenges of their fragile VWF-ADAMTS13 equilibrium. In these patients, the tiny amount of residual ADAMTS13 may be sufficient to prevent the excessive accumulation of ULVWF, until strong challenges (e.g., pregnancy) overcome the residual activity and trigger acute TTP (Figure 1B). This results in later age of disease onset and less frequent episodes in congenital patients (Figure 1B, 2 and 3). Occasionally, a strong challenge or other disease modifiers may determine early disease onset also in these patients (Figure 2). In contrast, patients with low residual activity are exposed to early disease and frequent episodes, because ADAMTS13 activity fluctuations occur at a level that is very close to the TTP-triggering threshold (Figure 1C). In congenital disease, early-onset disease and frequent recurrences are observed (Figure 2 and 3). A similar scenario occurs in patients with acquired TTP, with the complication that severe ADAMTS13 deficiency is not present since birth and may be corrected by the implementation of plasma exchange (PEX) and immunosuppressive therapy. Another contribution to the greater complexity of acquired TTP comes from the fact that antibody amount fluctuates as well, conceivably resulting in changes in the residual activity of ADAMTS13 of the patients. Nonetheless, a clear association between residual ADAMTS13 activity and risk of recurrence is observed also in acquired TTP, consistent with the proposed model (Figure 4).

The measurement of residual ADAMTS13 activity may help improve the knowledge of TTP-triggering mechanisms, which is necessary to develop adequate preventive strategies. The proposed model also has clinical implications. Monitoring of residual activity may assist in deciding on the implementation of prophylactic therapies aimed at preventing recurrences and may constitute a valuable surrogate endpoint for the preliminary evaluation of preventive strategies in clinical trials. This may improve the predictive value of ADAMTS13 beyond what currently attained by the detection of severe ADAMTS13 deficiency, ameliorating the management of this rare but life-threatening condition.

Figures

Figure 1. Panel A: fluctuations of ADAMTS13 activity over time in the healthy individual. Fluctuations are spontaneous or determined by environmental challenges. Infections, traumas, local inflammation may determine small deflections (broken arrow); pregnancy, infections, surgery or other currently unknown factors may constitute stronger challenges and determine great deflections in the activity (full arrow). Broken horizontal line close to 0 indicates the thrombotic thrombocytopenic purpura (TTP)-triggering threshold. Panel B: fluctuations of ADAMTS13 activity over time in individual with severe ADAMTS13 deficiency and relatively high (>6%) residual activity. Minor fluctuations do not result in the onset of TTP, whereas major challenges result in TTP onset. Time to onset is relatively long (horizontal line) and TTP episodes infrequent (arrow). The number on top of the arrow indicates the number of TTP episode. Panel C: fluctuations of ADAMTS13 activity over time in individual with severe ADAMTS13 deficiency and relatively low (<3%) residual activity. Minor fluctuations result in the onset of TTP. Time to onset is relatively short (horizontal line) and TTP episodes frequent (arrows). Numbers on top of the arrow indicate the number of TTP episode.

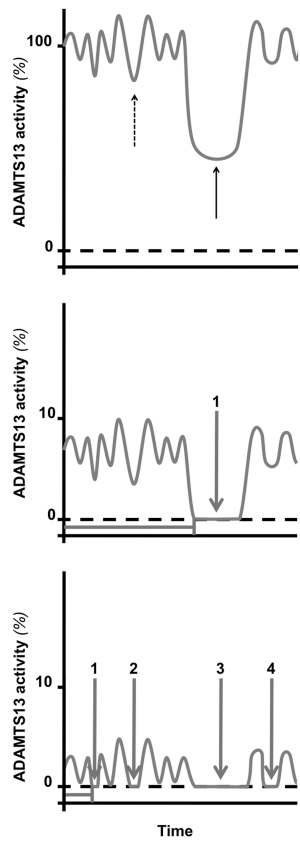


Figure 2. Relationship between age of disease onset and residual plasmatic activity of ADAMTS13 in congenital TTP patients. Areas indicate three different scenarios corresponding to the models reported above the plot. A: low residual ADAMTS13 activity and early age of onset. B: high residual activity and early age of onset. C: high residual activity and late onset.

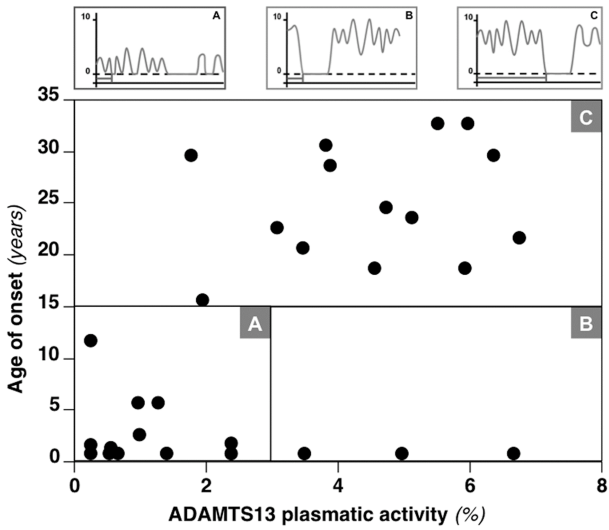


Figure 3. Relationship between frequency of TTP episodes and residual plasmatic activity of ADAMTS13 in congenital TTP patients. Areas indicate two different scenarios corresponding to the models reported above the plot. Dark grey: low residual activity and frequent recurrences. Light grey: high residual activity and infrequent recurrences.

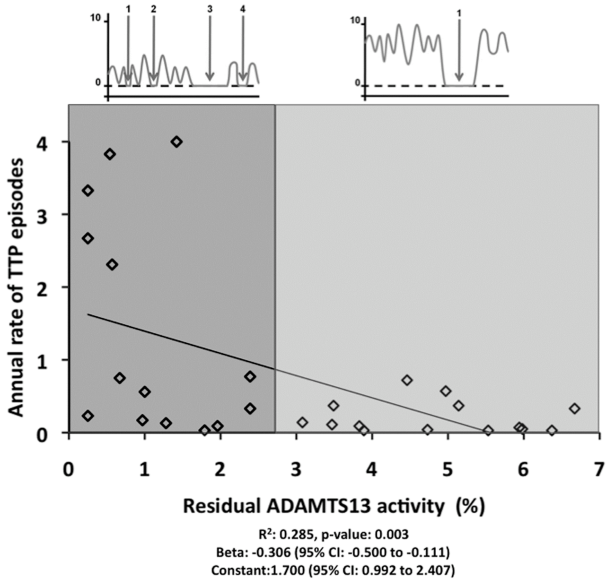
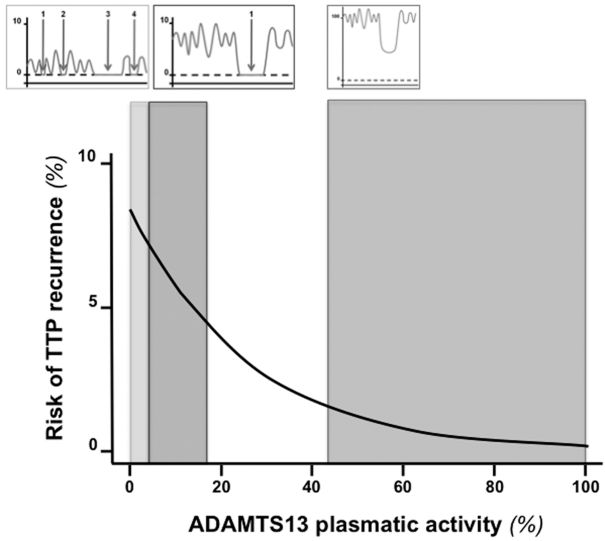


Figure 4. Relationship between risk of TTP recurrence and residual plasmatic activity of ADAMTS13 in acquired TTP patients. Increasing levels of plasmatic activity of ADAMTS13 are associated with exponentially decreasing risk of recurrence. Patients with low residual activity have the highest risk of recurrence (left), patients with high residual activity are at lower risk (middle), and patients with normal activity have virtually no risk (right). This is consistent with observations in congenital TTP patients.



Samenvatting

In dit proefschrift worden de resultaten van onderzoekingen gepresenteerd naar de pathofysiologie van Trombotische Trombocytopenische Purpura (TTP) en een ernstig tekort aan ADAMTS13. De resultaten gaan vergezeld van een overzicht van de reeds gepubliceerde literatuur en theoretische overwegingen die de nieuwe gegevens een plaats geven binnen wat reeds bekend was. Bovendien worden een aantal ziektebeschrijvingen opgevoerd ter illustratie. Het manuscript kent twee secties: sectie I betreft congenitale TTP, sectie II verworven TTP. Hieronder een korte beschrijving van de bevindingen in ieder hoofdstuk.

Sectie I: congenitale Trombotische Trombocytopenische Purpura

Hoofdstuk 1: dit hoofdstuk bevat een systematische analyse van ADAMTS13 genvariaties. Dit betreft analyses van de allelfrequentie en genlocalisatie van varianten die voorkomen in de algemene bevolking en bij patiënten met congenitale TTP. In dit hoofdstuk wordt aangetoond dat de leeftijd waarop acute TTP manifest wordt, geassocieerd is met bepaalde genvarianten.

Hoofdstuk 2: hier wordt een onderzoek gepresenteerd naar de residuele activiteit van ADAMTS13 in congenitale TTP en de samenhang met ziekteverschijnselen. Hierbij bleek dat er enige ADAMTS13 activiteit is bij de meeste patiënten met TTP, dat de mate van activiteit samenhangt met de ernst van de aandoening, en dat dit gebruikt kan worden om patiënten te identificeren met een slechte prognose. Bovendien bleek hierbij een samenhang met het genotype, waarbij mutaties in het geconserveerde N-terminale domein van ADAMTS13 leidden tot de meest ernstige vorm van de aandoening.

Hoofdstuk 3: ADAMTS13 spiegels werden gemeten bij een patiënt met congenitale TTP, zowel tijdens een acute fase als tijdens remissie. Tijdens de acute fase was de ADAMTS13 activiteit gedaald tot onder de detectiegrens (0.5% van normaal), terwijl de spiegels meer dan zes procent waren in remissie. Deze bevinding laat zien dat een acute fase wordt geïnitieerd door een sterke vermindering van de residuele activiteit, wat aangeeft dat een bijkomende oorzaak aanwezig moet zijn die de acute fase luxeert bij een patiënt met een ernstig tekort aan ADAMTS13.

Hoofdstuk 4: in dit hoofdstuk worden recente onderzoeksresultaten samengevat betreffende de rol van de residuele ADAMTS13 activiteit in TTP bij mensen met een erfelijk ADAMTS13 tekort, met de daaruit voortvloeiende implicaties voor de klinische praktijk.

Sectie II: verworven Trombotische Trombocytopenische Purpura

Hoofdstuk 5: in dit overzicht van de epidemiologie en pathofysiologie van verworven TTP ligt de nadruk op de rol van anti-ADAMTS13 auto-antistoffen. Tevens wordt besproken in hoeverre klinisch experimenteel onderzoek in TTP haalbaar is, gegeven de lage incidentie.

Hoofdstuk 6: in een groep patiënten met verworven TTP is nagegaan wat de prevalentie van de verschillende symptomen was ten tijde van de acute manifestatie van de ziekte, welke waarden in het laboratorium gevonden werden, en wat het overlijdensrisico was, zowel bij eerste TTP als bij recidieven. Hierbij bleek een milder klinisch beloop en een lagere sterfte bij recidieven van TTP dan bij een eerste acute manifestatie, hetgeen naar alle waarschijnlijkheid is terug te

voeren op snellere diagnostiek en een eerder aangevangen behandeling bij een tweede episode dan bij de eerste. Dit impliceert dat er met name bij een eerste episode enige tijd verstrijkt tussen het begin van stolselvorming bij TTP en de eerste symptomen, en het stellen van de diagnose.

Hoofdstuk 7: in het onderzoek dat beschreven wordt in dit hoofdstuk was het doel na te gaan of veranderingen in von Willebrandfactor, hetzij in de ruimtelijke vorm, de concentratie of het multimerenpatroon, samenhangen met het optreden van acute TTP bij een ernstig verworven tekort aan ADAMTS13. Het bleek dat met name de afwezigheid van ultragrote multimeren van von Willebrandfactor samenhing met de ernst van de acute ziekte. Dit onderzoek heeft daarmee aangetoond dat veranderingen in de hoeveelheid en multimeerpatronen van von Willebrandfactor acute trombose kunnen initiëren bij patiënten met een tekort aan ADAMTS13.

Hoofdstuk 8: dit hoofdstuk richt zich op metingen van verschillende auto-antistoffen tegen ADAMTS13 en hun samenhang met de ernst van acute episoden en het herhalingsrisico bij patiënten met verworven TTP. Hierbij bleek dat met name IGg klasse en subklassen van voorspellende waarde zijn voor de ernst van de acute episode.

Hoofdstuk 9: een patiënt met verworven TTP wordt besproken bij wie twee verschillende thienopyridines als geneesmiddel gebruikt waren zonder dat dit tot complicaties leidde, terwijl van deze groep geneesmiddelen bekend is dat zij TTP kan induceren. Dit suggereert dat TTP in samenhang met het gebruik van bepaalde geneesmiddelen een andere ontstaanswijze kent dan idiopathische

verworven TTP, ondanks de schijnbare overeenkomst door de aanwezigheid van antistoffen tegen ADAMTS13 bij beide vormen.

Het concept van residuele ADAMTS13 activiteit en het tweetrapsparadigma voor de pathofysiologie van trombotische trombocytopenische purpura

De aanwezigheid van een ernstig tekort aan ADAMTS13 is niet voldoende om de klinische heterogeniteit van TTP te verklaren. Volgens het 'tweetrapsparadigma' fluctueren de concentraties van ADAMTS13 rond een genetisch bepaald niveau. In gezonde individuen leiden deze fluctuaties, die het gevolg zijn van omgevingsfactoren, nooit tot bloedspiegels onder de kritische drempel voor TTP - dat is, een activiteit van ADAMTS13 waarbij ultragrote von Willebrandfactormultimeren aanleiding geven tot microvasculaire thrombose. Bij patiënten met een aangeboren of verworven tekort aan ADAMTS13 vinden deze fluctuaties plaats rond een spiegel die al dicht tegen deze drempelwaarde aanligt. Hoe dicht hangt af van de residuele ADAMTS13 activiteit. Hierdoor zijn patiënten met enige residuele ADAMTS13 activiteit bestand tegen de fluctuaties die milde stimuli opwekken, en zullen zij ondanks hun fragiele VWF-ADAMTS13 equilibrium nog geen TTP ontwikkelen. Daarentegen zijn patiënten met lage residuele activiteit zeer gevoelig voor een omgevingsstimulus (de "tweede trap") en zullen zij daardoor veelvuldig recidieven van acute episodes van TTP doormaken. Dit alles impliceert dat het bijhouden van de residuele ADAMTS13 activiteit waardevolle prognostische informatie kan opleveren bij patiënten met TTP en een ernstig tekort aan ADAMTS13.

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CURRICULUM VITAE

Luca Andrea Lotta was born in Milan (Italy) on May 16th 1983. In 2002, he obtained his high-school degree from the Giuseppe Parini Classical High-School with top marks. In the same year, he enrolled in the Medical School of the University of Milan. In 2008, he graduated *summa cum laude* in Medicine and Surgery from the University of Milan, ranking in the top 5% of the class. He then joined the Fondazione Luigi Villa research group in Milan, led by Dr Flora Peyvandi and started conducting research in the field of thrombotic and hemorrhagic diseases. In 2009, after obtaining the Medical Licence from the Board of Medical Doctors of Milan he started his PhD studies on the pathophysiology of thrombotic thrombocytopenic purpura (TTP) and on the genetic predisposition to common thrombotic diseases. In 2010-2011 he was a visiting Post-Doctoral Fellow at the Human Genome Sequencing Center, Baylor College of Medicine (Houston, Texas), where he conducted studies on the application of second-generation genome sequencing techniques in common thrombotic diseases under the supervision of Prof. Richard A Gibbs. In early 2012, he obtained a PhD degree in Methodology of Clinical Research from the University of Milan with a thesis on the genetic predisposition to common thrombotic diseases. He is currently a Research Fellow at the Fondazione IRCCS Ca' Granda – Ospedale Maggiore Policlinico, Milan.

In his three-year research experience, he was author or coauthor of several scientific publications in peer-reviewed scientific journals. He is the recipient of national and international prizes and awards, including the Bayer Hemophilia

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