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"ADAMTS13-RELATED ASSAYS IN ACQUIRED THROMBOTIC THROMBOCYTOPENIC PURPURA"

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CHAPTER I – INTRODUCTION

1. Von Willebrand Factor (VWF)

Von Willebrand factor (VWF) was first identified as an adhesive glycoprotein involved in haemostasis by Zimmerman in 1971 (1). Since then, two main haemostatic functions of VWF have been recognized. These are to mediate platelet adhesion and aggregation in the vessel wall, and to serve as a carrier and stabilizer of factor VIII in the circulation system (2). Both the size and the conformation of VWF are fundamental for its function and finely regulated by several mechanisms, including the cleavage by the metalloprotease ADAMTS13 under shear stress forces. Defects in VWF assembly, secretion and/or regulation of its activity lead to two diseases, opposite in their hemorrhagic and thrombotic nature: von Willebrand disease (VWD) and thrombotic thrombocytopenic purpura (TTP). VWD is the most common inherited human bleeding disorder and results from defects in plasma VWF quantity and/or quality (3). TTP is a thrombotic microangiopathic disorder associated with the spontaneous VWF-dependent platelet adhesion and aggregation, owing to hereditary or acquired deficiency of the VWF-cleaving protease, the metalloprotease ADAMTS13 (4).

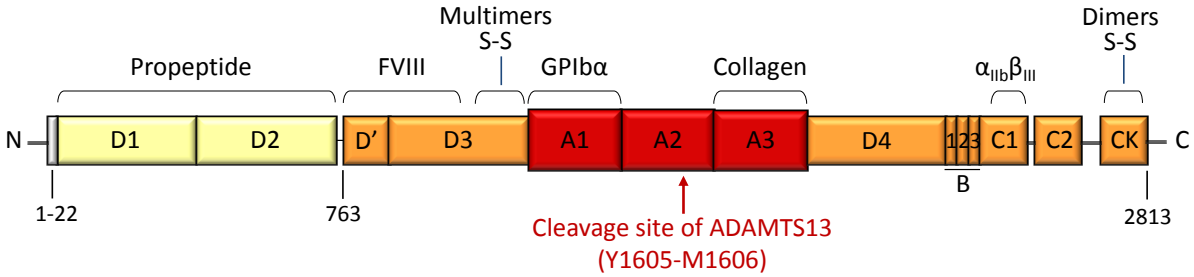

Figure 1. Scheme of von Willebrand factor monomer molecule with its functional domains. The cleavage site of ADAMTS13 and the binding sites for factor VIII (FVIII), platelet glycoprotein Ibα (GPIbα), collagen and integrin αIIbβIII are indicated.

1.1. Synthesis and secretion of VWF

VWF is an abundant plasma glycoprotein synthesized in all vascular endothelial cells and megakaryocytes as a precursor containing a signal peptide, a large propeptide and several structurally defined domains, each with specific function, arranged in the order of D1-D2-D3'-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (5, 6) (Figure 1). The D1 and D2 domains comprise the propeptide and are

cleaved during proteolytic processing to generate the mature VWF. The pre-pro-VWF is comprised of 2813 amino acids (aa) which encompass a 22-aa signal peptide, a 741-aa large propeptide and a 2050-aa mature subunit. Translocation of VWF into the endoplasmic reticulum is facilitated by the proteolytic processing of the signal peptide. In the endoplasmic reticulum, proVWF (275 kDa) forms dimers via disulfide bonds at the carboxyl terminus, in a process named "tail-to-tail" dimerization. The pro-VWF dimers are subsequently transported to the Golgi complex, where further modifications take place, such as the multimerization through an additional disulfide bond near the amino terminus of the mature subunit ("head-to-head" multimerization), the proteolytic removal of the large VWF propeptide by furin and the sulfation of N-linked oligosaccharides and O-linked glycosilation (7). The multimeric VWF produced by endothelial cells is either secreted constitutively or stored in highly ordered tubules within storage organelles named Weibel-Palade bodies, whereas VWF produced by megakaryocytes is stored in α-granules that are later partitioned into platelets (8). Secretion of stored VWF from endothelial cells occurs through both a constitutive and a regulated pathway, while platelets lack the regulatory pathway and, once stimulated, constitutively release VWF. Upon stimulation by a variety of agonists, such as thrombin, epinephrine and inflammatory cytokines, VWF is released from endothelial cells as ultra-large VWF (UL-VWF) multimers, which consist of several hundreds of VWF monomers and can reach 50000 kDa in size (7). Upon release from endothelial cells, UL-VWF multimers are kept on the cell surface through a molecular anchor whose identity remains a subject of debate, but both P-selectin and integrin αvβ3 have been implicated in *in vitro* studies on cultured endothelial cells (9, 10). The anchored UL-VWF multimers are believed to be rapidly cleaved by the metalloprotease ADAMTS13 (11), whose action appears to be critical in preventing thrombosis in the microvasculature. Without this cleavage, these UL-VWF could eventually form string-like structure that would rapidly recruit platelets and leukocytes to endothelial cells (11, 12). Under fluid shear stress, ADAMTS13 cleave UL-VWF strings at the peptide bond between Tyr1605 and Met1606 in the A2 domain to generate the range of VWF multimer sizes that normally circulate in the blood, from approximately 500 kDa to 20000 kDa (13).

1.2. VWF role in thrombus formation

Under physiological conditions, circulating platelets are recruited from the blood flow to injury sites, where they act to prevent excessive bleeding (14). To perform their haemostatic function, platelets have to bind irreversibly at site of vascular injury, opposing the blood flow adjacent to the luminal surface. VWF is essential in this process, especially at the high shear rate (above 500-1000 s^{-1}) found in small arterioles and capillaries, where it creates a bridge between platelets and the subendothelial

surface and acts to hold platelets together transiently until activation makes the platelet plug stable (15).

When a vessel membrane is injured and the sub-endothelial components are exposed, the secreted UL-VWF is in part bound locally to endothelial cells and partly to the exposed collagen through its A3 domain (Figure 2). Hydrodynamic forces cause conformational changes in VWF that expose its binding site in the A1 domain for platelet GPIbα, allowing platelets to attach and roll on the subendothelium. During this slow translocation, the continuous contact between platelets and VWF induces an activation process and another platelet surface receptor (integrin αIIbβ3 or GPIIb-IIIa) becomes available. This receptor is also able to interact with VWF with a tight binding, which arrests the rolling of platelets and stabilizes their adhesion to the damaged sub-endothelium (Figure 2A). Once platelets are immobilized to sites of vascular injury, they are activated by a range of agonists released or generated locally (such as collagen and thrombin). Platelet activation results in cytoskeleton and membrane changes/rearrangements associated with changes in platelet shape, from a discoid into a spherical, extrusion of extensive pseudopodia that help anchoring and exposure of negatively charged phospholipids for optimal binding of coagulation factors. The first layer of stable activated platelets becomes the substrate for accumulation of more platelets and thrombus growth (Figure 2B). Adhesive ligands, mainly fibrinogen and VWF, bind via activated GPIIb-IIIa on the membrane of the adherent platelets and become the substrate for the additional recruitment and attachment of incoming platelets. Also in this phase of thrombus growth, the high shear rate makes crucial the role of VWF since its multimeric conformation confers resistance to rheological forces of the circulating blood.

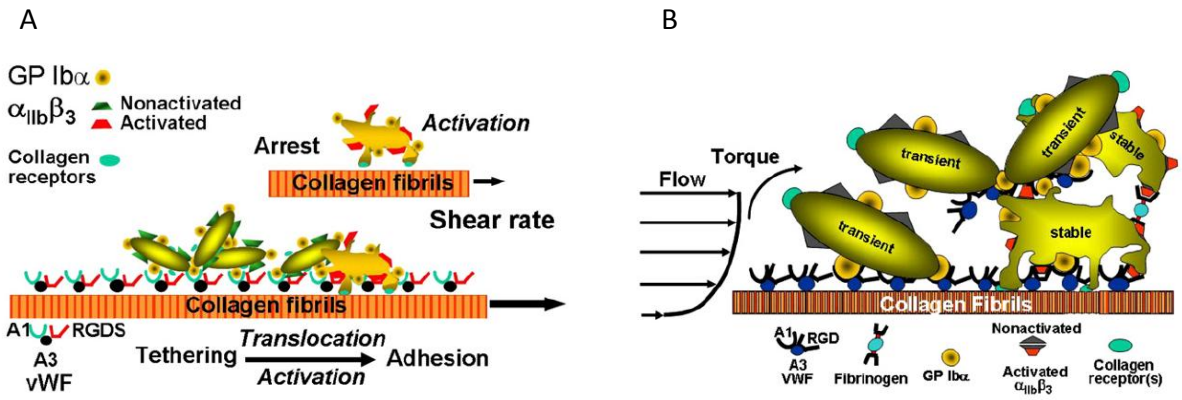

Figure 2. Schematic representation of the mechanisms of platelet adhesion (A) and aggregation (B) in flowing blood. Modified from (15).

1.3. Regulation of VWF size and conformation

The biological function of VWF depends largely on the size of its multimers. Larger multimers contain multiple sites of interaction with platelets and vessel wall components and undergo more marked conformational changes in response to high shear stress (53). A shear stress is defined as the force that is applied parallel or tangential to the face of a material or molecule, as opposed to normal stress, which is applied perpendicularly. Under normal flowing conditions, VWF multimers circulate in a "globular" form that does not bind tightly to GPIbα and is not cleaved by ADAMTS13. Fluid shear-stress changes the conformation of VWF from a globular shape to an elongated rope-like structure (16, 17) so that it binds to platelet GPIbα tightly and can be cleaved by ADAMTS13. A similar modulating effect can be induced *in vitro* by using the antibiotic ristocetin or denaturating agents as urea and guanidine-HCl (13, 18). Moreover, shear-dependent changes in VWF promote VWF self-association and multimerization, resulting in the formation of fibrillar VWF structures that are activated in binding platelets (19, 20).

The multimeric size of VWF has been shown to be regulated by a disulfide bond reducing activity associated with thrombospondin-1 (TSP-1) (21, 22). As VWF, TSP-1 is released by endothelial cells and megakaryocytes and tethers to sites of vascular damage. TSP-1 can then alter multimer size of proximal VWF molecules by splitting the disulfide-bonds that link VWF monomers at its N- and/or C-terminal ends; in addition, TSP-1 can compete with ADAMTS13 for the same VWF binding site in A2 and A3 domains (23) and has been shown to protect VWF from degradation by ADAMTS13, both in *in vitro* and *in vivo* (24). An inter-molecular disulfide bond reduction of VWF multimers has also been reported by ADAMTS13 under high shear conditions, suggesting a mechanism that might reduce self-association of plasma VWF multimers to fibrillar structures with enhanced binding to platelets (25).

Under fluid shear stress, the size of VWF is regulated by ADAMTS13 proteolysis. The unraveling of VWF due to high shear stress exposes the Tyr1605-Met1606 peptide bond in the A2 domain to ADAMTS13-mediated proteolysis. The VWF A2 domain crystal structure revealed a very rare vicinal disulphide bond between adjacent Cys1669 and Cys1670 at the C-terminus of the A2 domain, that contribute to form a "molecular plug" that directly interacts with hydrophobic residues in the core of the domain and stabilizing its fold. When VWF encounters elevated rheologic shear forces, this "molecular plug" is pulled out, allowing water molecules to enter and destabilize the hydrophobic core resulting in VWF A2 domain unfolding (26).

Under similar conditions, leukocyte proteinases including elastase, cathepsin G, matrix metalloprotease 9 and proteinase 3 are also known to clip VWF at or near the Tyr1605 - Met1606

bond (27, 28), although the *in vivo* relevance of these proteases in VWF multimers regulation is currently unknown.

Very recently, another protease belonging to the ADAM family of metalloproteases, ADAM28, has been shown to bind and cleave VWF, either in its native or unfolded conformation (29). ADAM28 is overexpressed in human cancers and associated with tumor growth and progression (30, 31) and may be involved in the degradation of VWF under pathological, rather than physiological conditions.

2. The metalloprotease ADAMTS13

As already stated, VWF platelet-tethering function depends on VWF multimeric size. The largest VWF multimers show an enhanced haemostatic potential. Consequently, the size of plasma VWF multimers needs to be physiologically regulated to prevent abnormal thrombus formation. This is finely modulated by the plasma metalloprotease ADAMTS13, which cleaves VWF between Tyr1605 and Met1606 within the A2 domain (13, 32). Mature ADAMTS13 is a 190 kDa zinc protease, which circulates in plasma as a constitutively active enzyme with a concentration of 1 μg/ml (~5 nM) and an estimated half life of two or three days (33-35).

ADAMTS13 was first identified in 1996 (13, 18) and initially called VWF-cleaving protease, due to its function of regulating VWF multimeric size. A few years later, following its cloning, purification and domain structure characterization, it was classified as the 13th member of the ADAMTS (A Disintegrin and Metalloprotease with TromboSpondin motif) metalloproteinase family (34-37), which is related to the large ADAM (A Disintegrin And Metalloprotease) family. The ADAMTS family of zinc metalloproteases contains 19 members that share the common structure of a hydrophobic signal sequence, a propeptide, a metalloprotease domain, a disintegrin-like domain, a thrombospondin type 1 (TSP1) repeat, a cysteine-rich domain, and a spacer domain (35, 36). In contrast to ADAM proteases, ADAMTSs lack EGF-like repeats and a transmembrane domain and therefore are secreted rather than membrane-bound enzymes. In addition, all ADAMTS family members possess one or more thrombospondin type 1 (TSP1) motifs (38) and variable additional C-terminal domains. The carboxyl terminus of ADAMTS13 contains seven more TSP1 repeats and two CUB domains, which are named after motifs first identified in Complement components C1r and C1s, sea urchin protein Uegf, and Bone morphogenetic protein-1 (39) (Figure 3).

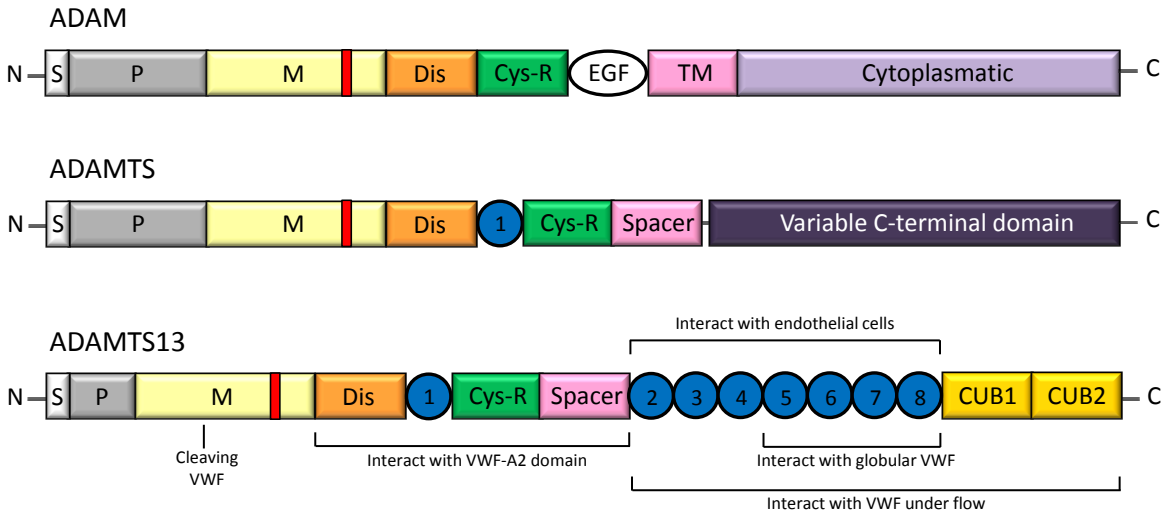

Figure 3. Schematic diagram of ADAM, ADAMTS and ADAMTS13 structure. The structural domains are indicated: signal peptide (S), propeptide (P), metalloprotease (M) (location of zinc-binding motif shown in red), disintegrin-like domain (Dis), first thrombospondin type 1 (TSP1) repeat (1), cysteine-rich domain (Cys-R), spacer domain (Spacer), the second to eighth TSP1 repeats (2-8) and two CUB domains (CUB1 and CUB2). The metalloprotease domain is the catalytic center that cleaves von Willebrand factor (VWF) The proximal carboxyl-terminal domains from Dis to Spacer interact with the A2 domain of VWF. More distal carboxyl-terminal domains interact with VWF under fluid shear stress. Interactions with globular VWF and endothelial cells have been mapped to the distal TSP1 repeats. EGF indicates epidermal growth factor-like repeat and TM indicates transmembrane domain.

2.1. ADAMTS13 gene, synthesis and secretion

The human ADAMTS13 gene is located on chromosome 9 at position 9q34. It spans 37 kb and contains 29 exons (36). ADAMTS13 mRNA is approximately 5 kb and encodes a 1427 amino acid protein, with a predicted molecular weight of 145 kDa, differing from the observed molecular mass of purified plasma ADAMTS13 (180-200 kDa) (34, 40) due to extensive glycosylation (41). ADAMTS13 is mainly synthesized in hepatic stellate cells (35, 42, 43) even though several later studies found ADAMTS13 mRNA and protein in megakaryocytes/platelets (44, 45) and endothelial cells (46). Given the large surface area of vascular beds, the endothelial cell-derived ADAMTS13 could be a major source of plasma ADAMTS13. A 2.4-kb alternative spliced product was also found in placenta and skeletal muscle (35). In endothelial cells, ADAMTS13 secretion is targeted to the vascular lumen, primarily mediated by an interaction between the CUB domains in ADAMTS13 and the detergent-resistant and cholesterol-enriched lipid rafts in the apical side of cells (47, 48). Glycosilation regulates ADAMTS13 secretion. Disrupting O-fucosylation by mutating conserved serine residues in the C-terminal TSP1 repeats is reported to reduce ADAMTS13 secretion from transfected HEK293T cells (49), and N-glycosylation in the endoplasmic reticulum is also critical for the efficient secretion of

ADAMTS13 (50). Differently from the other metalloproteinase, ADAMTS13 is constitutively secreted into blood as an active enzyme (51).

2.2. ADAMTS13 structure

The 1427 amino acids (aa) of the precursor of ADAMTS13 consists of a 33-aa signal peptide, a 41-aa propeptide, a metalloprotease domain, a Thrombospondin Type-1 (TSP1) repeat, a cystein-rich domain, a spacer domain, seven additional TSP1 repeats and two CUB domains (82) (Figure 3). In 2009, the X-ray diffraction map of the recombinant ADAMTS13 fragment comprising the disintegrin-like domain (D), the first TSP1 repeat (T), the cysteine-rich domain (C) and the spacer domain (S) (DTCS) has been reported, providing a considerable wealth of data demonstrating the relevance of noncatalytic exosites of ADAMTS13 for molecular recognition and cleavage of VWF multimers (52) (Figure 4).

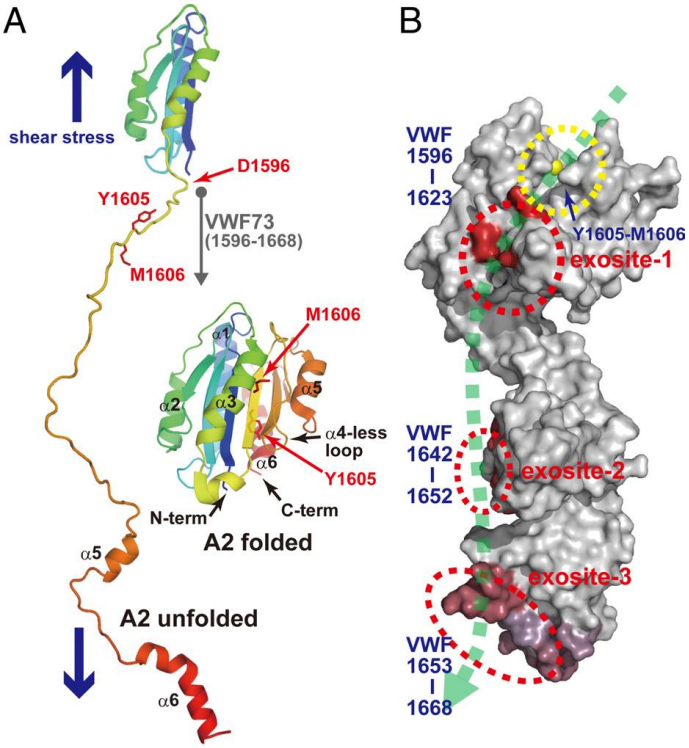

Figure 4. ADAMTS13-VWF interactions. (A) Folded and unfolded structures of the VWFA2 domain. The scissile peptide bond (Tyr1605-Met1606) is buried within the protein core under static conditions ("A2 folded"). The C-terminal region (residues 1596-1668, corresponding to the VWF73 peptide) (53) of the A2 domain must be unfolded to expose the scissile bond and the exosite-binding regions under shear-stress conditions ("A2 unfolded"). **(B) ADAMTS13-MDTCS-VWF binding model.** The molecular surface of the ADAMTS13-MDTCS model is shown in gray and the bound zinc ion is shown in yellow. Residues that mediate VWF binding are depicted and the exosites and the catalytic cleft are indicated by red and yellow dotted ellipsoids, respectively. The dotted green line represents a VWF molecule (residues 1596-1668) bound to ADAMTS-MDTCS. Structural and functional data suggest that the catalytic cleft plus exosite-1 (MD), exosite-2 (C), and exosite-3 (S) make cooperative, modular contacts with 3 discrete segments of the VWF A2 domain (see text for discussion). Adapted from (52).

2.2.1. Propeptide domain (residues 34-74)

The ADAMTS13 propeptide is shorter (41 residues) compared with other ADAMTS family propeptides (about 200 aa). Like other metalloproteases, ADAMTS13 propeptide presents a typical proprotein processing site (RQRR), which has been shown to be a furin cleavage site (35). ADAMTS13 propeptide seems to be dispensable for normal folding, secretion or enzymatic activity, since deletion of the propeptide does not affect secretion of active enzyme (54). Moreover, it does not confer enzyme latency, as it has been shown that mutation in furin consensus recognition site leads to secretion of an active pro-ADAMTS13 (54). Finally, detection of anti-propeptide antibodies in some patients with TTP suggests that not all plasma ADAMTS13 has this sequence removed (55).

2.2.2. Metalloprotease domain (residues 75-289)

No detailed knowledge of the atomic structure of the metalloprotease domain of ADAMTS13 is available, but, thanks to the high degree of similarity with other ADAMTS proteinases, especially ADAMTS4 and 5, a three-dimensional model of ADAMTS13 metalloprotease domain was built by homology modeling techniques (56). The catalytic domain of ADAMTS13 contains the highly conserved zinc-binding sequence, in which the catalytic Zn^{2+} ion is coordinated by three histidine residues, "H_{224}EXXHXXGXXHD_{235}", where "X" represents any amino acid residue and the aspartic acid distinguishes the ADAMs and ADAMTSs from other metalloproteinases. The glutamate following the first zinc-binding histidine has a catalytic role (57), polarizing through hydrogen bonding a water molecule, which is stabilized by coordination with the Zn^{2+} ion and it is responsible for the nucleophilic attack on the carbonyl of the substrate scissile peptide bond (58). The arrangement of the active site is facilitated by the conserved glycine 231, which permits a tight hairpin loop and enables the third histidine to occupy its correct position (59, 60). As in all metalloproteinases, the zinc-binding sequence is followed a short distance from the C-terminal end by a conserved methionine residue, that constitutes the so-called ''Met-turn'', a tight turn arranged as a right-handed screw that seems to serve an important function in the structure of the active site (59). Recently, deletion and mutagenesis studies by Xiang *et al.* and de Groot *et al.* showed that Leu151 and Val195, and Asp252-Pro256, are critical for VWF cleavage, as they probably shape the docking sites of VWF Tyr1605 and Met1606, respectively, contributing to scissile bond specificity (61, 62). Moreover, in the same mutagenesis study, residues Leu198, Leu232 and Leu274 have been identified as elements that together may make up another crucial subsite accommodating VWF Leu1603 which has an essential role in proteolysis of the scissile bond (62).

In addition to the Zn^{2+} ion in the active site, Ca^{2+} ions are also required for enzyme function. ADAMTS13 activity is cooperatively enhanced by the two ions and it is likely that ADAMTS13

circulating in normal plasma is fully saturated by both ions (63). The importance of zinc and calcium ions explains why ADAMTS13 function is completed inhibited by metal ions chelators, such as ethylenediaminetetraacetic acid (EDTA) (13). Two putative calcium binding sites (CaBSs), CaBS-I and CaBS-II can be identified in ADAMTS13. Through homology modeling techniques, Pozzi *et al.* recently showed that CaBS-I is formed by highly conserved aminoacids (Asp165, Asp173, Glu83) and by a connector loop consisting of Cys281 and Asp284 and can accommodate two Ca^{2+} ions (56). Alternatively, CaBS-II binds a single Ca^{2+} ion, is positioned opposite to CaBS-I, adjacent to the active site, and is formed by Asp182, Leu183, Arg190, Val192 and Glu212 (56). This model is consistent with biochemical and mutagenesis studies showing that ADAMTS13 contains a low-affinity CaBS-I (EC_{50} = 880 μM), which is occupied by two Ca^{2+} ions and mainly exerts a structural role, and a high-affinity site ($K_{D(app)}$ = 70 μM), CaBS-II, which plays a functional role, as mutations in the involved residues increased the $K_{D(app)}$ by more than 10 fold (64).

Different studies using C-terminal truncations of recombinant ADAMTS13 have shown that the metalloprotease domain alone was not able to cleave plasma VWF (65, 66) or a short A2 domain peptide, VWF73 (residues 1596-1668) (67). However, it was found that the metalloprotease domain alone could cleave the substrate after long incubation times (16-24 hours), but at a different site than the predicted Tyr1605-Met1606 (67), suggesting that the other downstream domains are responsible for determining substrate specificity of cleavage.

2.2.3. Disintegrin-like domain (residues 290-385)

Despite its name and high similarity with snake venom disintegrins, this domain does not actually have an RGD integrin binding sequence and it is not known to bind integrins. On the contrary, the crystal structure of the metalloprotease-disintegrin-like domains from other ADAMTS proteinases revealed a greater structural homology to cysteine-rich domains (52, 68, 69). Truncated ADAMTS13 variants suggested the importance of the disintegrin-like domain for both enzyme activity and specificity (70). Moreover, targeted mutagenesis of nonconserved regions in this domain identified three residues, Arg349, Leu350 and Val352, important for ADAMTS13 proteolytic function (71). In particular, the authors proposed a model in which Arg349 and Leu350 in ADAMTS13 interact with Asp1614 and Ala1612 in VWF, assisting in positioning the Tyr1605-Met1606 bond into the active site cleft of ADAMTS13 (71). Recent structural data confirmed this model, individuating Arg349 as part of exosite-1, one of the three exosites whose interaction with VWF A2 domain is pivotal for its proteolytic processing by ADAMTS13 (52) (Figure 4).

2.2.4. Thrombospondin Type 1-Like (TSP1) Repeats

ADAMTS13 has eight TSP1 repeats: the first one (residues 386-439) is located between the disintegrin-like domain and the Cys-rich domain; the other seven (residues 686-1131) are located between the spacer domain and the two C-terminal CUB domains. By analogy to thrombospondin 1 and 2, TSP1 repeats may play a role in binding glycosaminoclycans and/or CD36, localizing ADAMTS13 to the surface of endothelial cells (72). Vomund and Majerus showed that ADAMTS13 binds to endothelial cells in a specific manner (K_D = 58 nM) and that the binding requires the C-terminal TSP1 repeats of the protease (73). Moreover, Davis *et al.* reported that recombinant human ADAMTS13 binds to both recombinant human CD36 and CD36 expressed on platelets membrane *in vitro* (74). Taken together, these data suggest that the interaction between ADAMTS13 TSP1 repeats and CD36, or some other still unidentified receptor on endothelial cells and platelets, might localize ADAMTS13 at the endothelium surface, where cleavage of VWF multimers mainly occurs. Furthermore, supporting this hypothesis, anti-CD36 antibodies have been frequently found in patients with acquired TTP (75, 76). Finally, two groups independently showed that the C-terminal TSP1 repeats, TSP5-8, mediate the binding to the C-terminal domains of VWF in its quiescent/globular conformation (77, 78) (see section 2.3).

2.2.5. Cys-rich domain (residues 440-555)

This domain is a well-conserved, rich in cysteines domain, with 10 cysteine residues. The importance of this domain is highlighted by the identification of a polymorphism, Pro475Ser, in the Japanese population, that alters ADAMTS13 activity but not its secretion (41). Furthermore, a mutation found in a patient with inherited ADAMTS13 deficiency (Gln449stop) causing a premature stop after the first TSP1 repeat resulted in a truncated protein which was secreted normally, but possessed very low enzymatic activity (41). According to the DTCS crystal structure, the cys-rich domain contains a variable loop that might create another exosite (exosite-2) and mutations in the involved residues resulted in very low enzymatic activity (52) (Figure 4).

2.2.6. Spacer domain (residues 556-685)

This domain has been shown to be essential for efficient ADAMTS13 VWF-cleaving activity (65, 66). ADAMTS13 fragments truncated after the metalloprotease domain, the disintegrin domain, the first TSP1 repeat or the Cys-rich domain were not able to cleave full-length VWF, whereas addition of the spacer region restored protease activity (65, 66). Moreover, ADAMTS13 truncated after the first TSR bound full-length VWF with a K_D of 206 nM whereas ADAMTS13 truncated after the spacer domain bound to it with a K_D of 23 nM. This is comparable to the KD of 14 nM found for full-length

ADAMTS13 interaction with full-length VWF (54). Similar results were obtained testing the binding to the VWF peptide, VWF73: ADAMTS13 truncated after the spacer domain bound to VWF73 with a K_D of 7 nM, comparable to that obtained using full-length ADAMTS13 (K_D 4,6 nM), whereas further truncation drastically reduced the binding. Other studies reported that the spacer domain is able to bind a complementary site that includes residues Glu1660–Arg1668 at the carboxyl terminus of the A2 domain of VWF (52, 79, 80). Deleting the spacer domain from ADAMTS13 or deleting the carboxyl-terminal end of the A2 domain did indeed reduce the rate of cleavage by 20-fold (79). According to the DTCS crystal structure, the spacer domain contains a VWF-binding exosite (exosite-3), comprising residues Tyr659-Tyr665 (52) (Figure 4). Substitution of Arg660, Tyr661 and Tyr665 resulted in a 12-fold reduction in catalytic efficiency of peptide VWF proteolysis and a 25-fold reduction in its binding (81). Very recently, a region between residues Glu^{634} and Arg^{639} has been identified as a novel exosite necessary for recognition and cleavage of VWF and termed exosite 4 (82). Further insight into the role of the spacer domain derives from the characterization of anti-ADAMTS13 antibodies that arise in patients with acquired TTP. Indeed, nearly all adult idiopathic TTP patients with severely deficient plasma ADAMTS13 activity harbor polyclonal anti-ADAMTS13 antibodies that bind the Cys-rich and spacer domains, particularly the spacer domain of ADAMTS13 (65, 83-86). Recent studies have shown that exosite-3 (Tyr659-Tyr665) and several other adjacent amino acid residues (Arg568 and Phe592) in the spacer domain comprise a major antigenic epitope for anti-ADAMTS13 autoantibodies in idiopathic TTP (81, 87) (see section 4.3). If several modifications of these amino acids were found to impair VWF binding and cleaving by ADAMTS13 (81, 88, 89), a combination of amino acidic substitutions at positions 568, 592, 660 and 661 (Arg660Lys/Phe592Tyr/Arg568Lys/Tyr661Phe) generated an ADAMTS13 variant with dramatically enhanced specific activity toward VWF compared with wild type (89). These substitutions appear to increase hydrophobic interactions between exosite-3 in the spacer domain and VWF A2 domain, confirming once more the importance of the former in ADAMTS13-mediated proteolytic processing of VWF.

2.2.7. CUB domains (residues 1192-1408)

ADAMTS13 is the only member of the ADAMTS family to contain CUB domains, of which there are two located at the C-terminus. CUB domains are present on proteins known to be important for developmental regulation (39). CUB domains appear to determine ADAMTS13 secretion in endothelial cells, as their deletion abolishes the apical secretion of the enzyme in cell culture (47). Moreover, Zhou and colleagues have showed that naturally occurring mutations in the CUB-1 domain result in normal rates of synthesis, but lower rates of secretion to the luminal surface of transfected Hela cells (48). In a more recent study, the same authors demonstrated that cysteine residues in

CUB-1 domain are critical for ADAMTS13 secretion and stability, as conversion of cysteines 1192, 1213, 1236 and 1254 into serine residues reduced the secretion of the mutants to the conditioned medium, but not to extracellular matrix and resulted in a moderate increase in proteolytic degradation (90). To date, the role of ADAMTS13 CUB domains in ADAMTS13 function is controversial. Four different studies using C-terminal truncations of ADAMTS13 have shown that these domains are not required for VWF-cleaving activity under static (65-67) and flow (91) conditions. However, the CUB domains, along with the C-terminal TSP, have been claimed to be important for optimal cleavage activity of ADAMTS13 under flow conditions (92), against full-length murine VWF (93), for cleaving platelet decorated strings (94) and modulating thrombus formation (95) *in vivo*. Moreover, competitive binding studies under flow conditions have found that recombinant CUB1 domain inhibited VWF proteolysis (96). Autoantibodies against the CUB domains have been found in about one third of patients with acquired TTP, but it is unknown whether they compromise ADAMTS13 function or lead to ADAMTS13 depletion by the formation of antibody-antigen complexes (55, 86).

2.3. Recognition and cleavage of VWF by ADAMTS13

A fine balance between UL-VWF multimers and proteolysis is crucial to generate the VWF multimers forms that normally circulate in plasma and to maintain haemostasis under pathophysiological circumstances. Recognition of VWF by ADAMTS13 is complex, yet highly specific and involves multiple interactions between distinct domains of both ADAMTS13 and VWF, with some of the interaction sites on VWF only becoming exposed during shear. The fact that ADAMTS13, unlike the vast majority of haemostatic proteases, is a constitutively active secreted enzyme without any known specific inhibitor, and that VWF is its only known substrate, implies that VWF proteolysis is primarily regulated by the availability of the scissile bond of VWF and by the high specificity of the VWF-ADAMTS13 interaction. This lead to the intriguing concept that the regulation of VWF function (the substrate) by ADAMTS13 (the enzyme) is in turn controlled by the substrate itself and explain why so many efforts have been done to unravel the molecular mechanisms underlying the modulation of this central interaction over the past decade.

2.3.1. ADAMTS13 binding to globular VWF

As described above, much of the studies performed to investigate the molecular determinants of the interaction between VWF and ADAMTS13 exploited the generation of ADAMTS13 deletion mutants progressively truncated from the C-terminus. Despite the limitations of potential conformational changes induced by domain deletion, these mutants have provided valuable initial tools for exploring ADAMTS13 domain function. Early results established the importance of several exosites in the proximal C-terminal domains of ADAMTS13, in particular in the spacer domain, while providing inconsistent evidence for a functional role for the C-terminal TSRs and CUB domains. To address this issue, Zanardelli and colleagues prepared deletion mutants of both VWF and of ADAMTS13 and performed binding and activity studies under both static and flow conditions (77). These experiments showed that full-length ADAMTS13 bind with a K_D of 86 nM to VWF in its globular conformation and that this binding is mediated in part by the VWF D4 domain and the TSP5-8 and/or the CUB domains of ADAMTS13. Similar conclusions were drawn by Feys and colleagues using immunoprecipitation of VWF-ADAMTS13 complexes in solution (78). The authors determined a K_D of 79 nM and mapped the interaction in the ADAMTS13 TSP2-8 repeats. Both studies demonstrated the specific binding of ADAMTS13 to globular VWF and that, in the absence of shear-induced unfolding of VWF, this interaction is nonproductive in terms of VWF proteolysis. Importantly, Feys *et al.* demonstrated that about 3% of ADAMTS13 circulates bound to VWF in normal plasma. Even though this represents a small percentage of plasma ADAMTS13, this pool may be particularly effective at colocalizing VWF and ADAMTS13 to the site of vessel damage. Evidence that this may have a functional physiological

role was provided by Banno and collegues, who examined mice with an *Adamts13* gene that expresses a truncated form of the enzyme (ADAMTS13^{S}), lacking the TSP7-8 and CUB domains (95). Plasma VWF multimers from *Adamts13*$^{S/S}$ did not differ from those of *Adamts13*$^{+/+}$ mice, suggesting that the TSP7-8 and the CUB domains may not be necessary for modulating normal plasma VWF size. However, mice expressing the truncated ADAMTS13 were more thrombogenic in experimental thrombus formation under high shear conditions, suggesting that the loss of the TSP7-8 and CUB domains impaired the ability of ADAMTS13 to regulate VWF-dependent development of the platelet plug.

2.3.2. VWF proteolysis by ADAMTS13: a "molecular" zipper mode of action

As described above, VWF circulates in its globular/ADAMTS13-resistant form until it encounters a site of vessel damage that has resulted in the exposure of subendothelial collagen to the flowing blood. A binding site for ADAMTS13 within the D4-CK domains of VWF is nevertheless constitutively exposed, and a small proportion of circulating ADAMTS13 can reversibly associate with it through its TSP5-CUB domains. Under high shear stress conditions (when tethered to the site of vessel injury by its A3 domain, during passage through the microvasculature or when secreted), VWF undergoes a conformational change from its globular to its string-like conformation, exposing additional binding sites for exosites present in the proximal C-terminal domains of ADAMTS13 that collectively contribute to bring the scissile bond of VWF over the active site of the metalloprotease. First, an exosite comprising residues Arg660-Tyr665, Arg 568 and Phe 592 of ADAMTS13 spacer domain recognizes VWF residues Glu1660-Arg1668, revealed when the "molecular plug" is pulled out from the hydrophobic core of the A2 domain. Then, a variable loop in the Cys-rich domain of ADAMTS13 interact with residues 1642-1652 of VWF, followed by the binding of Arg349 in ADAMTS13 disintegrin-like domain to Asp1614 of VWF. Finally, essential contacts are made between the subsites in the metalloprotease domain of ADAMTS13 (involving Leu198, Leu232, Leu274, Leu151, Val195 and Asp252-Pro256) and VWF residues adjacent and composing the scissile bond (Leu1603, Tyr1605 and Met1606). All together, these interactions, that can be envisaged to represent a "molecular zipper", position the scissile bond into the ADAMTS13 active site pocket, where cleavage takes place (97).

2.3.3. Modulators of ADAMTS13 activity

The unique requirement of shear forces and the multiple interactions of remote exosites on both the enzyme and the substrate finely regulates ADAMTS13 activity. These mechanisms appear to be sufficient to impede uncontrolled VWF proteolysis, as no physiological specific inhibitor of ADAMTS13 activity is known. However, its activity might be negatively modulated by other factors, such as chloride ions, some coagulation proteases, haemolysis products and inflammatory cytokines.

De Cristofaro *et al.* showed that binding of chloride ions at physiological concentrations to the A1 domain of VWF induce conformational transitions in the A1-A2 VWF domains, which make the scissile bond unavailable to proteolysis by ADAMTS13 and that chloride affinity drastically decreased in the presence of ristocetin (98). The authors thus suggested that GPIbα binding could reverse the inhibitory effect of chloride ions, whose concentration gradient across the cell membrane may represent a physiological strategy to regulate ADAMTS13 enzymatic activity. Free haemoglobin (99, 100) and interleukin-6 (101) have been shown to inhibit ADAMTS13 function, but their physiological significance is unclear and they may have a role only under pathophysiological circumstances. Thrombin, factor Xa, plasmin (102, 103) and leukocyte proteases (28) can cleave and inactivate ADAMTS13 through defined cleavage inactivation sites, and therefore might regulate ADAMTS13 activity at sites of thrombus formation. However, as evidence of ADAMTS13 proteolysis *in vivo* has only been observed in patients with severe sepsis (104) or in a very rare case of 2-antiplasmin deficiency (105), it is probable that this remains a pathologic phenomenon rather than a normal control mechanism.

On the other hand, ADAMTS13 proteolytic activity have been shown to be positively regulated by platelets (106, 107) and factor VIII (107-109), suggesting that the binding of platelet GPIbα and factor VIII to the specific regions of VWF may accelerate the conformational transitions under fluid shear stress, thereby allowing ADAMTS13 to access the scissile bond and reduce VWF multimers size.

3. Thrombotic thrombocytopenic purpura (TTP)

3.1. History of TTP

Thrombotic thrombocytopenic purpura (TTP) is a rare thrombotic microangiopathy characterized by thrombocytopenia and haemolytic anemia due to disseminated microvascular thrombosis. TTP was first described in 1924 by Eli Moschcowitz at the Mount Sinai Hospital in New York City in a previously healthy 16-year-old girl who died after an acute illness presenting anemia, purpura, microscopic haematuria, and, at autopsy, disseminated microvascular thrombi (110). A similar disorder was reported in 1960 by Schulman *et al.* (111) and later on by Upshaw *et al.* (112). In 1966, the evaluation of 271 cases by Amorosi and Ultamann permitted this mysterious disease to be clinically established as a symptomatic pentad of signs and symptoms: anemia, thrombocytopenia, fever, hemiparesis and haematuria (113). It was postulated that massive thrombus formation in the terminal circulation was due to the presence of “poisonous substances” that aggregated platelets intravascularly but the putative toxic agent had remained unknown for a long time. A major breakthrough in understanding the pathogenesis of TTP was provided by Joel Moake and colleagues in 1982 when they observed that the plasma of a patient affected with TTP presented highly thrombogenic forms of the multimeric glycoprotein von Willebrand factor (VWF), a major adhesive protein contained in endothelial cells, platelets and plasma (114). The hypothesis that a deficiency of a cleaving protease was responsible for the presence of these particularly high molecular weight (ultra-large) VWF multimers was postulated by Moake, but it was not confirmed until 1996, when Furlan and Tsai independently isolated from human plasma a metal ion-dependent protease which cleaves the peptide bond between the tyrosine at position 1605 and the methionine at position 1606 in the central A2 domain of VWF (115, 116). Furthermore, the VWF-cleaving protease was found to be deficient in patients affected with TTP (115-117). In 2001, this protease was identified by Zheng and colleagues as a new (the thirteenth) member of the ADAMTS (a Disintegrin And Metalloprotease with Thrombospondins 1 repeats) family of metalloproteases and was, therefore, designated ADAMTS13 (35). ADAMTS13 regulates the size of normally circulating VWF multimers by cleaving ultra-large multimers as soon as they are secreted from endothelial cells into plasma, thereby preventing their circulation in plasma and spontaneous platelet aggregation and thrombus formation (118).

3.2. Epidemiology, clinical manifestations, therapy and pathofisiology of TTP

TTP is a rare life-threatening disorder with an estimated incidence of 2-10 cases per million per year (119). Demographic studies report a higher incidence in women (two-third of cases) and in african-american people (nine-tenth of cases in the USA) (120). Mortality was very high (80%-90%), but has dramatically decreased to 10-20% after the introduction of plasma exchange, which is the treatment of choice of acute TTP episodes (121-124). In most cases TTP occurs as a single, acute episode, but there are chronic recurrent forms developed in up to 40% of patients, which usually have a genetic basis or are associated to the persistence of autoantibodies (118).

TTP is characterized by the widespread formation in the microcirculation of platelet thrombi, associated with abundant intra-thrombus VWF. Microthrombi are found in several organs, mainly represented by brain, heart, kidney, lung and pancreas (118). TTP was originally described as a pentad of thrombocytopenia, microangiopathic haemolytic anemia, fluctuating neurological signs, renal impairment and fever (113). However, later on it was observed how neurologic signs, fever and impaired renal function were not consistently present in patients affected with this syndrome, especially in the early phase of the disease (122, 125). These observations lead to the currently accepted definition of TTP, whose diagnosis requires the association of microangiopathic haemolytic anaemia with fragmented erythrocytes and thrombocytopenia, with or without renal failure or neurologic abnormalities, and without alternative causes (122, 126, 127). Thrombocytopenia results from consumption of platelets in platelet-rich thrombi; haemolysis is the consequence of mechanical red blood cells fragmentation during flow through partially occluded high-shear small vessels.

There are two different forms of TTP: congenital TTP, which is caused by mutations in the ADAMTS13 gene (36), and acquired TTP, which is associated with the development of autoantibodies against ADAMTS13 (116). In the absence of ADAMTS13, ultra large VWF multimers secreted by activated endothelial cells are not cleaved properly and cause spontaneous platelet aggregates in conditions of high shear stress, leading to massive microvascular thrombosis and the above mentioned clinical manifestations characteristic of TTP (Figure 5).

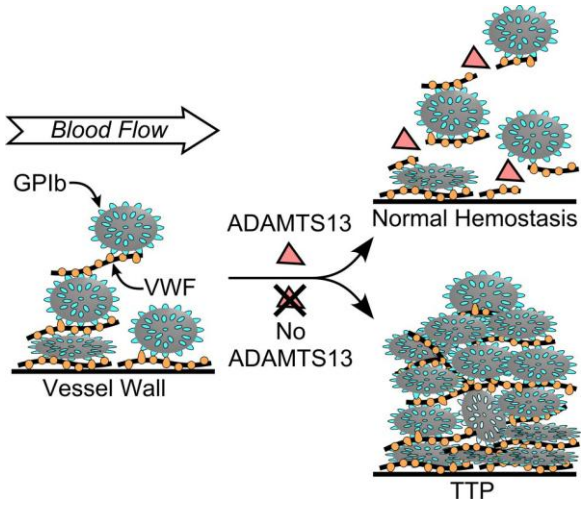

Figure 5. Schematic representation of TTP pathogenesis. Multimeric VWF adheres to endothelial cells or to connective tissue exposed in the vessel wall. Platelets adhere to VWF through platelet membrane GPIb. In flowing blood, VWF in the platelet-rich thrombus is stretched and cleaved by ADAMTS13, limiting thrombus growth. In the absence of ADAMTS13, VWF-dependent platelet accumulation continues, eventually causing microvascular thrombosis and TTP. Adapted from (128).

3.3. Congenital TTP

Congenital TTP, also known as Upshaw-Schulman syndrome, is very rare (1 in 1 million) and represents 5% of all TTP cases (119). It is caused by homozygous or double heterozygous mutations in the ADAMTS13 gene, that affect protein secretion or function. Congenital TTP is inherited as an autosomal recessive condition and is often clinically manifested at birth or during childhood, even though cases where the disease became manifest in adulthood have been described (129, 130). Also the clinical course of the disease is very wide: some patients experience asymptomatic episodes of thrombocytopenia and anemia, while others develop multiorgan failure; some patients have multiple recurrent episodes and necessitate prophylactic plasma infusions, whereas others may only experience a single episode or remain disease-free lifelong (130-132). To date, more than 100 mutations have been documented in patients with familial TTP (130, 133, 134). In a recent review of the literature, an analysis of the location of the mutations on the ADAMTS13 gene revealed no evident mutation hot spots (130).

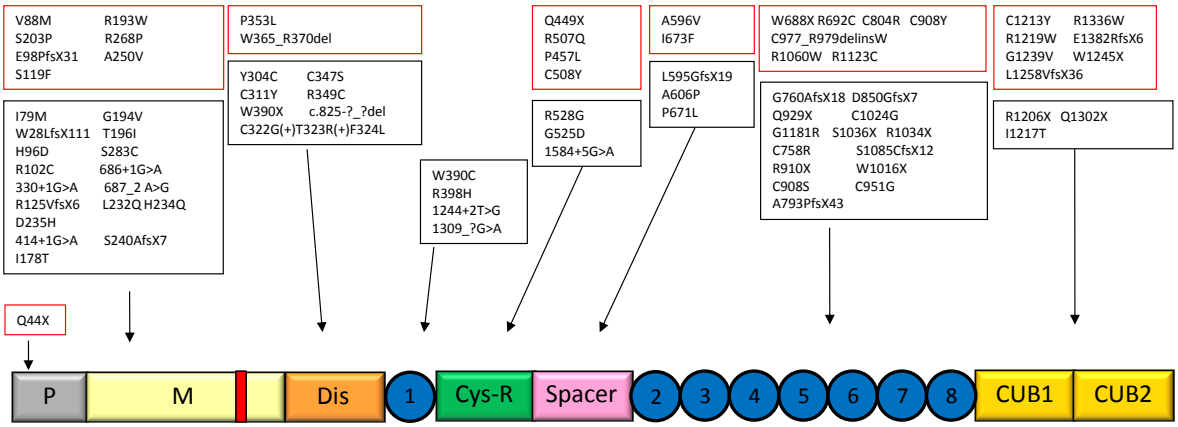

Figure 6. Distribution of ADAMTS13 missense mutations on the protein. Red boxes indicate ADAMTS13 mutations expressed *in vitro* (130).

However, 75% of the missense mutations are found in the N-terminal part of the molecule, up to the spacer domain (Figure 6). Most (60%) of the identified mutations are missense; the remaining 40% (nonsense, frameshift, splicing mutations) generates truncated forms of the protein. The majority of the mutations, among those analyzed by *in vitro* expression studies (about 20-30% of the reported gene mutations), cause reduced ADAMTS13 secretion (130). Lotta *et al.* showed that patients carrying the same ADAMTS13 gene mutations develop their first disease episode at a similar age, providing the first evidence of genotype-phenotype correlation in congenital TTP (130). In a subsequent study where ADAMTS13 activity was measured by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry in 29 congenital TTP patients, Lotta and colleagues demonstrated that different ADAMTS13 gene mutations influence the severity of clinical phenotype by determining different levels of residual plasmatic activity of ADAMTS13 and that mutations affecting the N-terminal domains of the protein are associated with more severe clinical phenotype (134).

Patients with congenital TTP usually respond well to plasma infusion. Despite that ADAMTS13 has a half-life of only 2-3 days (33), a periodic plasma infusion every 10-20 days is sufficient to supply enough ADAMTS13 to achieve normal platelet and haemoglobin level (127). Virally-inactivated intermediate purity factor VIII concentrate containing ADAMTS13 have also been used for the treatment of congenital TTP (135). Plasma-derived or recombinant concentrates of human ADAMTS13 are not yet available. However, a recombinant ADAMTS13 product for potential prophylaxis and treatment of TTP is currently under development (136-138).

3.4. Acquired TTP

Two types of acquired TTP can be distinguished: idiopathic TTP and secondary TTP. Autoimmune idiopathic usually occurs in adults between 20 and 60 years old, in the absence of any obvious triggering event (139). Secondary TTP is associated with other physiological or pathological conditions, such as pregnancy, autoimmune diseases, infections, drugs, tumors, bone marrow transplantation, pancreatitis (140-146). These various conditions may directly injure endothelial cells with release of ultra-large VWF multimers in amounts exceeding the system ability to degrade them, despite the presence of normal or slightly reduced ADAMTS13 activity. In contrast to congenital TTP, the therapy of choice for acquired TTP consists of plasma exchange, required to remove the inhibiting autoantibodies, in association with immunosuppressive therapy (118). Steroids are used as immunosuppression to attain complete remission and, more recently, the use of rituximab, a monoclonal antibody directed against CD20 antigen on B cells, has also been proved highly efficacious in treating acquired TTP (118, 147). Recent therapeutical approaches aim at developing inhibitors of the binding between GPIb and ultra-large VWF multimers, preventing intravascular platelet aggregation in the microcirculation (148-150). Finally, a recombinant ADAMTS13 preparation has been shown to restore ADAMTS13 activity in plasma of TTP patients with ADAMTS13 inhibitors *in vitro*, suggesting a potential use of recombinant ADAMTS13 in the treatment of acquired TTP patients as well (151).

Autoimmune TTP is caused by the severe deficiency of ADAMTS13 owing to autoantibodies that inhibit ADAMTS13 activity and/or bind the protease to accelerate its clearance from plasma through opsonization and/or yet unclear mechanisms (116, 152-154). Anti-ADAMTS13 antibodies were identified for the first time in 1998 in patients with acute acquired TTP (116, 117). Since then, autoantibodies inhibiting ADAMTS13 activity have been detected in most patients with acquired TTP (55, 116, 152, 153, 155-160) and much attention has been paid to the biochemical and functional characterization of these antibodies.

4. Anti-ADAMTS13 antibodies in acquired TTP

4.1. Neutralizing activity of anti-ADAMTS13 autoantibodies

In 1998, Tsai and Lian studied the activity of VWF-cleaving protease in plasma from patients with acquired TTP, patients with other autoimmune or blood disorders and in normal subjects. They found that all the samples obtained from patients during an acute episode of TTP had severe deficiency of the VWF-cleaving protease and that 67% of these presented inhibitory activity against the protease in mixing studies of heat-inactivated patient plasma and pooled normal plasma (116). They further characterized the source of the inhibitory activity and showed that it was due to antibodies, precisely to immunoglobulin (Ig) belonging to the G class. The same results were obtained by Furlan *et al.*, who reported a case of a patient with recurrent episodes of TTP who was finally treated with splenectomy and corticosteroid treatment, resulting in disappearance of the autoantibody and normalization of the protease activity and of the platelet count (117). In 2003, Scheiflinger and colleagues reported data on a patient with confirmed TTP who had severely reduced ADAMTS13 activity but surprisingly showed no ADAMTS13 inhibition *in vitro* (153). The authors developed an enzyme-linked immunosorbent assay (ELISA) using immobilized recombinant ADAMTS13 and they found high titers of IgM and IgG antibodies binding to ADAMTS13, without neutralizing its protease activity. Since then, anti-ADAMTS13 autoantibodies have been distinguished in two classes: neutralizing antibodies, or inhibitors, and non-neutralizing antibodies. If it is easy to assign particular antibodies to one type or another based on their functional activity in classical mixing studies, less straightforward is to explain the molecular mechanisms behind it (see section 4.3). Recent advances in our knowledge of the molecular interactions between ADAMTS13 and VWF and ADAMTS13 and anti-ADAMTS13 antibodies are making increasingly clear how the latters may directly inhibit ADAMTS13 proteolytic activity. On the other hand, only speculations have been made on the mode of action of non-neutralizing antibodies. It has been suggested that they might enhance ADAMTS13 clearance from the circulation through opsonization and/or yet unclear mechanisms or that they could disturb the interaction of ADAMTS13 with physiologic binding partners, such as endothelial cells (73, 153). Another possible explanation is that non-neutralizing antibodies bind to regions of the protein that are not necessary for exerting protease activity under the conditions of *in vitro* assays. Finally, weak inhibitory antibodies might be missed because of the low sensitivity of the assays.

Similarly to the quantification of anti-factor VIII inhibitors by Bethesda and Bethesda-modified assays (161, 162), anti-ADAMTS13 neutralizing autoantibodies can be titrated *in vitro* using mixing studies of heat-inactivated patient and pooled normal human plasmas at 1:1 dilution (116, 117). Immunoenzymatic assays like ELISA or Western Blot, using recombinant ADAMTS13, are used to

detect total anti-ADAMTS13 antibodies, regardless their neutralizing activity (153, 158). The clinical utility of these assays for the diagnosis, prognosis and the therapeutical management of TTP will be discussed later on in this dissertation (see section 5.2).

4.2. Immunoglobulin classes and subclasses of anti-ADAMTS13 antibodies

Anti-ADAMTS13 antibodies are usually immunoglobulin (Ig) G, although autoantibodies of IgA and IgM class have been described (152, 153, 159). In 2008, Dong *et al.* reported a fatal case of acquired TTP, in which the inhibitory activity of antibodies against ADAMTS13 rapidly escalated to extremely high levels despite daily plasma exchange and corticosteroid therapy (163). This increase was found to be because of a combination of higher antibody concentration and potency. Remarkably, the rising inhibitor level was associated with switching of the IgG subclasses, from IgG1 to IgG2 subclass, suggesting a Th1-type cytokine response and that an infection might have contributed to the exacerbation of TTP in this patient.

Given the diverse biological function of the different IgG subclasses, the characterization of the subclass profile of anti-ADAMTS13 antibodies could provide insights into the pathophysiology of acquired TTP. An analysis of the IgG subclass distribution of anti-ADAMTS13 antibodies in 58 patients diagnosed with acute acquired TTP was reported by Ferrari and colleagues (164). IgG4 was found to be the most prevalent IgG subclass (90%), followed by IgG1 (52%), IgG2 (50%), and IgG3 (33%). IgG4 was found either alone (33%) or with other IgG subclasses (67%), while it was not detected in 10% of the patients. The concurrent presence of several IgG subclasses indicate that the autoimmune response against ADAMTS13 is polyclonal and heterogeneous. Remarkably, absolute levels of IgG1 and IgG4 were always inversely correlated (r^2 = -0,927, P < 0,01) and patients with high IgG4 levels and undetectable IgG1 were found to be more prone to relapse than patients with low IgG4 levels and detectable IgG1. On the other hand, despite a higher number of patients is needed to reach statistical significance, the authors observed that patients were less likely to survive their first TTP event (four out of seven died) if they had IgG1 and very low or undetectable IgG4 levels, plus higher titres of other classes of anti-ADAMTS13 antibodies, particularly IgA.

The distribution of Ig class and A/G subclasses was also studied in a more recent study by Pos *et al.* in 48 acquired TTP patients (87). Anti-ADAMTS13 IgG was found in all samples tested, whereas anti-ADAMTS13 IgM was found in 10% of the patients. Approximately 19% of plasma samples were positive for anti-ADAMTS13 IgA, precisely for anti-ADAMTS13 IgA1. IgG1 and IgG4 were present in 73% and 69% of samples, respectively, whereas low levels of IgG2 and IgG3 were observed in 15% and 8%, respectively. Overall, these results confirmed the previous observation that IgG4 and IgG1

predominate in plasma of acquired TTP patients. No association analysis with disease state, episode severity or risk of relapse was performed.

Further studies on a wider number of patients are needed to confirm these early data and establish a possible role of anti-ADAMTS13 antibodies classes and subclasses as prognostic biomarkers in acquired TTP.

4.3. Epitope mapping and mode of action of anti-ADAMTS13 antibodies

Epitope mapping studies showed that anti-ADAMTS13 antibodies are polyclonal, with a primary epitope in the spacer domain (55, 81, 83-87, 165) (Figure 7).

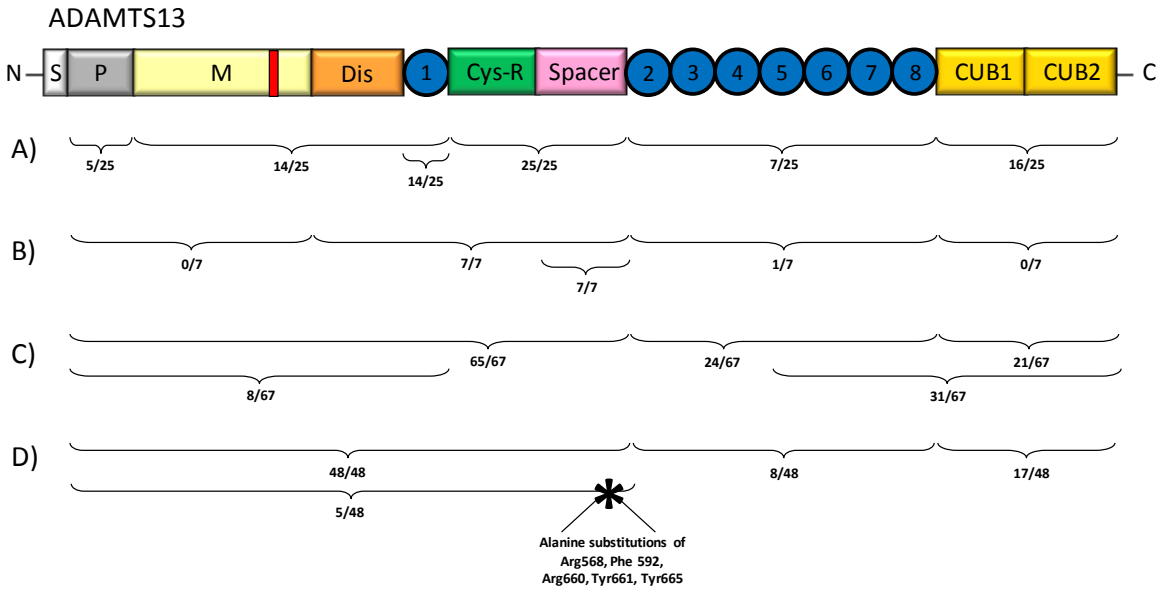

Figure 7. Schematic protein domain structure of ADAMTS13 and localization of anti-ADAMTS13 autoantibody epitopes. The results of the main epitope-mapping studies are reported (see text for discussion) (A-D). Braces depict the ADAMTS13 fragments used for epitope mapping of anti-ADAMTS13 Antibodies. Number of positive out of total cases are indicated. **Row A:** Klaus *et al.* investigated 25 patients using E coli–derived recombinant (r) ADAMTS13 fragments (55). **Row B:** Luken *et al.* performed epitope mapping in 7 patients using full-length ADAMTS13 and ADAMTS13 fragments produced in insect cells (83). **Row C:** Zheng *et al.* performed epitope mapping on 67 patient samples using rADAMTS13 and rADAMTS13 fragments produced in mammalian cell lines (86). **Row D:** Pos *et al.* analyzed 48 patients using wild-type and mutant rADAMTS13 fragments produced in mammalian cells (87).

In the first study by Klaus and co-workers, a series of ADAMTS13 domains were expressed in *Escherichia coli* and the reactivity of purified recombinant fragments with anti-ADAMTS13 autoantibodies from 25 patients with severe ADAMTS13 deficiency and neutralizing anti-ADAMTS13 antibodies was evaluated by Western Blot analysis (55). All TTP plasmas contained antibodies directed against the cysteine-rich and spacer domains of ADAMTS13. Remarkably, the plasma of 3 patients reacted exclusively with these domains. In 64%, 28% and 56% of the plasmas, antibodies recognizing the CUB domains, the TSP2-8 variant and the fragment consisting of the metalloprotease/disintegrin-like/TSP1 domains were detected, respectively. Unexpectedly, antibodies reacting with the propeptide region were present in 20% of the plasmas, confirming that its cleavage is not required for proper folding and secretion of ADAMTS13.

A second study by Luken *et al.* analyzed the epitope mapping profile of 7 TTP patients with ADAMTS13 activity below 5%, using recombinant ADAMTS13 fragment expressed in insect cells and immunoprecipitation plus Western Blot analysis (83). All samples tested were found to react towards a fragment consisting of the disintegrin-like/cysteine-rich/spacer domains and towards the isolated spacer domain. No binding to propeptide, metalloprotease and CUB domains was detected, whereas one patient plasma reacted to the TSP2-8 domains. Despite discrepancies that could be due to the different number of samples analyzed or to differences in the experimental approaches, the results of this study confirmed the previous one, inasmuch as the spacer domain was identified to harbor a major epitope for anti-ADAMTS13 antibodies in acquired TTP.

The studies by Klaus and Luken have limitations since they employed non-physiological antigens or detection methods or involved a small case series of patients. To circumvent the potential pitfalls of the first two epitope mapping studies, Zheng and colleagues used recombinant ADAMTS13 variants expressed in human embryonic kidney (HEK)-293 cells and immunoprecipitation plus Western Blotting to detect anti-ADAMTS13 antibodies in a large cohort of acquired TTP patients (86). Sixty-seven patients with acute acquired autoimmune TTP and severe deficiency of ADAMTS13 in 88% of the cases were included in the study. Anti-ADAMTS13 IgG from all patients bound full-length ADAMTS13 and a variant deleted of the CUB domains. Approximately 97% (65/67) of patients harbored antibodies targeted against a variant truncated after the spacer domain. However, only 12% of patient samples reacted with a variant lacking the Cys-rich and spacer domains, confirming these domains as the most targeted by anti-ADAMTS13 antibodies. In addition, approximately 37%, 31%, and 46% of patient IgG interacted with the ADAMTS13 fragment containing TSP2-8 repeats (T2-8), CUB domains, and TSP5-8 repeats plus CUB domains, respectively. Importantly, Zheng *et al.* investigated for the first time whether there was an association between the positive or negative recognition of the C-terminal domains (TSP2-8 and/or CUB domains) by anti-ADAMTS13 antibodies and clinical and laboratory markers of acute episode severity, such as neurological symptoms, lactate

dehydrogenase (LDH) and creatinine levels and platelet count. If no correlation was found with the frequence of neurological symptoms, LDH and creatinine levels, the presence of IgG targeted against the T2-8 and/or CUB domains was inversely correlated with platelet count on admission.

The reactivity of anti-ADAMTS13 antibodies against the C-terminal domains of ADAMTS13 was also recently investigated by Pos *et al.* in 48 acquired TTP patients with severe ADAMTS13 deficiency and high inhibitor titre (87). Anti-TSP2-8 antibodies were found in 17% of the samples, whereas anti-CUB antibodies were detected in 35% of patients analyzed, percentage that is similar to the value of 31% observed by Zheng and co-workers.

A different experimental approach was used by Yamaguchi and co-workers to examine the epitope recognized by anti-ADAMTS13 antibodies (165). In this study, purified IgG from 13 acquired TTP patients was screened by a phage display library expressing approximately 30 to 50 amino acids of the ADAMTS13 peptide sequence. Different peptide sequences were obtained from almost all of the ADAMTS13 domains, in particular an identical amino acid sequence in the C-terminus of the spacer domain from Gly662 to Val687 was recognized by autoantibodies from 5 TTP patients.

Given the striking evidences supporting the primary role of the spacer domain in the interaction between anti-ADAMTS13 antibodies and the metalloprotease, several studies have been performed to define the recognized epitopes in this domain at the amino acidic level. A first study employing a panel of ADAMTS13-ADAMTS1 hybrids identified residues 572-579 and 657-666 as crucial for binding of anti-ADAMTS13 antibodies (85). A subsequent study fine-tuned the details of the interaction, showing that replacement of Arg660, Tyr661 and Tyr665 with alanine in the spacer domain of ADAMTS13 reduced or abolished the binding of two human monoclonal antibodies previously isolated (84) and polyclonal antibodies from plasma of 6 patients with acquired TTP (81). Moreover, surface plasmon resonance analysis of the binding between this mutant ADAMTS13 and short VWF peptides demonstrated that residues Arg660, Tyr661 and Tyr665 identify a binding site for the A2 domain of VWF, explaining why antibodies against this region may interfere with ADAMTS13-mediated VWF proteolysis (81). In a further study by the same researchers, Arg568 and Phe592 were also found to contribute to the antigenic surface in the spacer domain of ADAMTS13, as 90% of the acquired TTP patients tested (43 out of 48) lost reactivity towards the spacer domain following introduction of multiple alanine substitutions of these residues, in addition to Arg 660, Tyr661 and Tyr665 (87).

All together, the results from epitope mapping studies using recombinant ADAMTS13 truncated variants demonstrate that the immune response against ADAMTS13 is polyclonal and engages multiple domains of the metalloprotease. Anti-ADAMTS13 antibodies recognizing a primary epitope on the Cys-rich/spacer domains are present in 97%-100% of patients with acquired TTP, and up to 64% of these patients also have antibodies against other ADAMTS13 domains. In particular,

fine-mapping studies have shown that exosite-3 (i.e. Tyr659-Tyr665) and several other adjacent aminoacid residues (i.e. Arg568 and Phe592) in the spacer domain constitute a major antigenic epitope for anti-ADAMTS13 autoantibodies in idiopathic TTP. As yet, the epitopes of ADAMTS13 antibodies binding to other domains have not been identified.

Deletion, mutagenesis and structural studies aimed at discovering the molecular mechanisms that regulate VWF binding and cleavage by ADAMTS13 identified several crucial exosites spanning the whole molecule of ADAMTS13 (see sections 2.2, 2.3). Besides the above mentioned exosites-3 in the spacer domain, subsites adjacent to the active site of the enzyme and exosites involved in ADAMTS13-mediated proteolysis of VWF were indeed found in the disintegrin-like and Cys-rich domains at the N-terminal half of the metalloprotease (REF). Recently, it has been shown that infusion of an inhibitory murine monoclonal antibody directed towards the metalloprotease domain of ADAMTS13 results in severe thrombocytopenia and microangiopathic haemolytic anemia in baboons (166). On the other hand, ADAMTS13 C-terminal domains were showed to play a role in the recognition and proteolytic cleavage of native VWF under fluid shear stress (77, 92). Moreover, the distal TSP1 repeats bind the endothelial cell surface, which may enhance proteolytic cleavage of newly released UL-VWF on endothelial cells by ADAMTS13 under flow conditions (73). Thus, antibodies against the C-terminal domains of ADAMTS13 might cause ADAMTS13 deficiency by directly inhibiting its interaction with VWF *in vivo* or by interfering with ADAMTS13 binding to the endothelial surface. Furthermore, the inverse correlation between anti-ADAMTS13 IgG positivity for the middle and distal C-terminal domains of ADAMTS13 and the patients' initial platelet counts observed by Zheng *et al.* further supports the hypothesis that anti-ADAMTS13 autoantibodies against the more distal C-terminal domains in conjunction with those against the proximal C-terminal domains may have a synergistic effect on ADAMTS13 function *in vivo* (86).

4.4. Molecular characterization of anti-ADAMTS13 antibodies genes and etiology of autoimmune TTP

Antigen driven selection governs the production of antibodies; a limited number of gene segment rearrangements generates a large repertoire of antibodies with virtually unlimited specificities. The molecular characterization of human monoclonal anti-ADAMTS13 antibodies isolated using immunoglobulin (Ig) V-gene phage-display libraries derived from acquired TTP patients, showed that antibodies directed towards ADAMTS13 preferentially incorporate heavy chain gene segment VH1-69 (84, 167, 168). The heavy chain complementarity determining region (CDR) 2 of the VH1-69 germline contains a unique hydrophobic 5 amino acid-long motif which may interact with hydrophobic

residues Tyr661 and Tyr665 on the antigenic surface of ADAMTS13 spacer domain. Based on this observation, it was hypothesized that shape complementarity between VH1-69 encoded peptide sequence and ADAMTS13 spacer domain explained the frequent usage of this Ig gene segment in anti-ADAMTS13 antibodies (169).

The etiology of autoimmune TTP is unknown. Interestingly, a case report described the development of anti-ADAMTS13 antibodies in two identical twins, suggesting that a genetic predisposition can be present even in the acquired form of the disease (170). Moreover, the presence of HLA-DRB1*11 was recently identified as a risk factor for acquired TTP, while HLA-DRB1*04 was underrepresented in this patients compared with controls and might have a protective role (171, 172). This observation suggests that ADAMTS13-derived peptides might be presented on HLA-DRB1*11 on the surface of antigen presenting cells promoting the activation of ADAMTS13-specific CD4+ T cells. Sorvillo *et al.* recently demonstrated that recombinant ADAMTS13 is efficiently internalized by human immature monocyte-derived dendritic cells in a mannose receptor dependent manner. Furthermore, very recently, the same researchers have shown that pulsing of dendritic cells of HLA-DRB1*11 positive donors with 100 nM ADAMTS13 resulted in presentation of a single CUB2 derived ADAMTS13 peptide, suggesting that presentation of this CUB2 domain derived peptide on DRB1*11 may provoke proliferation of low affinity self-reactive CD4+ T cells that have escaped negative selection in the thymus and contribute to the onset of acquired TTP (173). Besides the immune dysregulation of the HLA system, additional support for a role of T cells in the pathogenesis of acquired TTP derives from the clinical observation that exacerbation of acute TTP episodes was less frequent in patients who received cyclosporine A, a T cell immunosuppressant, in addition to plasma exchange therapy (174). Moreover, isotype switching of antibodies, which is a common feature of acquired TTP, is dependent on the presence of antigen specific CD4+ T cells.

5. ADAMTS13-related laboratory measurements and their clinical utility

Since the discovery of the VWF-cleaving protease, several assays have been developed to quantify ADAMTS13 and anti-ADAMTS13 antibodies in human plasma. Such tests includes assays of ADAMTS13 antigen, ADAMTS13 activity and neutralizing or non-neutralizing anti-ADAMTS13 antibodies.

5.1. ADAMTS13 antigen assays

Several immunoassays, in-house developed or commercial kits, are available for the quantification of ADAMTS13 antigen level (158, 175-177). The most commonly used assays are represented by Enzyme-Linked Immunosorbent Assays (ELISA), in which plasma ADAMTS13 is "sandwiched" between specific monoclonal or polyclonal antibodies. ADAMTS13 antigen determination can help the diagnosis of congenital TTP after the presence of anti-ADAMTS13 antibodies has been excluded. In acquired TTP, despite low ADAMTS13 activity, antigen levels may be normal (due to the fact that antigen assays may not distinguish between free ADAMTS13 and immune complexed ADAMTS13) or reduced following increased clearance from the circulation. Therefore, ADAMTS13 antigen measurement is not usually helpful in the management of acquired TTP. However, recently, a severe deficiency of ADAMTS13 antigen level in the acute phase of the disease, was found to be associated with unfavorable short-term outcomes, such as death and disease exacerbation (178).

5.2. ADAMTS13 activity assays

ADAMTS13 activity assays are based on the cleavage of VWF (full-lenght VWF or synthetic VWF peptides comprising the scissile bond) by ADAMTS13 in patient plasma, followed by the direct or indirect detection of the VWF cleavage products (179). The assays based on full-lenght VWF are sensitive (3%-6% of the reference standard ADAMTS13 activity) and reproducible, but cumbersome, time-consuming (2-3 days) and performed in non-physiological conditions, using denaturing agents. On the other hand, the assays based on VWF peptides are very sensitive (1%-3% of the reference standard ADAMTS13 activity), reproducible, easy and rapid (1–4 h), performed in the absence of denaturing agents, but they use non-physiologic VWF substrates (179). Several studies have been performed to compare and validate ADAMTS13 assays (180-185). In the frame of an international multicentre study, eight functional assays were compared by testing six different test plasmas ranging from 100% to 0%, prepared centrally by dilutions of one ADAMTS13 congenital deficient

plasma (arbitrarily set at 0%) into one normal pooled plasma (100%) (184). Comparative analysis showed that functional assays employing modified VWF peptides as substrate for ADAMTS13 offer the best performance characteristics.

Direct assays are based on the detection of cleavage products of a given substrate, whether it is a full-lenght VWF, a VWF A2 domain or short VWF peptides, whereas indirect assays depend on measuring the residual substrate or its disappearance. Agarose or polyacrylamide gel electrophoresis (PAGE), Western blotting and fluorescence resonance energy transfer (FRET) technique are the most commonly used methods for direct detection of cleavage substrate, whereas ELISA (that includes the collagen-binding assay) and platelet aggregation techniques are the most widely used techniques to detect residual VWF.

5.2.1. SDS-Agarose gel electrophoresis and Western Blotting

This assay was initially developed by Furlan *et al.* (18, 115) to measure ADAMTS13 activity in plasma. Purified VWF is incubated with citrated plasma samples for approximately 24 hours in the presence of urea and $BaCl_2$, then agarose gel electrophoresis and Western blotting with peroxidase-conjugated anti-VWF antibody are used to determine the proteolytic degradation of purified VWF multimers. The relative amount of ADAMTS13 activity is expressed as percentage of serially diluted normal human plasma. This assay permitted the identification and characterization of ADAMTS13 and it is currently used to evaluate plasma or recombinant ADAMTS13 activity in research studies. However this assay is very complex and requires technical expertise in handling agarose gel electrophoresis, transfer and Western blotting, therefore it cannot be used in clinical practice.

5.2.2. SDS-PAGE and Western Blotting

Tsai *et al.* (13, 116) initially developed this assay based on detection of dimeric VWF fragments of 176 and 140 kd on denatured, but unreduced SDS-PAGE and Western blotting. Alternatively, the same biochemical techniques may be applied using epitope-tagged VWF fragments (His-A2 domain or GST-VWF73) as substrate (53, 67, 186), whose cleavage is detected with an anti-His (186) or anti-GST (53, 67) antibody. The cleavage of a small recombinant domain or a peptidyl substrate does not require denaturing reagents.

5.2.3. FRET assay

Förster (or Fluorescence) resonance energy transfer (FRET) is a physical phenomenon that relies on the distance-dependent transfer of energy between two dyes or fluorophore, named donor and acceptor. If the donor emission spectrum and the acceptor absorbance spectrum overlap, the energy that is initially absorbed by the donor molecule is subsequently transferred to the acceptor molecule.

Due to its high sensitivity to distance, FRET has been used to investigate molecular interactions or, as in this case, proteolytic reactions. FRETS-VWF73 is a chemically synthesized, fluorogenic peptide, containing the 73-amino acids from Asp1596 to Arg1668 of VWF, the region of VWF A2 domain that provides the minimal substrate for ADAMTS13 (53, 187). Within this peptide, the Gln1599 residue at the P7[1] position is converted to a 2,3-diaminopropionic residue (A2pr) modified with a 2-(N methylamino)benzoyl group (Nma). The N1610 residue of the P5'[1] position is converted to A2pr modified with a 2,4-dinitrophenyl group (Dnp) (Figure 8). When the Nma group, the acceptor fluorophore, is excited at 340 nm, fluorescence resonance energy is transferred to the neighboring donor fluorophore, Dnp. If the bond between Tyr1605 and Met1606 is cleaved, the energy transfer quenching the fluorescence does not occur, allowing the emission of fluorescence at 440 nm from Nma. Proteolysis of FRETS-VWF73 by ADAMTS13 can be followed in real-time as an increase of fluorescence over time within a 1-h period, using a 96-well format in commercial plate readers with 340/450 nm filters (187).

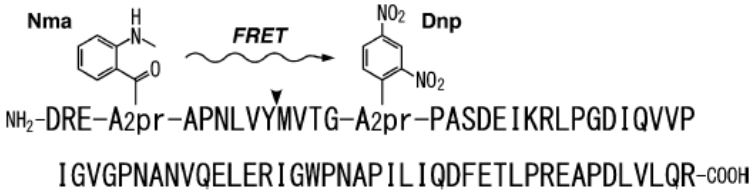

Figure 8. Structure of FRETS-VWF73. Within the 73-amino-acid peptide sequence, corresponding to the region from D1596 to R1668 of VWF, Q1599 and N1610 were substituted with A2pr(Nma) and A2pr(Dnp) respectively. The arrowhead indicates the site cleaved by ADAMTS13. Adapted from (187).

As in other ADAMTS13 activity assays, the assay buffer contains divalent metal ions to activate ADAMTS13 (25 mM $CaCl_2$ being the optimal ion concentration), and no chloride ions, as higher NaCl concentrations inhibit cleavage (18). FRETS-VWF73 assay is performed at a pH of 6, differing from previous studies reporting an optimal pH of 8-10 for the cleavage reaction (18). Finally, as in other assays involving short VWF peptides as substrate, denaturating agents are not required. Compared with the collagen binding assay (described below), the FRET-VWF73 assay has several advantages: it is more sensitive (limit of detection (LOD) = 3% VS 6%), it is easier and shorter to perform (1 h VS 20 h of substrate digestion) and does not require the use of denaturating agents to expose VWF A2 domain. On the other hand, it does not employ a physiological multimeric VWF substrate. Moreover,

[1] In an proteolysis reaction, residues are commonly indicated by the string "P/Snumber". The letter "P" or "S" indicate the substrate or the enzyme, respectively, whereas the number indicates the position of the residue from the scissile bond; the prime indicates residues C-terminal to the scissile bond.

plasma proteins, hemoglobin and bilirubin may interfere with FRETS-VWF73 assay because of their spectral properties at 340/450 nm. For example, bilirubin in hyperbilirubinemic patients' plasma (at concentrations higher than 100 uM) interferes with fluorescence evolution in this assay by acting as a quencher at the emission wavelength of 450 nm, potentially leading to a misdiagnosis of a severe deficiency of the enzyme (188). Modifications of the assay to avoid plasma autofluorescence have been recently proposed, using fluorophores that absorb/emit at higher wavelengths (189) or in the near infrared (190).

5.2.4. Surface Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF-MS)

An ADAMTS13 activity assay based on the detection of VWF cleaved fragments by mass spectrometry has been developed by Jin and colleagues (191, 192). This type of assay is not commonly used, nor applicable for routine laboratory practice because of the technology used, but it is worth-describing due to its extremely high sensitivity, being able to detect ADAMTS13 activity levels as low as 0,5% (192). A recombinant VWF73 peptide containing a His6 tag at the C-terminus is incubated with plasma samples for 16 hours. The cleavage products are bound to a Nickel-coated chip through their His6 tag and quantified using SELDI-TOF-MS. This assay was recently used to determine ADAMTS13 activity values in congenital TTP patients with undetectable activity by commonly used assays, permitting to establish a genotype-phenotype correlation based on residual ADAMTS13 activity levels (134).

5.2.5. Collagen-Binding Assay (CBA)

Initially described by Gerritsen *et al.* (193) and modified by many others (194-197), the CBA assay for the measurement of ADAMTS13 activity derives from an enzyme-immunoassay for measuring the collagen-binding activity of human VWF in plasma (198, 199) and is based on preferential binding of large VWF multimers to type III human collagen. Purified VWF or ADAMTS13-depleted plasma is incubated with patient plasma or normal human plasma (as control) in the presence of 5 mM Tris-HCl (pH 8.0), 1,5 M urea, and 3 mM $BaCl_2$ for 2-24 hours at 37 or 25 °C. The proteolytic degradation leads to low molecular weight forms of VWF, which show impaired binding to microtitre plates coated with human collagen type III. Bound VWF is quantified by peroxidase-conjugated anti-VWF antibodies, followed by a chromogenic reaction and absorbance measurement (193). Among assays using full-length VWF as substrate, this assay is relatively simple to perform and can accommodate many samples simultaneously.

5.2.6. Ristocetin-induced aggregation

After digestion of the purified VWF in the buffer (similar to that in collagen-binding assay), the residual VWF is determined by a ristocetin cofactor activity (RiCof) in a platelet aggregometer (200, 201).

5.2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

This assay uses N- and C-terminal tagged recombinant fragment (VWF A2 domain or VWF73) as a substrate (202, 203). The substrate is immobilized to a microtitre plate with an antibody to one tag. After incubation of the immobilized substrate with plasma samples, an antibody to another tag detects the residual substrate. The proteolytic activity of ADAMTS13 is inversely proportional to the residual substrate concentration. In a variation of this method, a single N-terminal GST-tagged VWF73 is captured on a microtitre plate and incubated with plasma sample (204). The cleaved product is directly detected by a monoclonal antibody specific for the Tyr1605 residue of VWF73 scissile bond.

5.2.8. Flow-based assays

Three types of assays using flow conditions for measuring ADAMTS13 activity have been described (11, 205, 206). First, Shenkman *et al.* used a cone and plate(let) analyzer to evaluate the ability of TTP plasma to increase deposition on a polystyrene surface from normal plasma at a shear rate of 1800 s^{-1} (205). Second, Dong *et al.* described that ULVWF multimers, upon release from cultured endothelial cells, form extremely long, platelet-decorated string-like structures on the surface of endothelial cells under fluid shear stress (11). When perfused with normal human plasma, the string-like structures disappear rapidly, and proportionally to plasma concentration (207), within a few minutes, indicating the cleavage of ULVWF by ADAMTS13. On the contrary, ULVWF strings persist when plasma from TTP patients with severe ADAMTS13 deficiency is perfused (11). Under flow conditions, cleaving ULVWF occurs at 1000-fold faster kinetics than that observed under static conditions. This assay is performed in more physiological conditions compared with the other described assays: it uses a physiological substrate, the interaction between ADAMTS13 and VWF is regulated by shear stress and the environment is represented by endothelial cells on a collagen-coated slide. However, the evaluation of this assay in a multicentre study showed that it may only be reliable in discriminating ADAMTS13 levels higher or lower than 20%, without measuring a precise value of ADAMTS13 activity (184). Moreover, this assay is cumbersome and depend on endothelial cells culturing, thus it is not suitable for routine laboratory tests. Recently, a third assay performed at high shear conditions has been described by Han and colleagues (206). A mixture of purified VWF and patient plasma is subjected to

vortexing for 60 minutes at the rotation rate of 2,500 rpm, which generates a shear-stress corresponding to that found in the arteries. The cleavage products are then detected by Western blotting following a separation on an agarose SDS-gel. This assay showed a good agreement with a FRET-based assay in detecting ADAMTS13 activity and inhibitors, however its sensitivity and specificity for the diagnosis of TTP remain to be established (206).

In all the reported activity assays, a number of variables may interfere with assay results. First, all the validated assays measure ADAMTS13 activity in static conditions which do not reflect the *in vivo* physiologic flow conditions. Furthermore, the assays based on full-length VWF, in order to unfold VWF A2 domain, use denaturing agents as urea or guanidine-HCl, that could also affect the enzymatic activity of ADAMTS13 and/or antibody-ADAMTS13 interactions, artifactually changing the assay results. The use of short VWF peptides does not require denaturing agents, but the lack of VWF domains (as the C-terminal domains), that have been found to be important for VWF recognition by ADAMTS13 (see sections 2.2, 2.3) may alter the proteolytic cleavage of VWF in a way that does not reflect the *in vivo* situation. For example, anti-ADAMTS13 antibodies against the C-terminal domains of ADAMTS13, which have been shown to interact with VWF D4-CK domains, may lose their effect in such assays. All these reported factors could influence the clinical utility of each assay.

5.2.9. Discrepancies in ADAMTS13 activity levels between CBA and FRET assays

CBA and FRET assay are two of the most widely used methods for the measurement of ADAMTS13 activity in thrombotic microangiopathies. As described above (see section 5.2), CBA and FRET assay differ for the substrate used (full-length VWF VS VWF73 peptide), for the use of denaturing agents (urea VS none) and for the detection method (indirect detection of cleavage products through absorbance measurement VS direct detection of cleavage products through fluorescence measurement). Several studies have been perform to evaluate the performance and clinical utility of ADAMTS13 activity assays based on full-length or VWF peptides, most of them on a small number of patients (154, 182, 183, 185, 208). In a recent study by Palla *et al.*, a large number of plasma samples (400) obtained from patients affected with different thrombotic microangiopathies were analyzed to investigate whether or not there was a concordance between CBA and FRET assays (185). An excellent correlation between the two assays (Spearman's rho = 0.90, $p = 1x10^{-5}$) was observed in all samples tested. After categorizing the samples into subgroups based on ADAMTS13 activity level (Table 1), agreement was found in 72% of the cases. A particularly good agreement (90%) was found in samples with ADAMTS13 activity lower than the detection limit of the assay. However, in some samples, the results of the two assays were considerably discordant (Table 1), ADAMTS13 activity measured by FRET being consistently lower than that measured by CBA. Most importantly in 2,5%

(n=10) of the samples ADAMTS13 activity was higher than 10% by CBA, but undetectable by FRET. On the whole, a considerable discrepancy between normal ADAMTS13 by CBA and undetectable levels by FRET was observed in 1% (n=4) of samples, all obtained from patients with TTP.

ADAMTS13 activity (%)		FRET					Total
		below detection limit (<3)	3–10	11–20	21–45	normal range (> 45)	
CBA	below detection limit (<6)	93	10	0	0	0	103
	6 – 10	8	4	3	0	0	15
	11–20	4	11	9	4	0	28
	21–50	2	15	19	35	9	80
	normal range (>50)	4	2	4	18	146	174
Total		111	42	35	57	155	400

Table 1. Cross-tabulation of the distribution of ADAMTS13 activity results by CBA and FRET assays after arbitrary categorization into five subgroups according to the plasma levels of ADAMTS13 activity: i) less than the detection limit, ii) from the detection limit to 10% (the plasma level previously adopted by us and others to define severely reduced activity), iii) from 11–20% (moderate deficiency), iv) from 21% to the lower limit of the normal range (mild deficiency), and v) the normal range of the assays. Adapted from (185).

Modifications of FRET assay experimental conditions, such as pH buffer (pH 8 instead of 6) and prolongation of the digestion time (4 hours instead of 1 hour) were tested, but none of these variations affected the degree of discrepancy. A chromogenic activity-ELISA (Technoclone), which employs the VWF73aa peptide as FRET, and the chromogenic detection method as CBA, was performed on 97 samples, including all the discordant ones. This assay confirmed the FRET assay, indicating that the observed discrepancy was not related to the detection method. Finally, Palla and colleagues analyzed those conditions/features that might interfere with ADAMTS13 measurement, such as the type of the disease (TTP VS no TTP), the state of the disease (acute or remission), the type of treatment at the time of blood sampling (plasma treatment, corticosteroids or antiplatelet agents), hyperbilirubinaemia (188), high levels of endogenous VWF antigen and the presence of anti-ADAMTS13 antibodies. None of these conditions seems to explain the obtained discrepancy, except for the presence of anti-ADAMTS13 antibodies which was more frequent in discordant samples than in concordant samples ($p<0.01$) (185).

Discrepancies between full-length VWF- and VWF peptide-based assays in measuring ADAMTS13 activity in samples from TTP patients were also found in other studies (154, 160, 209). So far, the cause of these discrepancies are unknown. The clinical helpfulness of ADAMTS13 activity testing in the diagnosis and prognosis of TTP, in particular as a prognostic marker for relapse, is

well-established. Thus, understanding the reason of these discrepancies might avoid a potential misdiagnosis or wrong management during follow-up of this rare, but life-threatening disease.

5.2.10. Clinical utility of ADAMTS13 activity assays

A severe deficiency of ADAMTS13, define as an ADAMTS13 activity level below 10%, strongly suggest a diagnosis of TTP, although it has been reported in 13% of atypical haemolytic uremic syndrome (HUS) cases (210) and in 16% of disseminated intravascular coagulation (DIC) (104). After the first retrospective studies (13, 18), who found a severe ADAMTS13 deficiency in 87%-100% of idiopathic TTP patients, subsequent investigations challenged the implications of the original findings, as severe ADAMTS13 deficiency was variably found in 18% to 72% of patients clinically diagnosed with TTP (157, 194, 211-216). The variability of the results might be due to differences in the criteria used to select the cohort of patients. Idiopathic TTP patients are well identified by severe ADAMTS13 deficiency, whereas secondary TTP cases, such as those associated with tumors, bone marrow transplantation or HIV infection, are more frequently associated with normal or moderately reduced ADAMTS13 activity (157).

A critical issue for the management of TTP patients is whether or not ADAMTS13-related assays may be prognostic biomarkers of short-term outcomes in the acute phase of the disease. Results from two prospective studies showed that patients with ADAMTS13 activity below 10% had more favorable short-terms outcomes, in terms of better response to plasma therapy and low mortality rate (157, 194). In a more recent prospective study by Kremer-Hovinga *et al.*, survival was not statistically different between patients with ADAMTS13 activity level below and above 10% (p=0.11), however it was higher in the first group of patients (160). These results may reflect the severity of the associated diseases or conditions that are usually found in TTP patients with detectable levels of the metalloprotease (157, 217).

Since up to 40% of TTP patients develops a recurrent form of the disease, a second major aspect of ADAMTS13 testing is represented by its potential utility as predictor of relapse. Peyvandi *et al.* reported that survivors of an acute TTP episode with severely reduced ADAMTS13 activity in remission had an approximately threefold greater likelihood of relapsing (158). The association between severe ADAMTS13 activity during remission and higher risk of recurrence was found also by Ferrari *et al.* (159) and Jin *et al.* (192). Kremer-Hovinga and colleagues recently reported a higher relapse rate among patients with ADAMTS13 activity level below 10% than among those with ADAMTS13 activity level above 10% (160). On the whole, several prospective and retrospective studies had clearly established that a severe deficiency of ADAMTS13 activity is associated with a higher risk of relapse both in the acute (157, 160, 194) and in the remission phase (158, 159, 192).

5.3. Anti-ADAMTS13 antibodies assays

Anti-ADAMTS13 autoantibodies have been distinguished in two classes: neutralizing antibodies, or inhibitors, and non-neutralizing antibodies, depending on whether or not the antibodies block proteolytic cleavage of VWF in an *in vitro* assay. Similarly to the quantification of anti-factor VIII inhibitors by Bethesda and Bethesda-modified assays (161, 162), anti-ADAMTS13 neutralizing autoantibodies can be titrated *in vitro* using mixing studies of heat-inactivated patient and pooled normal human plasma at a 1:1 or several dilutions (116, 117). Residual ADAMTS13 activity is then measured by one of the functional assay described above. Inhibitor assays are far from being optimized and generally lack sensitivity. Different functional assay to determine residual ADAMTS13 activity and different experimental conditions, in terms of ratios of patient plasma and pooled normal human plasmas, incubation time and incubation temperatures (160, 194, 201, 208, 218) have been employed to measure ADAMTS13 inhibitors. A comparison between the assays used has never been performed.

Immunoenzymatic assays like ELISA or Western Blot are used to detect total anti-ADAMTS13 antibodies, regardless their neutralizing activity (153, 158). This methods are based on the binding of anti-ADAMTS13 antibodies to recombinant ADAMTS13, either captured on an ELISA plate or subjected to SDS-PAGE and Western blot, followed by detection by enzyme-linked anti-human immunoglobulin antibodies. Western Blot method seems to be more sensitive than ELISA, although is not a quantitative assay and may miss conformational antibodies. On the other hand, ELISA methods may vary in relation to the dilution of plasma used, the ADAMTS13 antigen captured on the plate and the coating antibody.

The results of studies assessing the clinical utility of the measurement of ADAMTS13 inhibitor titre provide conflicting results. Most studies (159, 194, 219, 220) reported poorer responses to plasma exchange and higher mortality rates in patients with high inhibitor titres, whereas others (157, 159) did not. Recently, a neutralizing autoantibody titre of 2 or more Bethesda units per ml was found to be associated with lower survival among patients with severe ADAMTS13 deficiency (160). In a prospective cohort study, Ferrari *et al.* investigated 32 patients who had low plasma levels of ADAMTS13 activity at the time of the first acute episode and subsequently achieved remission. In them, the presence of high levels of anti-ADAMTS13 inhibitors at presentation was associated with the persistence of undetectable ADAMTS13 activity, which was in turn predictive of relapses within 18 months (159). In a study by Peyvandi *et al.*, the presence of anti-ADAMTS13 antibodies during remission was predictive of recurrent disease, whereas Jin *et al.* found no predictive value for anti-ADAMTS13 IgG levels (192). Overall, it appears that the presence of anti-ADAMTS13 antibodies at

high titres during acute TTP or during remission are associated with a worse prognosis and with a higher risk of relapse, although larger prospective studies are needed to confirm these findings (217).

CHAPTER II – AIMS

Thrombotic thrombocytopenic purpura (TTP) is a rare but life-threatening microangiopathic disorder, characterized by thrombocytopenia, hemolytic anemia and signs and symptoms of ischemic organ dysfunctions due to the persistence of highly thrombogenic ultra large (UL) von Willebrand factor (VWF) multimers in the microcirculation. TTP is associated with the severe deficiency of ADAMTS13, the metalloprotease responsible for the proteolytic regulation of VWF multimers size. In the acquired form of the disease, the severe deficiency of ADAMTS13 is caused by anti-ADAMTS13 antibodies that inhibit ADAMTS13 activity (neutralizing anti-ADAMTS13 antibodies or inhibitors) and/or increase its clearance from the circulation through yet unknown mechanisms (non-neutralizing anti-ADAMTS13 antibodies).

Since the role of ADAMTS13 in the pathogenesis of TTP has been discovered, many different assays have been developed to measure ADAMTS13 and anti-ADAMTS13 antibodies. These assays have been compared in the frame of international multicenter studies and further standardization is necessary. Moreover, the studies performed to assess the utility of ADAMTS13 measurements in clinical practice provided conflicting results.

With this background, the present research has two main objectives: to evaluate, in our cohort of acquired TTP patients, the clinical utility of ADAMTS13-related biomarkers and to investigate which ADAMTS13 assay may be more reliable in the management of acquired TTP.

Specific aims are described below:

1. ADAMTS13-related assays at different times of the disease clinical course have been evaluated for the prediction of clinical outcomes in TTP (death, response to plasma exchange and disease recurrence). Severe ADAMTS13 deficiency has been shown to be predictive for recurrence both at presentation and during remission. The predictive value of anti-ADAMTS13 antibodies, inhibitory activity and immunoglobulin (Ig) class subtype for disease recurrence is still to be firmly established. Based on these data, we analyzed, during both conditions, the acute and the remission phase, all the biomarkers associated with ADAMTS13 (activity, antigen and class, subclass and titre of anti-ADAMTS13 autoantibodies) in acquired TTP patients referred to our centre and we correlated them to episode severity and recurrence. The aims of the study were to verify if any of the investigated biomarkers could be of prognostic value on the long- and on short-term and to identify differences between subgroups of patients that could allow us to focus resources on patients at higher risk of recurrence.

2. Anti-ADAMTS13 neutralizing autoantibodies can be titrated *in vitro* using mixing studies of heat-inactivated patient and pooled normal human plasmas (NHP) at 1:1 dilution. Reported inhibitor assays are far from being optimized and use several different experimental conditions. This lack of standardization may in part explain the conflicting results obtained in studies assessing the clinical utility of ADAMTS13 inhibitors testing. With this background, the aim of this study was to compare CBA and FRET assay for the measurement of ADAMTS13 inhibitors.

3. Collagen binding assay (CBA) and fluorescence resonance energy transfert (FRET) assay are two of the most widely used methods for the measurement of ADAMTS13 activity. CBA and FRET assays differ for the substrate used (full-length VWF VS VWF73 peptide), for the use of denaturing agents (urea VS none) and for the detection method (indirect detection of cleavage products through absorbance measurement VS direct detection of cleavage products through fluorescence measurement). In comparative studies, these two assays showed a good correlation, although relevant discrepancies that might lead to misdiagnosis, have been reported in about 10% of TTP samples. So far, the cause for the observed discrepancies is unknown, but a role of anti-ADAMTS13 antibodies has been suggested.

   Based on these results, we decided to investigate whether the CBA or the FRET assay results reflect the real ADAMTS13 activity level *in vivo* in a discordant group of samples from acquired TTP patients. The experimental conditions were evaluated as possible causes of the reported discrepancies. The goal of this study was to determine which assay can be considered the most reliable in clinical practice.

CHAPTER III – Adapted from:

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

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1. SUMMARY

Background. Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening disease. Of surviving patients, 45% develops an exacerbation or a late recurrence. Severe ADAMTS13 deficiency, both during the acute episode and remission is a well-established predictor of recurrence. The predictive value of anti-ADAMTS13 antibodies, their inhibitory activity and Ig class subtype for disease recurrence is still to be established.

Objectives. To analyze ADAMTS13 related biomarkers (ADAMTS13 and anti-ADAMTS13 immunoglobulins, classes and subclasses) and their potential relationship with prognosis.

Patients/Methods. In 115 patients with TTP we assessed the association between levels of these biomarkers and the severity of acute episodes; we analyzed also the hazard ratio and 95% confidence interval of recurrence in association with biomarkers levels retrieved at the previous acute episode or during remission, using Cox regression models.

Results. During the acute phase, higher IgA, IgG1 and IgG3 titres showed the strongest association with acute episode severity. In the survival analyses the only biomarker significantly associated with a high hazard of recurrence following an acute episode was presence of IgG. Conversely, low ADAMTS13 activity or antigen levels (<10%), presence of ADAMTS13 inhibitor or IgG during remission were all significantly associated with a higher hazard of recurrence.

Conclusions. 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.

2. INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a rare (annual incidence 5-10 per million) life threatening disease characterized by widespread hyaline thrombi, composed primarily of platelets and rich in von Willebrand factor (VWF), accompanied by variable fibroblastic infiltration and endothelial overlay. It manifests in the terminal arterioles and capillaries of multiple organs, most extensively in the heart, brain, kidney, pancreas, spleen, mesentery and adrenal gland (1, 2). The disorder is associated with a severe deficiency of the VWF-cleaving protease ADAMTS13 causing persistence of highly adhesive ultra-large VWF (ULVWF) (3-7). Within the microcirculation unfolded ULVWF multimers spontaneously seize platelets and promote platelet thrombi formation, eventually leading to life-threatening microvascular thrombosis and the clinical manifestations typical of TTP. In patients with acquired TTP, a deficiency of ADAMTS13 is caused by auto-antibodies against ADAMTS13 (3-7). Neutralizing antibodies that inhibit proteolytic activity (6, 7) but also non-neutralizing antibodies that may enhance clearance or disturb interaction with physiologic binding partners have been described (8). In most cases, anti-ADAMTS13 autoantibodies are of the IgG isotype, with IgG4 as the most prevalent subclass followed by IgG1 (9, 10). IgM and IgA autoantibodies have also been identified (8).

The currently accepted definition of TTP is based on clinical criteria only and requires the presence of mechanical hemolytic anemia with fragmented erythrocytes and thrombocytopenia (platelet count $<100 \times 10^9$/L) without alternative causes (11). This is frequently accompanied by symptoms such as fever, neurological abnormalities and renal impairment, but since these are not invariably present they are not part of the defining symptoms. Note that low levels of ADAMTS13 are also not part of the definition and, indeed, are not always observed.

Demographic studies have shown a preponderance of women (2- to 3-fold risk compared to males) and black race (9-fold risk compared to whites) (12). Without timely intervention, TTP is nearly always fatal; however, plasma exchange treatment has dramatically decreased the mortality rate to 10-20%. Exacerbation within 30 days of remission occurs in 22 to 45% of patients, and late recurrences (after 30 days) have been reported in 13 to 40% of cases (13-16). Most recurrences occur during the first year, but they have also been documented up to 13 years after the first acute event (14, 17-21).

After identifying the diagnostic utility of ADAMTS13 to characterize TTP patients among other thrombotic microangiopathy (5, 6, 13, 22-29), 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 (death, response to plasma exchange and disease recurrence) (10, 30-36). Although both low ADAMTS13 activity and antigen level were associated with higher

disease mortality (33, 35), data on anti-ADAMTS13 inhibitor levels are contradictory (31, 33). Patients with severe ADAMTS13 deficiency at presentation often develop recurrent disease, whereas patients without severe deficiency rarely relapse (33). Severe ADAMTS13 deficiency during remission has also been shown to be predictive of recurrence (30-32), although some studies did not observe a similar association (33). The predictive value of anti-ADAMTS13 antibodies, their inhibitory activity and Ig class subtype for disease recurrence is still to be established. Some authors (32) reported that the presence of anti-ADAMTS13 antibodies and the titer of ADAMTS13 inhibitor were predictive of recurrent disease, others (30) did not observe such an association. Recently, Ferrari and colleagues (10) reported an association between anti-ADAMTS13 IgG subclass 4 and recurrent disease. Factors that would allow to predict a diagnosis of an isolated or a recurrent form of TTP are thus not well established.

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.

3. MATERIALS AND METHODS

3.1. Patients and definition of clinical categories

Clinical centers from seven countries (Italy, Hungary, Lebanon, Romania, Slovenia, Serbia and Russia) that manage patients diagnosed with TTP participated in the study. In the frame of an international collaborative study the centers collected clinical information in local registries and stored plasma samples, during the acute TTP episode and during remission, pertaining to the years 1994-2010. The centers provided the Milan Hemophilia and Thrombosis Center (core center) with information on the clinical and laboratory data (plasma samples) of these patients, using a specially-tailored collection form.

The local ethical committees in each center approved the study and all patients or their legal guardians gave written informed consent. The original diagnosis of an acute TTP episode was considered confirmed when at least three of the following criteria were met: thrombocytopenia ($<150 \times 10^9$/L) with no other apparent cause, Coombs-negative hemolytic anemia with schistocytes in the blood smear, high (>2 standard deviations over the upper limit) serum levels of lactate dehydrogenase and signs and symptoms compatible with organ ischemia, mainly but not exclusively in the central nervous system. Alternative diseases that may present with similar signs and symptoms – such as the enterohemorrhagic form of the hemolytic uremic syndrome, the catastrophic antiphospholipid syndrome, disseminated intravascular coagulation, pre-eclampsia and related syndromes, collagen vascular diseases, metastatic cancer and Evans' syndrome – were excluded using currently accepted clinical and laboratory criteria (37). Data of all patients were included in a digital database (URL: http://www.ttpdatabase.org).

The first acute TTP episode was classified as secondary when associated with one or more of the following conditions: (i) pregnancy/postpartum, (ii) use of drugs reported to be associated with TTP, (iii) additional disease associated with TTP (autoimmune diseases or human immunodeficiency virus infection). Patients who did not fit the aforementioned categories were classified as having idiopathic disease (38). 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 (22). Acute episode severity was assessed in terms of platelet counts and the number of plasma exchanges required to achieve remission based on previous studies (38) and in light of the observation that lower platelet counts are associated with consumption of VWF multimers in acute TTP (39). This would also facilitate comparison with previous studies evaluating the prognostic values of ADAMTS13-related biomarkers (36). 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 (32).

3.2. Samples collection

Venous blood samples for the measurements of ADAMTS13 (activity and anti-ADAMTS13 antibodies) were collected into evacuated tubes containing 0.109 M trisodium citrate at concentration 9:1 as anticoagulant. Blood was then centrifuged at 4000 x g for 20 min at 4°C and plasma samples were stored in aliquots at -80°C. Samples available for this study were collected for events occurring form the year 2000 onwards, with 90% of samples being collected from 2005 onwards. All patients in this study had at least one sample retrieved. No more than one sample per patient was collected during the same acute event or remission period for an individual patient, although patients had multiple samples collected from different acute events or remission periods throughout the study window. All ADAMTS13-related measurements were performed at a central laboratory, the Milan Hemophilia and Thrombosis Center, within one month of blood collection.

3.3. ADAMTS13 activity

ADAMTS13 activity was measured using the residual VWF collagen binding activity (CBA) assay, as previously reported (32) with minor modifications: serially diluted pooled normal human plasma (NHP) and plasma samples were diluted 1:10 in assay buffer and incubated 1:1 with VWF substrate, in a final volume of 100 µL. A detailed description of the assay is provided in chapter IV, paragraph 3.5.

Our most recent laboratory evaluation of assay reproducibility yielded an intra-assay coefficient of variation of 9% and an inter-assay coefficient of variation of 12%. The lower limit of sensitivity was 6% of protease levels in NHP taken as the reference standard. The lower value of the reference interval (45%) was set at the 5th percentile of the distribution of the values obtained in 72 healthy individuals. Severe ADAMTS13 deficiency was arbitrarily considered as levels of 10% or less of ADAMTS13 activity in plasma. Previous *in vitro* experiments showed that, using this assay, high plasma levels of VWF do not influence the measurement of ADAMTS13 activity to a major degree (40).

3.4. ADAMTS13 antigen

ADAMTS13 antigen levels were measured by an ELISA method previously described by Feys and colleagues (41) with minor modifications (32). The murine monoclonal anti-ADAMTS13 antibody 20A5 was used to immobilized the plasma protease on a microtitre plate. Bound ADAMTS13 was detected by using the biotinylated anti-ADAMTS13 monoclonal antibody 19H4 and peroxidase-labelled streptavidin (Roche Mannheim, Germany). Test plasmas (1/2-1/4) and NHP (1/2 to 1/32) were diluted in phosphate buffered saline (PBS), pH 7.4. ADAMTS13 antigen values were expressed

as percentage of NHP. The lower limit of detection was 1.6% of the reference standard, the intra- and inter-assay coefficient of variations were 4% and 8% respectively. The lower limit of the normal laboratory range was 45%.

3.5. Anti-ADAMTS13 inhibitory activity

Anti-ADAMTS13 inhibitory activity was measured by a CBA-based mixing assay as previously reported (32). Heat-inactivated (1 hour incubation at 56°C) patient plasma and pooled NHP were mixed at 1:1 dilution and incubated for 2 hours at 37°C. ADAMTS13 residual activity was measured using CBA as described above. Anti-ADAMTS13 inhibitor titre was considered positive when the residual activity of ADAMTS13 in heat-inactivated patient plasma and NHP mixture was less than 50% of that in the control mixture (a 1:1 mixture of buffer and NHP). The inhibitor concentration that neutralized 50% of ADAMTS13 activity in an equal volume of NHP was defined as 1 BU mL^{-1}.

3.6. Anti-ADAMTS13 antibody detection by ELISA

The ELISA method was performed as previously reported (42) with minor modifications. A 96-well plate was coated with mouse anti-V5 antibody (Invitrogen, Grand Island, NY, USA) at 4 μg/ml final concentration, overnight at 4°C. After blocking with phosphate-buffered saline (PBS), pH 7.4, containing 3% skimmed milk powder, the plate was incubated with cell-culture conditioned media containing recombinant V5-tagged ADAMTS13 at a final concentration of 1 μg/ml, followed by incubation with plasma samples at various dilutions (1/50, 1/100 and 1/200). For each dilution a blank was performed using assay buffer (PBS, pH 7.4, containing 2.5% skimmed milk powder) instead of cell-culture conditioned media. Bound antibodies were detected with a sheep HRP-labeled anti-human IgG/IgA/IgM/IgG1,2,3,4 and OPD as substrate. Incubation steps were performed at 37°C for 1 hour, washing the plate with PBS, pH 7.4, containing 0.1% (v/v) Tween-20 between each step. The optical density (OD) values were read at 492 nm using a reference filter at 620 nm in a Multiskan Ascent spectrophotometer (Thermo Labsystems, Thermo Electron Corporation, Finland). For IgG, after subtracting the blank OD values, the amount of anti-ADAMTS13 autoantibodies was calculated against a reference curve made of serial dilutions of a high inhibitor titre patient plasma, arbitrarily set as 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).

3.7. Normalization of the absorbances measured in IgG subclass ELISA

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 (10) with minor modifications.

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%).

3.8. Statistical analysis

To take into account the intra-individual correlation of repeated measurements over time, linear regression random intercept models (43) 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 required to achieve remission. 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 analyzed 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 (44). Two separate analyses were performed, for measurements during acute episode admissions and remission visits, respectively. All the analyses were performed with Stata, version 11 (StataCorp LP, College Station, TX, USA).

4. 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.

N			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 activity at acute episode (n=62)	*idiopathic*	*Secondary*	*Total*	
<10	34	9	43	69%
≥10,<46	9	5	14	23%
≥46	1	4	5	8%

Table 1. Characteristics of patients.

Of the 115 patients, 54 entered the study (first sample retrieved) with an acute TTP episode. Among these, 43 entered the study with a first acute TTP event and were followed from then; of them, 7 developed at least one recurrent event during follow-up. Eleven patients entered the study with a recurrent acute TTP event and were followed from then; of them 3 developed at least one subsequent recurrent event. The remaining patients (n=61) had their first samples retrieved during remission and were followed up from then; of them, 20 had at least one subsequent acute episode of TTP (Figure 1). 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.

54 patients enrolled at acute episode 4 patients dead 10 patients recurrent (1 pt dead after recurrence) 5 patients lost to follow up 35 patients in remission during follow up (at least 24 months) Follow-up time: median 5.8, mean 7.3, max 32.8 years	61 patients enrolled during remission 2 patients dead 20 patients recurrent (2 pts dead after recurrence) 6 patients lost to follow up 33 patients in remission during follow up (at least 24 months) Follow-up time: median 3.4, mean 3.8, max 10.5 years

Follow up summary (number of patients)	
lost to follow up	11
Died at first episode	4
no recurrence	70
one recurrence	20
two recurrences	6
three recurrences	2
five recurrences	1
six recurrences	1
Total	115

Figure 2. Information on study entry and follow-up.

4.1. Acute phase analysis

Data from 62 patients were available for acute phase analysis. 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 achieve 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.

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

*p-values from random effect models

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

4.2. Survival analyses

Data on the number of patients with samples available for survival analysis according to biomarker measurements are found in Tables 3 and 4. Patients with low levels of ADAMTS13 (<10%) during the acute TTP 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 TTP 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 patients who entered the study with a 'first' acute TTP 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).

	No. patients	No. patients with recurrences	Hazard Ratio	95% CI	p-value
ADAMTS13 activity (%)					
≥10	71	18	1.00	(ref)	
<10	19	9	4.89	2.00-11.99	0.001
ADAMTS13 antigen (%)					
≥10	77	19	1.00	(ref)	
<10	5	3	5.66	2.10-15.24	0.001
Inhibitor (U/ml)					
Absent	43	9	1.00	(ref)	
Present	13	9	4.30	2.00-9.21	<0.001
IgG (%)					
≤1.18	50	7	1.00	(ref)	
>1.18	26	11	4.99	2.08-12.00	<0.001
IgM					
≤0.034	74	16	1.00	(ref)	
>0.034	2	2	2.67	0.39-18.44	0.32
IgA					
≤0.014	59	16	1.00	(ref)	
>0.014	16	1	0.30	0.07-1.27	0.10
IgG1					
≤0.006	25	6	1.00	(ref)	
>0.006	17	6	1.29	0.61-2.71	0.50
IgG2					
≤0.013	30	6	1.00	(ref)	
>0.013	12	6	1.85	0.86-3.98	0.12
IgG3					
≤0.019	29	7	1.00	(ref)	
>0.019	13	5	1.45	0.59-3.58	0.42
IgG4					
≤0.003	11	2	1.00	(ref)	
>0.003	31	10	1.49	0.49-4.49	0.48

*Calculated with Cox frailty models to take into account within-subject correlation.

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

5. 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 (studied also in relation to a number of autoimmune diseases such as lupus erythematodes (45), rheumatoid arthritis (46), bullous dermopathies (47), celiac disease (48), type I diabetes (49), acquired hemophilia (50), sclerosing pancreatitis, autoimmune nephropathy (51), multiple sclerosis (52). These studies showed how each IgG subclass seems to have a distinct biologic property 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 (33). It has also been demonstrated that a severely reduced ADAMTS13 activity is predictive of recurrence even during remission (30-32), although some authors failed to observe such an association (33). 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).

Reference	Study 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. 2006 (36)	Cohort	33	Acute TTP	Anti-ADAMTS13 inhibitor	Acute episode severity	Presence of inhibitor associated with more severe acute espidoe
Ferrari et al. 2007 (31)	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 (32)	Case-control	109	Remission	ADAMTS13 activity	Recurrence	ADAMTS13 activity <10% associated with recurrence
		77	Remission	ADAMTS13 antigen	Recurrence	No association
		97	Remission	Anti-ADAMTS13 autoantibodies	Recurrence	Presence of antibodies associated with recurrence
Jin et al. 2008 (30)	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 (10)	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 (33)	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 level (≥2 Bethesda units) associated with mortality
		37	Remission	ADAMTS13 activity	Recurrence	No association

Zheng et al. 2010 (34)	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 (35)	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 for some studies.
TTP, thrombotic thrombocytopenic purpura; Ig, immunoglobulin; PEX, plasma exchange.

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

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 controversial. The presence of anti-ADAMTS13 autoantibodies during remission is predictive of recurrence (32). However, Jin and colleagues (30) did not find any predictive value for the levels of IgG anti-ADAMTS13 measured during remission and the risk of recurrence. Our data add to these findings by showing that the presence of anti-ADAMTS13 inhibitors during remission also predicts the risk of recurrence. They also confirm, contrary to previous reports (30), 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, as was reported in previous studies (10) (Table 5).

We did not find an association between ADAMTS13 activity or antigen level and acute disease severity in acquired TTP, although both parameters were previously associated with higher disease mortality (33, 35). Moreover, we did not observe an association between anti-ADAMTS13 inhibitor levels and acute episode severity which is in disagreement with previous reports (36) yet echoes others evaluating the outcome of mortality (31). 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 the complement-mediated inflammation (53). In fact IgA levels have been associated with increase mortality from acute phase TTP (31). In this study, we identified that the IgG class and subclasses are also predictive of the severity of the acute TTP episode. High IgG titres were associated with a higher number of plasma exchanges; and of the IgG subclasses, IgG1 and IgG3 were the most strongly 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, and maybe in the future also useful for immunotolerance therapy using recombinant ADAMTS13.

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, nor clarity whether over the full period of admissions we have included all patients. 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 anlayses that showed a preponderance of specific biomarkers in patients with severe events or recurrence is reliable. The large number of patients, given the low incidence of the disease, the extended observation time available for most patients and the performance of assays for ADAMTS13 and related biomarkers in the same center, make these the most precise estimates available. Lastly, we used platelet counts and the number of plasma exchanges required to achieve remission as markers of acute episode severity to facilitate comparisons with similar studies, and based on biological evidence of increased consumption of VWF multimers associated with low platelet counts (36, 38, 39). However, one previous report showed that low platelet counts do not predict response to plasma exchange during acute TTP (54). Thus, studies evaluating the association between ADAMTS13-related biomarkers and acute episode mortality may provide a more objective assessment of the association between ADAMTS13-related biomarkers and acute episode severity.

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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CHAPTER IV – Adapted from:

Measurement of anti-ADAMTS13 neutralizing autoantibodies: a comparison between CBA and FRET assays

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1. SUMMARY

Background. Acquired thrombotic thrombocytopenic purpura (TTP) is associated with the severe deficiency of ADAMTS13 due to neutralizing (i.e. inhibitors) and non-neutralizing anti-ADAMTS13 autoantibodies. The comparison of different ADAMTS13 activity assays to detect inhibitors has never been evaluated. *Objectives.* To compare the anti-ADAMTS13 neutralizing autoantibodies titre results obtained with CBA and FRET assays in a series of plasma samples from acquired TTP patients.

Samples/Methods. 30 plasma samples from acquired TTP patients were analysed. ADAMTS13 inhibitor was measured using either CBA and FRET assay after mixing heat-inactivated patient's and pooled normal human plasmas (NHP). ADAMTS13 residual activity was measured under three different conditions: CBA was performed after 2 hours of incubation of NHP and patient's plasma at 37 °C (condition 1); FRET was performed after 2 hours of incubation of NHP and patient's plasma at 37 °C (condition 2) and at 25 °C (condition 3). Results were analysed by the Spearman rank order correlation and a p-value ≤0.05 was considered statistically significant.

Results. Spearman rank order correlation analysis revealed a good agreement between ADAMTS13 inhibitor titres measured by the CBA method and the FRET assay performed after incubation at different temperatures (CBA vs FRET–condition 2, Spearman's rho=0.92, p<0.0001; CBA vs FRET–condition 3, Spearman's rho=0.94, p<0.0001). Interestingly, when plasma ADAMTS13 activity was measured under condition 2, pre-incubated NHP showed a decrease of enzyme activity about one third compared to non-incubated NHP. By contrast, no difference was observed in both conditions 1 and 3.

Conclusion. FRET and CBA-based inhibitor measurement performed similarly. FRET, which is more rapid and easier to perform than CBA, could hence be the assay of choice for inhibitor quantification. Moreover, incubation between heat-inactivated patient plasma and NHP could be performed at 25 °C, where ADAMTS13 recovery is maintained.

2. INTRODUCTION

Acquired thrombotic thrombocytopenic purpura (TTP) is associated with the severe deficiency of ADAMTS13 due to anti-ADAMTS13 autoantibodies, which have been distinguished in two classes, neutralizing (i.e. inhibitors) and non-neutralizing antibodies. Neutralizing autoantibodies inhibit ADAMTS13 proteolytic activity (1), while non-neutralizing autoantibodies have been proposed to enhance its clearance (1, 2). Anti-ADAMTS13 neutralizing autoantibodies can be titrated *in vitro* using mixing studies of heat-inactivated patient and pooled normal human plasmas (NHP) at 1:1 dilution. The measurement of inhibitor titre is of clinical relevance. High inhibitor titres at presentation were found to be associated with persistent undetectable ADAMTS13 activity in remission, the latter being predictive of relapse at 18 months (3). Recently, a neutralizing autoantibody titre of 2 or more Bethesda units per ml (BU mL^{-1}) was found to be associated with lower survival among patients with severe ADAMTS13 deficiency (4). The comparison of different ADAMTS13 activity assays to detect neutralizing autoantibodies has never been evaluated. The importance to determine which assay at which experimental conditions should be used to measure the anti-ADAMTS13 neutralizing autoantibodies titre in clinical practice has been recently underlined by Plaimauer *et al.* The authors showed that recombinant ADAMTS13 overrides inhibitory antibodies and normalizes deficient ADAMTS13 activity *in vitro*, proposing recombinant ADAMTS13 as a new therapeutic tool in the treatment of acquired TTP patients (5).

With this background, we compared the anti-ADAMTS13 neutralizing autoantibodies titre results obtained with the two most commonly used methods, the residual collagen binding activity (CBA) (6) and the fluorescence resonance energy transfer (FRET) assays (7), in a series of 30 plasma samples of acquired TTP patients. Because different experimental conditions might influence the performance of the test (1, 4, 8), we also assessed FRET inhibitor titration at different temperatures (25 °C and 37 °C).

3. MATERIALS AND METHODS

3.1. Patient samples

Thirty citrated plasma samples obtained from patients with acquired TTP, referred to our centre and enrolled in the Milan TTP Registry (www.ttpdatabase.org) were analyzed. Samples were collected during different stages of the disease: 23 samples on disease remission and 7 samples during the acute phase. None of the patients were on plasma therapy at the time of sample collection. The study was carried out with patient informed consent on the experimental nature of this study. All tested samples presented anti-ADAMTS13 autoantibodies by Western Blot analysis and IgG ELISA assay.

3.2. ADAMTS13 antigen

ADAMTS13 antigen levels were measured by an ELISA method previously described by Feys and colleagues (9) with minor modifications (10). The murine monoclonal anti-ADAMTS13 antibody 20A5 was used to immobilized the plasma protease on a microtitre plate. Bound ADAMTS13 was detected by using the biotinylated anti-ADAMTS13 monoclonal antibody 19H4 and peroxidase-labelled streptavidin (Roche Mannheim, Germany). Test plasmas (1/2-1/4) and NHP (1/2 to 1/32) were diluted in phosphate buffered saline (PBS), pH 7.4. ADAMTS13 antigen values were expressed as percentage of NHP. The lower limit of detection was 1.6% of the reference standard, the intra- and inter-assay coefficient of variations were 4% and 8% respectively. The lower limit of the normal laboratory range was 45%.

3.3. Anti-ADAMTS13 antibodies by Western Blot and ELISA

For Western Blot analysis, HEK293 cells conditioned media containing recombinant ADAMTS13 (100 ng/lane) was separated by SDS-PAGE and transferred onto a pure nitrocellulose membrane (GE Healthcare). After blocking, membranes were incubated with citrate plasma samples (1:100 in TBS, 5% skimmed milk, 0.05% Tween 20, pH 7.4) and bound anti-ADAMTS13 IgGs were detected using a peroxidase-conjugated anti-human IgG antibody (Sigma-Aldrich) and a chromogenic substrate kit (Biorad).

The ELISA method was performed as previously reported (11) with minor modifications. See chapter III, paragraph 3.6 for a detailed description of the assay.

3.4. Anti-ADAMTS13 inhibitory activity

Anti-ADAMTS13 neutralizing antibody was titrated measuring ADAMTS13 residual activity in a 1:1 mixture of heat-inactivated (1 hour incubation at 56 °C) patient plasma and pooled NHP. ADAMTS13 residual activity was measured using the two different assays under three different conditions: CBA was performed after 2 hours of incubation of NHP and heat-inactivated patient plasma mixture at 37 °C (condition 1); FRET was performed after 2 hours of incubation of NHP and heat-inactivated patient plasma mixture at 37 °C (condition 2) and at 25 °C (condition 3). ADAMTS13 residual activity was measured against a reference curve of serial dilutions of NHP, using the same preincubation time and temperature conditions of the sample mixtures. A non-incubated NHP reference curve is included in each assay to verify whether ADAMTS13 activity in preincubated plasma samples is preserved. Anti-ADAMTS13 neutralizing autoantibodies titre was considered positive when the residual activity of ADAMTS13 in heat-inactivated patient plasma and NHP mixture was less than 75% of that in the control mixture (a 1:1 mixture of buffer and NHP). The inhibitor concentration that neutralized 50% of ADAMTS13 activity in an equal volume of NHP was defined as 1 BU mL^{-1}. CBA and FRET were performed as previously described below.

3.5. Collagen binding assay (CBA)

The source of protease substrate was a purified VWF concentrate (Facteur Willebrand Humain Tres Haute Purité) provided by “Laboratoire Francais du Fractionnement et des Biotechnologies” (Lille, France). After reconstitution, at a concentration of 100U/ml, the product was aliquoted and stored at -80°C. After thawing, the VWF concentrated was diluted to a concentration of 3 U/mL in 5 mM Tris-HCl (pH 8.3), 5 M urea and incubated for 10 min at room temperature. For digestion of VWF substrate, test samples were first diluted 1/10, 1/20, and 1/40 with 2.5 M urea, 5 mM Tris (pH8.3) and 1mM Pefabloc SC (Roche,Mannheim, Germany). Then, 50 µL were transferred into a microtitre plate and incubated with 8 mM BaCl2 for 60 min at 37° C to achieve partial degradation of endogenous VWF. Subsequently, 50 µl of VWF substrate were added to test samples and incubated for 20 hours at 25°C. The day after, the incubation mixtures were centrifuged for 3 min at 2500 g, and the supernatants were used for the collagen binding assay of digested VWF substrate. Fifty µL of pooled normal human plasma (NHP) diluted 1/10, 1/20, 1/40, 1/80, 1/160, and 1/320 in 2.5 M urea, 5 mM Tris, pH 8.3 were used for the calibration curve. The collagen binding activity of the digested VWF substrate was then determined according to a previously described method (6) with minor modifications. Forty µL of digested samples were diluted with 160 µL PBS containing 0.5% BSA, 0.05% Tween 20, using a microtitre plate (Sterilin). Subsequently, 100 µL of each dilution was transferred into wells of a second multiwell plate (CovaLink) precoated with 100 µL human collagen type III (3

µg/ml) in PBS overnight at 4°C and blocked with 220 µL of PBS containing 2.5% BSA for 30 min. After incubation for 2 h, the bound VWF multimers were incubated for 1 h with 100 µL of a solution of a peroxidase-conjugated anti-VWF antibody (dilution 1/4000 in PBS containing 0.5% BSA and 0.05% Tween 20). Between each step, the microtitre wells were washed three times with 220 µL PBS. The chromogenic reaction was performed by addition of 100 µL of 0.5 mg/ml 1,2- phenylenediamine dissolved in 7.6 mM citrate, 13.2 mM Na_2HPO_4, pH 5.0, containing 0.5 µL/ml 30% (v/v) H_2O_2. The reaction was terminated by addition of 50 µL 3 M H_2SO_4 and the absorbance was read at 492 nm using a referencefilter at 620 nm on Multiskan Ascent spectrophotometer (Thermo Labsystems, Thermo Electron Corporation, Finland). ADAMTS13 activity values were expressed as percentage of NHP standard curve (100% assigned value).

Our most recent laboratory evaluation of assay reproducibility yielded an intra-assay coefficient of variation of 9% and an inter-assay coefficient of variation of 12%. The lower limit of sensitivity was 6% of protease levels in NHP taken as the reference standard. The lower value of the reference interval (45%) was set at the 5th percentile of the distribution of the values obtained in 72 healthy individuals. Severe ADAMTS13 deficiency was arbitrarily considered as levels of 10% or less of ADAMTS13 activity in plasma. Previous *in vitro* experiments showed that, using this assay, high plasma levels of VWF do not influence the measurement of ADAMTS13 activity to a major degree (12).

3.6. Fluorescence resonance energy transfer (FRET) assay

Test plasma samples (1/15 to 1/30) and NHP (1/15 to 1/480) were diluted in 5 mM Bis-Tris, 25 mM $CaCl_2$, 1mM Pefabloc SC (Roche,Mannheim, Germany), 0.005% Tween-20, pH 6 and then mixed in a 96-well white plate (Greiner) with FRETS-VWF73 substrate (Peptide International, Osaka, Japan) at a final concentration of 2 µM. Fluorescence was measured every 5 min at 37°C in a Tecan Genios spectrofluorimeter (Tecan, Vienna, Austria) equipped with a 340-nm excitation filter and a 450-nm emission filter. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min. ADAMTS13 activity values were expressed as percentage of NHP standard curve (100% assigned value). Assay reproducibility yielded an intra-assay coefficient of variation of 6% and an inter-assay coefficient of variation of 9.5%. The lower limit of sensitivity was 3% of the reference standard, the lower value of the reference interval (45%) was set at the 5th percentile of the distribution of the values obtained in 72 healthy individuals.

4. RESULTS AND DISCUSSION

Results of CBA and FRET inhibitor measurements are reported in Table 1. Spearman rank order correlation analysis revealed a good agreement between anti-ADAMTS13 neutralizing autoantibodies titres measured by the CBA method and the FRET assay performed after incubation at different temperatures for all 30 samples (CBA VS FRET–condition 2, Spearman's rho=0.92, p<0.0001; CBA VS FRET–condition 3, Spearman's rho=0.94, p<0.0001). Since the majority of acquired TTP patients with anti-ADAMTS13 neutralizing autoantibodies (around 65% in our registry) present a low titre (less than 5 BU mL^{-1}), the correlation between assays in this subgroup of samples was analyzed. The Spearman rank order correlation was still significant (CBA VS FRET-condition 2, Spearman's rho=0.78, p<0.0001; CBA VS FRET–condition 3, Spearman's rho=0.85, p<0.0001). One sample (n. 21) was found to be positive for anti-ADAMTS13 neutralizing autoantibodies using either FRET assays, but negative using CBA assay.

When plasma ADAMTS13 activity was measured under condition 2, preincubated NHP showed a decrease of enzyme activity about one third compared to non-incubated NHP. By contrast, no difference in plasma ADAMTS13 activity level between preincubated and non-incubated NHP was observed in both conditions 1 and 3 (Figure 1). To investigate the cause of ADAMTS13 temperature-dependent stability in our FRET assay, plasma pH was checked. Incubation at variable temperatures did not cause any pH variation. Then, a different source of the enzyme was tested. Recombinant ADAMTS13 was incubated for 2 hours at 37 °C or 25 °C and residual ADAMTS13 activity was measured using FRET assay. After the incubation at both temperatures, no loss of activity was observed compared to non-incubated recombinant ADAMTS13. The cause of decreased plasma ADAMTS13 activity at 37 °C observed in FRET assay may be due to the effect of the high preincubation temperature of plasma using a fluorescence detection based assay. Importantly, the anti-ADAMTS13 neutralizing autoantibodies detection by FRET assay was not affected, as demonstrated by the correlation analysis (Table 1). Moreover, the similar results obtained at 37 °C and 25 °C suggest that the reaction between ADAMTS13 and anti-ADAMTS13 neutralizing autoantibodies is not temperature-dependent *in vitro*.

Table 1. Anti-ADAMTS13 neutralizing antibodies titres in 30 plasma samples as determined by the different assays. Values of anti-ADAMTS13 IgGs and ADAMTS13 antigen as determined by ELISA assay and ADAMTS13 activity as determined by CBA assay are also reported. All samples presented anti-ADAMTS13 autoantibodies by Western Blot analysis.

Sample	Anti-ADAMTS13 Inhibitor (BU mL^{-1})			Anti-ADAMTS13 IgG (%)	ADAMTS13 antigen (%)	ADAMTS13 activity (%)
	CBA 37°C	FRET 37 °C	FRET 25 °C			
	≥0.4[a]	≥0.4[a]	≥0.4[a]	>1.18[a]	40-155[b]	50-158[b]
1	2	1.8	2	6	49	<6
2	2	2.2	3.9	12	25	<6
3	12.5	14	22	117	52	<6
4	16	11	12.5	130	72	<6
5	2.5	3	2.2	14	43	<6
6	2.7	4	3.3	21	41	<6
7	6	5.8	10.5	75	40	<6
8	1.4	1.5	1.9	16	18	<6
9	1	2.3	2.2	15	47	<6
10	1	0.8	0.6	2.8	95	<6
11	13	25	17	285	29	<6
12	3	3.5	7	15	40	9
13	10	13	12	65	63	<6
14	5.5	5	4	15	176	<6
15	6	5.8	10	38	55	<6
16	12.5	12.5	12.5	128	36	<6
17	0.6	1.1	1.2	18	<1	10
18	1.1	3.8	3	10	68	<6
19	2.5	5.2	6	20	20	<6
20	4	8.3	11	60	102	<6
21	<0.4	1.6	1.2	2	70	27
22	1	0.7	1	4.8	53	55
23	18	20	27	35	14	<6
24	4.5	5	6	31	52	<6
25	2	2	2.3	6	82	<6
26	3	3	4.1	38	59	<6
27	2	2.5	2.7	18	33	<6
28	3.7	3.3	3.1	21	33	<6
29	20	20	28	135	38	<6
30	3	3.6	4.1	28	97	<6
Median	3	3.7	4.1	20.5	48	3
Min	0.2	0.7	0.6	2	0.5	3
Max	20	25.0	28.0	285	176	55
All samples (n=30)	R_s*	0.92	0.94			
	Slope*	1,04	1,26			
	y-intercept*	0,72	0,63			
CBA Inhibitor ≤ 5 BU mL^{-1} (n=20)	R_s*	0.78	0.85			
	Slope*	1.16	1.61			
	y-intercept*	0.46	-0.04			

* Spearman's rho, slope and y-intercepts of the regression lines between CBA assay and each type of FRET assay. Values below the detection limit of 0.4 BU mL^{-1} (ADAMTS13 inhibitor assays), 6% (ADAMTS13 activity assay, CBA) and 1% (anti-ADAMTS13 IgG ELISA assay) were arbitrarily set to 0.2 BU mL^{-1}, 3% and 0.5% for calculations, respectively. Analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA, USA). [a] Cut-off value, [b] Normal range

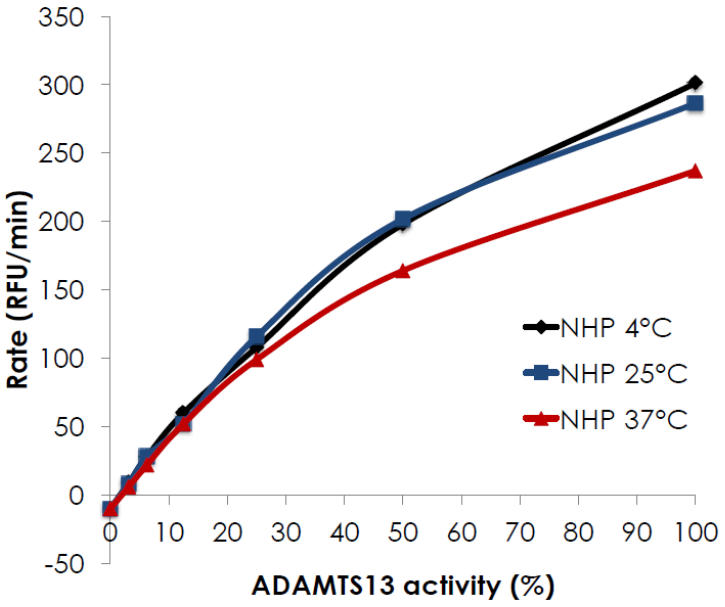

Figure 3. Representative NHP standard curves obtained by FRET assay. Non-incubated NHP (black), NH preincubated for 2 h at 25°C (blue) and NHP preincubated for 2 h at 37°C (red) are depicted.

In conclusion, FRET and CBA-based inhibitor measurement performed similarly. FRET, which is more rapid and easier to perform than CBA, could hence be the assay of choice for inhibitor quantification. Moreover, incubation between heat-inactivated patient plasma and NHP could be performed at 25 °C, where ADAMTS13 recovery is maintained. Considering the therapeutic potential of restoring the enzyme deficiency in acquired TTP patients (5), the rapid and precise measurement of anti-ADAMTS13 neutralizing antibodies would be pivotal for the success of a therapeutic approach using recombinant ADAMTS13 to overcome the inhibitory activity of anti-ADAMTS13 neutralizing autoantibodies.

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CHAPTER V

FRET rather than CBA results reflect ADAMTS13 activity level *in vivo* in samples from acquired thrombotic thrombocytopenic purpura (TTP) patients with discordant results between the two assays

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1. SUMMARY

Background. Collagen binding assay (CBA) and fluorescence resonance energy transfer (FRET) are two widely adopted methods for the measurement of the plasmatic activity of ADAMTS13, the von Willebrand factor (VWF) cleaving-protease. Accurately measuring ADAMTS13 plasmatic activity is essential in the management of thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy (TMA) often characterized by severely reduced ADAMTS13 activity. The finding of severely reduced ADAMTS13 activity (activity below 10% of normal) has important prognostic implications, being associated with 10-fold increased risk of disease recurrence. Despite a good agreement between the two assays in the measurement of normal plasmas, discrepant results have been reported in up to 10% of TMA cases. The cause for the observed discrepancies is unknown, but it has been suggested that the use of a denaturing agent (i.e. urea) in CBA may dissociate complexes between ADAMTS13 and anti-ADAMTS13 antibodies, thus generating spuriously-high ADAMTS13 activity results.

Aims. To determine whether FRET or CBA results reflect ADAMTS13 activity level *in vivo* in samples from autoimmune TTP patients with discordant results between the two assays and to evaluate the role of denaturing agents in the determination of discrepant results.

Methods. Twenty discordant samples (FRET <10% and CBA >20%) and 11 concordant samples (FRET and CBA <10%) with anti-ADAMTS13 antibodies from patients with autoimmune TTP were selected from the Milan TTP Registry (URL:http://www.ttpdatabase.org/). The analysis of the VWF multimeric pattern was performed on discordant samples collected during disease remission (13/20). ADAMTS13 activity was measured under flow conditions in 10/20 discordant and 4/11 concordant samples. FRET assay in presence of urea 1,5 M was performed in 19 discordant and 11 concordant samples.

Results. All discordant samples showed a ratio of high molecular weight VWF multimers higher than normal, due to the presence of ultra large VWF multimers. Under flow conditions, all tested samples showed reduced ADAMTS13 activity (range: 0%-30%). In FRET experiments performed in presence of urea, ADAMTS13 activity levels became detectable and/or overcame the 10% activity cut-off in 11 out of 19 discordant samples, whereas it remained undetectable in all 11 concordant samples tested (Fisher's exact test, p=0.0016).

Conclusions. The presence of ultra large VWF multimers and the reduced ADAMTS13 activity observed under flow conditions in discordant TTP samples indicate a deficiency of the enzyme, supporting FRET over CBA results. This study showed that the presence of urea in CBA could lead to spurious and misleading results in measuring ADAMTS13 activity. ADAMTS13 activity assays which do

not require any denaturing agents should be considered more reliable when assessing the presence of severe ADAMTS13 deficiency in patients with autoimmune TTP.

2. INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a rare life-threatening microangiopathy characterized by thrombocytopenia, hemolitic anemia and signs and symptoms of ischemic organ dysfunctions due to the persistence of highly thrombogenic ultra large (UL) von Willebrand factor (VWF) multimers in the microcirculation (1, 2). TTP is associated with the severe deficiency of the VWF-cleaving protease ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif) (3-5), owing to mutations in *adamts13* gene (congenital TTP) (6) or to the development of anti-ADAMTS13 autoantibodies (acquired TTP) (4). In the absence of ADAMTS13, highly adhesive ULVWF, that spontaneously aggregate platelets, accumulates promoting platelet thrombi formation and eventually leading to the clinical features typical of TTP (2).

Since the discovery of the molecular mechanisms involved in the pathogenesis of TTP, many assays for the measurement of ADAMTS13 activity have been developed (7) and several studies have been carried out to assess their performance (8, 9) and utility as diagnostic and prognostic tools in the treatment of this disease (10-19). Severe ADAMTS13 deficiency (activity below 10% of normal) has been reported to be predictive of a risk for relapse either at the time of the initial episode (11, 14, 18) and during remission (16, 19, 20). In the recent update of the Oklahoma TTP Registry conducted by Kremer-Hovinga *et al.*, relapse rate was shown to be greater among patients with ADAMTS13 activity <10% at presentation, with an estimated risk for relapse at 7.5 years of 41% (18). In a retrospective study conducted on the Milan TTP Registry, patients with severe ADAMTS13 deficiency during remission had a 5-times higher hazard of recurrence, compared with patients with ADAMTS13 activity ≥10% (19) (see chapter III). Therefore, accurately measuring ADAMTS13 plasmatic activity is essential in the management of TTP patients.

Collagen binding assay (CBA) and fluorescence resonance energy transfer (FRET) are two widely adopted methods for the measurement of ADAMTS13 activity. CBA and FRET assay differ for the substrate used (full-length VWF VS VWF73 peptide), for the use of denaturing agents (urea VS none) and for the detection method (indirect detection of cleavage products through absorbance measurement VS direct detection of cleavage products through fluorescence measurement). Despite a good agreement between the two assays (21, 22), discordant results (severe ADAMTS13 deficiency detected by only one of the assays) have been reported in up to 10% of TMA cases (22). The cause for the observed discrepancies is currently unknown, but an association with the presence of anti-ADAMTS13 antibodies was reported (22). It was speculated that the use of a denaturing agent (i.e. urea) in CBA may dissociate complexes between ADAMTS13 and anti-ADAMTS13 antibodies, thus generating spuriously-high ADAMTS13 activity results (22).

The aim of this study was to investigate whether the CBA or the FRET assays results reflect the real ADAMTS13 activity level *in vivo* in samples from acquired TTP patients with discordant results between the two assays and circulating anti-ADAMTS13 antibodies. In order to determine if ADAMTS13 activity was indeed suppressed (FRET) or not (CBA), an analysis of the multimeric pattern of VWF and the measurement of ADAMTS13 activity under flow conditions were performed. Moreover, the role of denaturing agents in the determination of the observed discrepancies was evaluated.

3. MATERIALS AND METHODS

3.1. Patients, samples and definition of discrepancy

A cohort of 372 patients with acquired TTP was retrospectively enrolled in the International Registry of TTP (www.ttpdatabase.org) of the Milan Hemophilia and Thrombosis Centre from 1994 to June 2012. The diagnosis of TTP was made on the basis of previously reported criteria (23). The study was carried out with patient informed consent on the experimental nature of this study and after approval by the Institutional Review Board of the IRCCS Ca' Granda, Ospedale Maggiore Policlinico.

Forty-nine samples with discordant values between CBA and FRET assays and 143 concordant samples with severe ADAMTS13 deficiency by both assays were selected for this study. All samples were positive for anti-ADAMTS13 antibodies. To define discrepancy, a 10% ADAMTS13 activity level was assumed as cut-off, being 10% the plasma level that indicates severely reduced activity (22). Samples were considered discordant when ADAMTS13 activity was below 10% using only one of the two assays. Conversely, samples were defined concordant when ADAMTS13 activity level was below or above 10% by both assays. In our cohort, all discordant samples presented ADAMTS13 activity below 10% by FRET and above 10% by CBA. Due to plasma availability, 20 out of 49 discordant samples were analyzed for VWF multimers pattern, FRET experiments in presence of urea and for ADAMTS13 activity measurement under flow conditions.

All acute plasma samples were collected before plasma therapy. None of the selected samples presented high level of bilirubin that could interfere with FRET assay (24).

3.2. Sample collection and preparation

Venous blood samples for the measurements of ADAMTS13 (activity and antigen, anti-ADAMTS13 antibodies) were collected into evacuated tubes containing 3.2% trisodium citrate at a 9:1 volume/volume ratio, whereas EDTA was used as anticoagulant to collect samples for the evaluation of VWF multimers pattern. Platelet-poor plasma was obtained by centrifugation at 3200 g for 20 minutes at 4°C, aliquoted and stored at -80°C until tested. A pooled normal human plasma (NHP) collected from 70 healthy donors was used as the reference standard.

For ADAMTS13 activity measurement under flow conditions, citrated patient plasma samples and NHP were recalcified by the addition of 25 mM $CaCl_2$. Samples were incubated for 3 h at room temperature, centrifuged for 10 minutes at 13000 rpm to remove the clot and diluted in Zn^{2+} and Ca^{2+}-containing Hepes buffered saline (HBS; 50 mM HEPES, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 150 mM NaCl, pH 7.4) buffer at 1:1 v/v. As source of platelets, blood was drawn into acid-citrate dextrose anticoagulant from healthy volunteers who gave informed consent. Washed platelets were prepared as previously described (25), diluted at a platelet count between 20,000 and 30,000 platelets per µL

in Hepes Tyrode's buffer (137 mM NaCl, 2 mM KCl, 3 mM NaH2PO4, 1 mM MgCl2, 5.5 mM glucose, 5 mM Hepes, 12mM NaHCO3, pH 7.4) and fluorescently labeled with 200 nM 3,3'-dihexyloxacarbocyanine iodide (DIOC6, Invitrogen) before flow assay.

3.3. Endothelial Cell Culture

Blood outgrowth endothelial cells (BOECs) were isolated from human healthy volunteers and characterized by immunofluorescent microscopy as described previously (26, 27). BOECs (split rate 2–6) were grown on collagen-coated cover slides in EBM-2/EGM-2 culture medium (Lonza, San Diego, CA) as reported (26). In brief, nitrogen stored aliquots were quickly defrosted at 37°C and transferred into tissue-culture flasks, previously coated with rat tail collagen type I (Becton Dickinson, San Diego, CA). The flasks were incubated at 37°C with 5% CO2. After 24 hours, nonadherent cells were removed by washing with EBM-2/EGM-2 culture medium (Clonetics, San Diego, CA). Thereafter, medium was changed every other day until the isolated BOECs reached near confluency. Cells were passed by lifting the cells with 0.05% trypsin (Gibco) and plated onto collagen-coated cover slides for flow experiments.

3.4. Laboratory measurements

3.4.1. ADAMTS13 activity

ADAMTS13 activity was measured using three different assays, two performed under static condition (CBA and FRET) and one performed under flow conditions (flow-based assay). CBA (16, 28) and FRET (22, 29) assays were performed essentially as described with minor modifications. For a detailed description of CBA and FRET assay, see chapter IV, paragraph 3.5 and 3.6, respectively. In some FRET experiments, urea was added to assay buffer at a final concentration of 1,5 M. After a 2 h incubation, FRET assay was performed as described before. Results were expressed as the mean of three independent determinations. A plasma sample derived from a healthy donor was used as control in each test. In preliminary experiments, different concentrations of urea ranging from 1 to 2,5 M were tested for assay set-up. A final concentration of 1.5 M was chosen for TTP samples testing. For statistical analysis, ADAMTS13 activity values measured in presence of urea were considered increased compared with those measured in absence of urea according to the following criteria: (1) when ADAMTS13 activity became detectable or (2) when ADAMTS13 activity value was equal or above 10% (the level assumed to define severe deficiency).

The flow-based assay was performed as previously reported (25). Briefly, blood outgrowth endothelial cells (BOEC) were grown on a collagen-coated coverslip and stimulated with 25 µM histamine for 10 min at room temperature. Coverslips were mounted in a parallel-plate flow

chamber and perfused with DIOC6-fluorescently labeled washed platelets at a shear stress of 2,5 dynes/cm^2 for 120 s, followed by HBS buffer, NHP or TTP patient plasmas for additional 180 s. The formation and disappearance of platelet-decorated VWF strings were monitored in real time using an Eclipse TE200 inverted fluorescence microscope (objective 20X, 0.4 NA) (Nikon Instruments, Melville, NY) coupled to a Hamamatsu CCD camera (ORCA®-R2, Hamamatsu Photonics, Hamamatsu City, Japan). Pictures were taken every 200 ms using the Hokawo software (Hamamatsu Photonics) for 240 s starting 60 s after the initiation of flow. Endothelial cell-anchored VWF strings were counted every 10 seconds and the percentage of remained VWF strings in function of time was calculated. ADAMTS13 activity of TTP samples was expressed as the percentage of disappeared VWF strings compared with NHP after 240 s of perfusion, after blank (buffer) subtraction. Due to the large volume of plasma requested, samples could not be tested more than two times. Results were expressed as the mean of two determinations.

In control experiments, 4 acquired TTP plasma samples collected in the acute phase, with undetectable ADAMTS13 activity by both static assays were tested. A plasma sample from a congenital TTP patient with undetectable ADAMTS13 activity and antigen levels was also tested.

3.4.2. ADAMTS13 antigen

ADAMTS13 antigen levels were measured by an ELISA method previously described by Feys and colleagues (30) with minor modifications (16). The murine monoclonal anti-ADAMTS13 antibody 20A5 was used to immobilized the plasma protease on a microtitre plate. Bound ADAMTS13 was detected by using the biotinylated anti-ADAMTS13 monoclonal antibody 19H4 and peroxidase-labelled streptavidin (Roche Mannheim, Germany). Test plasmas (1/2-1/4) and NHP (1/2 to 1/32) were diluted in phosphate buffered saline (PBS), pH 7.4. ADAMTS13 antigen values were expressed as percentage of NHP. The lower limit of detection was 1.6% of the reference standard, the intra- and inter-assay coefficient of variations were 4% and 8% respectively. The lower limit of the normal laboratory range was 45%.

3.4.3. Anti-ADAMTS13 antibodies by Western Blot and ELISA

For Western Blot analysis, HEK293 cells conditioned media containing recombinant ADAMTS13 (100 ng/lane) was separated by SDS-PAGE and transferred onto a pure nitrocellulose membrane (GE Healthcare). After blocking, membranes were incubated with citrate plasma samples (1:100 in TBS, 5% skimmed milk, 0.05% Tween 20, pH 7.4) and bound anti-ADAMTS13 IgG was detected using a peroxidase-conjugated anti-human IgG antibody (Sigma-Aldrich) and a chromogenic substrate kit (Biorad).

The ELISA method was performed as previously reported (31) with minor modifications. See chapter III, paragraph 3.6 for a detailed description of the assay.

3.4.4. Anti-ADAMTS13 antibodies inhibitory activity

Anti-ADAMTS13 antibodies inhibitory activity was measured by a CBA-based mixing assay as previously reported (32). Heat-inactivated (1 hour incubation at 56°C) patient plasma and pooled NHP were mixed at 1:1 dilution and incubated for 2 hours at 37°C. ADAMTS13 residual activity was measured using CBA as already described. Anti-ADAMTS13 inhibitor titre was considered positive when the residual activity of ADAMTS13 in heat-inactivated patient plasma and NHP mixture was less than 50% of that in the control mixture (a 1:1 mixture of buffer and NHP). The inhibitor concentration that neutralized 50% of ADAMTS13 activity in an equal volume of NHP was defined as 1 BU mL^{-1}.

3.4.5. VWF antigen and multimeric analysis

VWF antigen (VWF:Ag) was quantified using the commercial Kit "von WILLEBRAND FACTOR Antigen" (Instrumentation Laboratory) on the ACL TOP 500 Analyzer (Instrumentation Laboratory). VWF multimers were evaluated in samples collected during disease remission, as they are known to be consumed in the acute phase (32). The multimeric pattern of VWF molecule was analyzed by sodium dodecyl sulfate-agarose gel electrophoresis followed by luminographic visualization as previously described (32). Samples were diluted in order to obtain a final VWF antigen concentration of 10% and electrophoresis was carried out in 1% high gelling temperature agarose (SeaKem® HGT). After electrophoresis, proteins were electrotransferred to an immobilon polyvinylidene fluoride membrane (Millipore, Billerica, MA) 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 membranes were stained with a luminol-iodophenol solution and the densitometric analysis was performed with Typhoon 8600 Variable Mode Imager (Image Quant TM Software; Amersham Biosciences, Uppsala, Sweden). Quantitative evaluation of multimers distribution was performed according to Budde and Scheneppenheim (Rev Clin Exp Hematol. 2001;5:335-68) by dividing the plasma lane from the dye front into: small (1–5), intermediate (6-10) and large (> 10) multimers. Results were expressed as the ratio between the proportion of large (or high molecular weight, HMW) multimers in each sample and that of the reference pooled normal plasma within the same gel. The normal range for HMW multimers ratio in 36 healthy subjects was 0.85-1.21.

3.4.6. Statistical analysis

The Fisher's Exact test and the Mann-Whitney U-test were used to compare categorical and continuous variables in different study groups, respectively. Values below the limit of detection (LOD) were assigned a value equal to 50% of the LOD. A P value of 0.05 was considered statistically significant.

4. RESULTS

4.1. Discordant samples and anti-ADAMTS13 antibodies measurements

Over the study period (1994-2012), 569 samples from acquired TTP patients collected during the acute phase before plasma exchange or during disease remission were analyzed for ADAMTS13 activity using both CBA and FRET assays and for the presence of anti-ADAMTS13 antibodies (Figure 1). Among these, 59 discordant samples (10%) having ADAMTS13 activity <10% by FRET assay and >10% by CBA were individuated. The frequency of anti-ADAMTS13 antibodies by Western Blot analysis or ELISA was higher in discordant samples compared with concordant samples (47/59 VS 316/510, p=0.0066), confirming previous observations (22). Anti-ADAMTS13 IgG titres measured by ELISA and the presence of anti-ADAMTS13 inhibitors were assessed in 47 discordant samples with anti-ADAMTS13 antibodies. The results were compared with those obtained from 143 concordant samples with ADAMTS13 antibodies and severe ADAMTS13 deficiency by both CBA and FRET assays. Discordant samples presented lower anti-ADAMTS13 IgG titres (Mann-Whitney test, p<0.0001) and a lower anti-ADAMTS13 inhibitor frequence (Fisher's exact test, p<0.0001) compared with concordant samples.

Due to plasma availability, further analysis were performed on 20 discordant samples (Figure 1). Laboratory data of the selected samples are reported in Table 1.

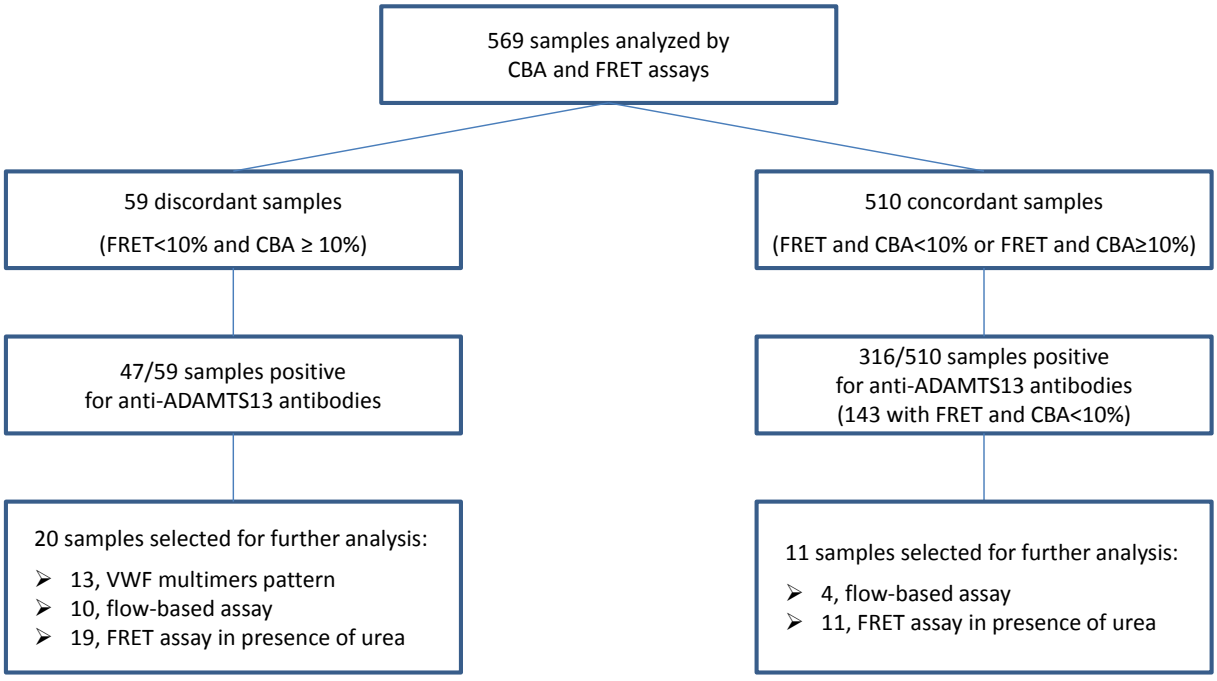

Figure 4. Study flow chart.

			ADAMTS13 activity measurements					Anti-ADAMTS13 antibodies measurements			VWF measurements		
Patient	**Sample**	**Disease phase**	**CBA (%, 50-158)**	**FRET (%, 45-138)**	**FRET Urea (%)**	**Flow assay (%)**	**ADAMTS13:Ag (%, 40-155)**	**WB**	**ELISA (%, >1.18)**	**CBA-Inhibitor (BU/mL, <1)**	**VWF:Ag (%, 40-169[1])**	**ULVWF**	**HMW ratio (0.85-1.21)**
A	1	A	21	<3	<3	ND	3	+	<1.18	<1	102	NA	NA
B	2	R	21	9	9	ND	28	+	107	<1	98	+	1.52
C	3	A	22	7	**18**	ND	24	+	<1.18	<1	246	NA	NA
D	4	R	22	5	<3	ND	63	+	10	<1	139	+	1.32
E	5	R	23	<3	<3	ND	33	+	32	<1	177	+	1.52
F	6	R	24	<3	**5**	ND	38	+	7	<1	95	ND	ND
G	7	R	26	7	9	ND	31	+	6	<1	72	+	1.22
H	8	R	26	6	**10**	ND	21	+	2.8	ND	162	+	1.46
I	9	R	28	<3	<3	ND	70	+	6	<1	135	+	1.35
D	10	R	30	<3	<3	17	54	+	1.2	<1	145	+	1.51
L	11	A	40	<3	**11**	19	49	+	11	<1	159	NA	NA
M	12	A	40	<3	**9**	0	3	+	<1.18	<1	209	NA	NA
N	13	R	42	5	5	29	49	+	<1.18	<1	158	-	1.27
O	14	A	48	<3	**7**	21	64	+	<1.18	<1	193	NA	NA
P	15	R	56	<3	ND	14	100	+	6	<1	213	ND	ND
Q	16	R	80	4	**30**	24	68	-	2.2	ND	105	+	1.43
Q	17	R	93	<3	**30**	26	70	-	3	ND	72	+	1.47
Q	18	R	93	<3	**11**	23	85	+	3.2	ND	93	+	1.23
Q	19	R	97	<3	**20**	12	62	-	1.2	ND	66	+	1.38
R	20	R	101	5	**27**	ND	51	+	2	<1	404	+	1.40

Table 1. ADAMTS13 and VWF-related laboratory data of discordant samples from acquired TTP patients. 20 samples (1-20) from 16 patients (A-R; patients D and Q present multiple samples) were selected for this study. Samples are ordered according to CBA results, from lowest to highest value. Normal ranges or cut-offs are indicated between parentheses. Results of FRET assay that significantly increased in presence of urea ("FRET urea") are indicated by bold numbers.
Disease phase: A, acute; R, remission. WB: Western Blot. +, positive; -, negative; ND, not done (due to lack of plasma); NA, not applicable (samples collected at acute phase).
1 Normal range for individuals with blood group O is reported. Normal range for individuals with blood group non-O was 55%-165%.

4.2. Multimeric analysis of VWF

To investigate which assay results reflect the real ADAMTS13 activity level *in vivo* (i.e. severely reduced activity in case of FRET or moderately reduced to normal activity in case of CBA), the multimeric pattern of VWF was evaluated in 13 discordant samples collected during disease remission. All tested samples but one (n. 13) presented UL-VWF (Figure 2), indicating a deficiency of ADAMTS13. Due to the presence of UL-VWF, densitometric analysis of multimers distribution in discordant plasma samples showed a HMW multimers ratio higher than normal range (mean ± SD, 1.40 ± 0.10; normal range: 0.85-1.21) (Table 2). Despite the absence of UL-VWF, a pathologically high HMW multimers ratio (1.27) indicating an abnormal proteolysis of VWF by ADAMTS13 was also found in sample n. 13.

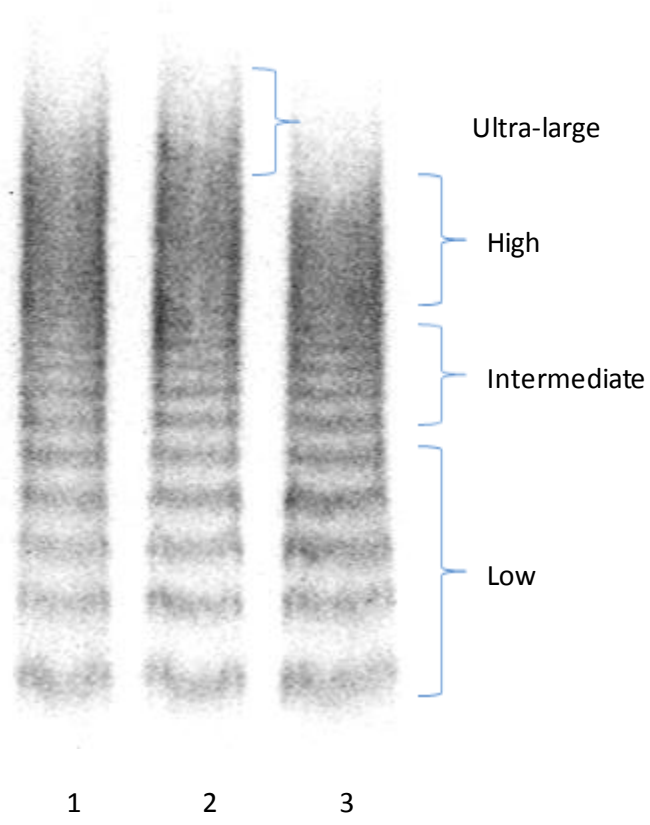

Figure 2. Representative pattern of VWF multimers in discordant TTP samples. Lane 1: positive control, sample collected during disease remission presenting undetectable ADAMTS13 activity by both assays; **lane 2:** discordant sample n. 8; **lane 3:** pooled normal human plasma. Low, intermediate, high molecula weight and ultra-large VWF multimers are highlighted by parentheses at the right side of the figure.

Among the 13 samples in which VWF multimers pattern was evaluated, sample n. 20 showed an extremely high VWF antigen level (404%) (Table 2). In this particular case, the presence of UL-VWF

might be also explained by a massive secretion of VWF, with consequent unbalancement of the VWF-ADAMTS13 axes. No clinical data at the time of sample collection was available to explain such high VWF antigen value, however the patient died for brain cancer a year later.

4.3. ADAMTS13 activity assay under flow conditions

A flow-based assay, in which endothelial cell-anchored VWF strings are used as ADAMTS13 substrate, was used to evaluate ADAMTS13 activity in 10 discordant samples (Table 2) and 4 concordant samples collected in the acute phase before plasma treatment. In a preliminary set-up series of experiments, we determined the disappearance of VWF strings in the presence or absence of NHP, by calculating the percentage of remaining strings in function of time. In the presence of buffer alone, 91±4% (n=10) of the released VWF strings remained attached to the endothelial surface after 240 s of perfusion. Conversely, perfusion with NHP resulted in the gradual disappearance of VWF strings, 49%±12% (n=11) after 240 s of perfusion. As negative control, a congenital TTP plasma sample with undetectable ADAMTS13 antigen and activity was used: 92% of VWF strings persisted after 240 s of perfusion, indicating that the VWF strings cleavage was ADAMTS13-specific.
ADAMTS13 activity of TTP samples was then expressed as the percentage of disappeared VWF strings compared with NHP. All samples tested showed reduced ADAMTS13 activity (range: 0%-29%), similar to those obtained in concordant samples (range: 0%-16%) (Figure 5). Moreover, all samples, with the exception of number 13 (29% activity), presented an ADAMTS13 activity value below that of a 1/10 mixture of NHP and heat-inactivated NHP (27% activity).

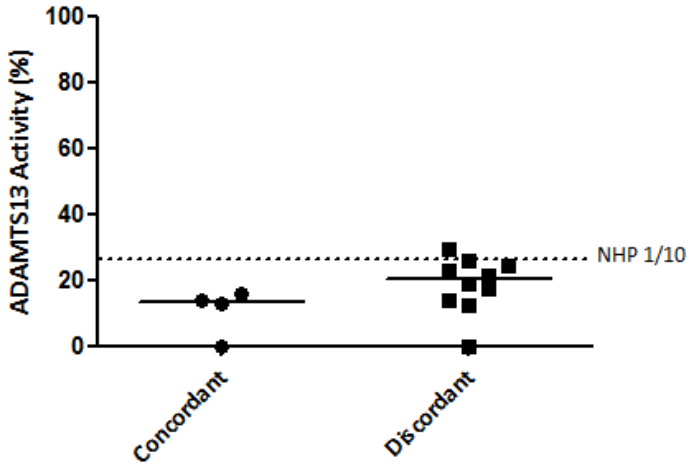

Figure 5. Scatter plot of ADAMTS13 activity values measured under flow conditions. ADAMTS13 activity values of concordant samples collected in the acute phase (n=4) and discordant samples (n=10) are depicted. Median values are indicated by solid lines. The dashed line indicates the ADAMTS13 activity value obtained diluting NHP 1/10 in heat-inactivated NHP (27% of VWF strings disappeared compared with NHP).

4.4. ADAMTS13 activity measured by FRET assay under denaturing conditions

Preliminary experiments were performed to evaluate the influence of different concentration of urea on the measurement of ADAMTS13 activity of NHP by FRET assay. Three concentrations of urea were tested: 1.5 M, 2 M and 2.5 M (Figure 3). In the presence of 2.5 M urea, which is the concentration used in CBA assay to unfold VWF, ADAMTS13 activity was completely abolished. A concentration of 1.5 M urea, which gave an approximately 30% reduction of ADAMTS13 activity, was chosen for further experiments (Figure 3). A normal plasma sample was used as control in each assay. No difference was observed in absence and presence of urea (n=6, 128% ± 4% VS 126% ± 4%).

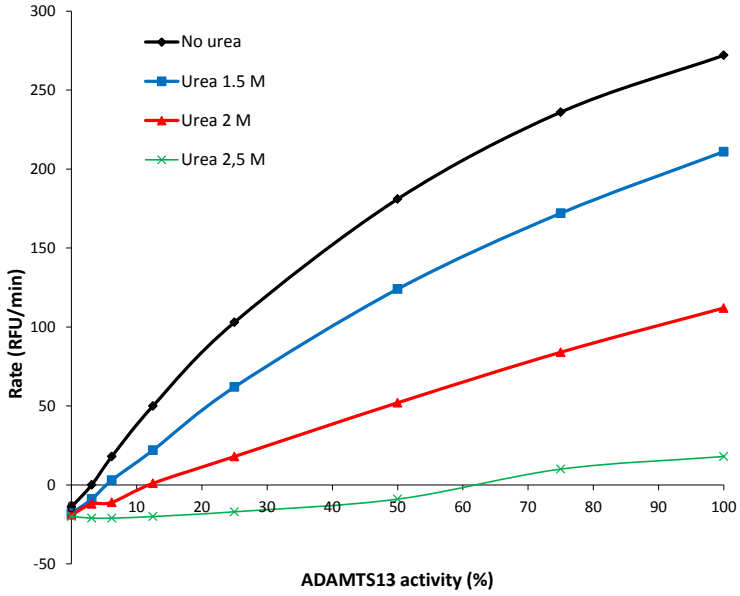

Figure 3. NHP standard curves obtained by FRET assay in absence (black) and presence of 1.5 M (blue), 2 M (red) and 2.5 M (green) urea.

The results of FRET experiments in presence of urea 1.5 M are reported in Table 2 and depicted in Figure 4. Under this condition, ADAMTS13 activity levels became detectable and/or overcame the 10% activity cut-off for severe ADAMTS13 deficiency in 11 out of 19 discordant samples (Table 2, bold numbers), whereas it remained undetectable in 11 concordant samples tested for comparison (p=0.0016). However, increased ADAMTS13 activity levels in discordant samples were always lower than those measured in CBA. This result may depend on the different concentrations of urea used (2.5 M in CBA VS 1.5 M in FRET).

Among discordant samples, 6 derived from the same 2 patients (n. 4, 10, patient D; n. 16-19, patient Q). The difference between discordant and concordant samples was still significant when only one sample per patient was considered (7/15 VS 0/11, p=0.0103).

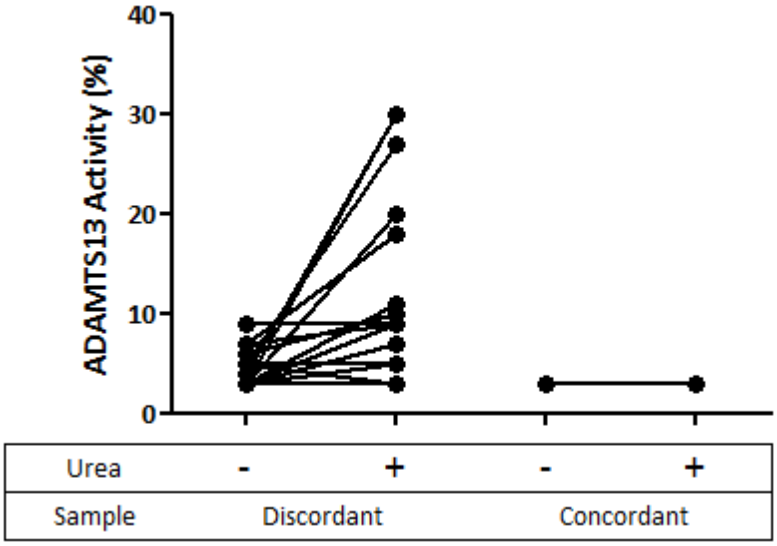

Figure 4. Paired comparisons of ADAMTS13 activity values measured by FRET assay in absence (-) and presence (+) of urea in discordant (left) and concordant (right) samples. Samples with the same ADAMTS13 activity values are superimposed. A value of half the limit of detection (1.5% activity) has been arbitrarly used to depict samples with ADAMTS13 activity values below FRET assay limit of detection (3% activity).

5. DISCUSSION

An ADAMTS13 activity level below 10% of normal, either at acute episode (11, 14, 18) and during disease remission (16, 19, 20), is associated with a higher risk of recurrence in TTP patients. Thus, the availability of a specific, sensitive and reliable assay for the assessment of ADAMTS13 severe deficiency is pivotal for the management of this life-threatening disease. CBA and FRET assays are two widely used methods for the quantification of ADAMTS13 activity. The two assays showed a good agreement in comparative studies (21, 22), nevertheless important discrepancies have been reported in a small but relevant percentage of acquired TTP cases (22). It was suggested that the denaturing agent (i.e. urea) required in CBA to unfold VWF, could dissociate ADAMTS13/anti-ADAMTS13 antibodies immune complexes, artifactually increasing ADAMTS13 activity measured in CBA (22). The aim of this study was to investigate whether CBA or FRET assay results reflect ADAMTS13 activity *in vivo* in a large number of discordant samples (FRET <10% and CBA ≥10%) presenting anti-ADAMTS13 antibodies and if urea is actually a cause of this phenomenon.

In order to assess whether ADAMTS13 activity is suppressed (FRET) or not (CBA) in discordant TTP samples, the analysis of VWF multimeric pattern and the measurement of ADAMTS13 activity in a flow-based assay were performed. All discordant samples collected during the remission phase (13 out of 18 tested) presented a proportion of HMW VWF multimers higher than normal, due to the presence of UL-VWF except for one case. This result indicates a deficiency of the enzyme, as demonstrated in a previous study (32). Lotta *et al.* indeed showed that, during remission, plasma from TTP patients with severely reduced ADAMTS13 activity presents a higher ratio of HMW multimers compared with patients having normal levels of the metalloprotease, who in turn display a HMW multimers ratio similar to that obtained in healthy donors (32).

In the flow-based assay, secreted UL-VWF multimers are anchored to endothelial cells and stretched by flow, which render the VWF scissile bond accessible to ADAMTS13 cleavage. As previously reported, this assay may only be reliable in discriminating ADAMTS13 levels higher or lower than 20%, without measuring a precise value of ADAMTS13 activity (9). Nevertheless, it has the great advantage of employing a physiological substrate (full-length VWF) in a physiological environment (platelets and endothelial cells on a collagen-coated surface), under physiological experimental conditions (flow). In presence of discordant plasmas (n=10, ADAMTS13 activity <10% by FRET and >30% by CBA), 0%-29% of VWF strings disappeared (compared with pooled normal plasma). Similarly, 0-16% of VWF strings disappeared when concordant plasmas with undetectable ADAMTS13 activity by both static assays were perfused. Moreover, 9 out of 10 discordant samples presented an ADAMTS13 activity value below that of a 1/10 mixture of NHP and heat-inactivated NHP (27% of

VWF strings disappeared). Flow-based assay results are consistent with a reduced ADAMTS13 activity in discordant samples.

All together, the analysis of VWF multimeric pattern and the measurement of ADAMTS13 activity under flow conditions support FRET over CBA results. Flow-based assay results suggest also that the different type of substrate, full-length- (CBA and flow-based assay) or peptide-based (FRET assay) VWF, is not the cause of the observed discrepancies, at least in our cohort of samples.

To investigate the role of denaturing agents, FRET assay was performed in presence of 1.5 M urea. Using this modified assay, no difference was observed in concordant samples with undetectable ADAMTS13 activity by both CBA and FRET assays. On the contrary, in 50% of discordant samples, ADAMTS13 activity levels became detectable and/or overcame the 10% activity cut-off for severe ADAMTS13 deficiency, suggesting urea as one of the possible cause of discordant results.

It was previously speculated that urea may act dissociating the immune complexes formed between ADAMTS13 and anti-ADAMTS13 antibodies (22). These immune complexes could vary in terms of affinity and/or avidity between different patients or within the same patient during the course of the disease. It has been formerly shown that anti-ADAMTS13 antibodies of different Ig class and IgG subclass are present in patients with autoimmune TTP and that the prevalence of each Ig class/subclass vary between different patients (those experiencing recurrencies and those who do not) and between the two phases of the disease (acute and remission) (19, 33). Furthermore, several epitope mapping studies showed that anti-ADAMTS13 antibodies target multiple domains of the metalloprotease: 97-100% of acquired TTP patients present antibodies against a major epitope in the spacer domain of ADAMTS13, however antibodies directed towards the C-terminal domains have been described (34-37). Zheng *et al.* reported that patients whose antibodies bound only to the N-terminal domains presented higher IgG titres than patients with antibodies against both the N-terminal and the C-terminal domains of ADAMTS13 (36). In another study, Pos *et al.* reported that 1% Triton X-100 (a commonly used detergent for solubilizing proteins and reducing non-specific interactions) disrupted the binding of anti-ADAMTS13 antibodies to ADAMTS13 C-terminal domains (37). These observations suggest that autoantibodies directed towards the C-terminal domains could be less affine than those recognizing the spacer domain. On the whole, these data support the hypothesis that ADAMTS13/anti-ADAMTS13 antibodies immune complexes with different affinity may be present in patients with acquired TTP. In our cohort, the frequency of anti-ADAMTS13 inhibitors and anti-ADAMTS13 IgG titres were lower in discordant samples compared with concordant samples with severe ADAMTS13 deficiency, suggesting that anti-ADAMTS13 antibodies with lower affinity/avidity may be present in the first group of samples. Consequently, it is reasonable to conclude that urea present in CBA may more easily dissociate such weaker immune complexes, thus generating spuriously-high ADAMTS13 activity results. It is as much

worth-considering that not all discordant samples tested turned concordant in FRET assay after the addition of 1.5 M urea. On the other hand, CBA was performed at a higher concentration of urea, 2.5 M, which could not be replicated in FRET assay because it completely abolished ADAMTS13 activity. This allow us to suppose that the interaction between ADAMTS13 and anti-ADAMTS13 antibodies may be variably susceptible to urea and that a concentration of 1.5 M may not be enough to dissociate immune complexes of all patients. It is tempting to speculate that the reported discrepancies may reflect the complexity and the heterogeneity of the immune response against ADAMTS13, which may be rather qualitatively than quantitatively different among TTP patients. The future biochemical and functional characterization of purified anti-ADAMTS13 antibodies from discordant TTP plasma samples may clarify the molecular mechanism underlying this phenomenon.

In conclusion, this study showed that FRET rather than CBA results reflect ADAMTS13 activity level *in vivo* in samples with discordant results between the two assays. Moreover, the presence of urea in CBA is a likely cause of these discrepancies. FRET assay, despite employing a non-physiological peptide-based VWF substrate, should be considered more reliable than CBA in the clinical management of acquired TTP patients. In general, the use of an assay which does not require any denaturing agents is advised when assessing the severe deficiency of ADAMTS13.

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CHAPTER VI – CONCLUSIONS

Thrombotic thrombocytopenic purpura (TTP) is a rare but life-threatening microangiopathic disorder, due to the persistence of highly thrombogenic ultra large (UL) von Willebrand factor (VWF) multimers in the microcirculation. TTP is associated with the severe deficiency of ADAMTS13 (activity <10%), the metalloprotease responsible for the proteolytic regulation of VWF multimers size. In the acquired form of the disease, the severe deficiency of ADAMTS13 is caused by anti-ADAMTS13 antibodies that inhibit ADAMTS13 activity (neutralizing anti-ADAMTS13 antibodies or inhibitors) and/or increase its clearance from the circulation (non-neutralizing anti-ADAMTS13 antibodies). In the absence of ADAMTS13, highly adhesive ULVWF, that spontaneously aggregate platelets, accumulates promoting platelet thrombi formation and eventually leading to the clinical features typical of TTP.

In the past decade, the increasing knowledge regarding the molecular mechanisms involved in the pathogenesis of TTP, yielded to a proliferation of assays for the measurement of ADAMTS13 and anti-ADAMTS13 antibodies. Despite the numerous studies conducted to assess the clinical utility of ADAMTS13-related markers, their prognostic value is still controversial. The finding that severe ADAMTS13 deficiency and the presence of anti-ADAMTS13 antibody is associated with an increased risk of relapse is rather consistent, but studies on larger cohort of patients are needed. More uncertain is the predictive value of immunoglobulin (Ig) class subtype of anti-ADAMTS13 antibodies, whether or not inhibitory. Controversial results may be partially due to differences in the assays used to measure ADAMTS13 activity and anti-ADAMTS13 antibodies, which require further standardization.

The possibility of using ADAMTS13 testing to manage TTP patients depends on ADAMTS13 assays becoming widely available, rapid, reliable and feasible for most clinical laboratories. Only a few laboratories have acquired sufficient experience in such a rare disease, allowing them to give helpful advice to less specialized laboratories. This doctoral thesis has been conducted in one of these specialized laboratories. Our research group at The Hemophilia and Thrombosis Centre of Milan has indeed an internationally recognized reputation in research and treatment of TTP. An international TTP registry, containing information on hundreds of cases of the disease, has been developed and curated in our centre. Based on these premises, the research conducted during this doctorate had two main objectives: to evaluate, in our large cohort of acquired TTP patients, the clinical utility of ADAMTS13-related biomarkers and to investigate which ADAMTS13 assay may be more reliable in the management of acquired TTP.

In the first part of this study, we analyzed, during both acute and remission phase, all the biomarkers associated with ADAMTS13 (activity, antigen and class, subclass and titre of anti-ADAMTS13 autoantibodies) in acquired TTP patients referred to our centre and we correlated them to episode severity and recurrence. We found that both the Ig class and subclass are of predictive value for acute episode severity in patients with TTP. Disease recurrence seemed to be associated with the presence of IgG antibodies during the acute phase, while alterations in several ADAMTS13-related biomarkers (ADAMTS13 activity or antigen levels <10%, presence of ADAMTS13 inhibitor or IgG) could predict recurrence risk when measured during disease remission.
The results of this study confirmed and extended previous data on the prognostic value of ADAMTS13 testing, providing valuable information for the identification of patients at higher risk requiring closer follow up.

The studies conducted in the second part of this doctorate intended to investigate the performance of two widely used methods, the collagen-binding assay (CBA) and the more recent fluorescence resonance energy transfer (FRET) assay, for the measurement of ADAMTS13 activity and anti-ADAMTS13 inhibitors. Given the prognostic value of ADAMTS13-related markers (as demonstrated in the first part of this thesis), it is essential to determine which ADAMTS13 assay is more reliable in the management of patients suffering from this rare but life-threatening disease.
CBA and FRET assay strongly differ from a methodological point of view: they use different substrates (full-length VWF VS peptide VWF), different experimental conditions (urea VS none) and different detection methods (indirect detection of cleavage products through absorbance measurement VS direct detection of cleavage products through fluorescence measurement). Several studies have been performed to evaluate the performance and utility of the two assays for the measurement of ADAMTS13 activity, but they have never been compared for the detection of anti-ADAMTS13 inhibitors in mixing studies. Moreover, despite the good correlation in the quantification of ADAMTS13 activity in normal plasmas, important discrepancies have been described in a small but relevant percentage of TTP cases.
First, we decided to compare anti-ADAMTS13 inhibitors titres measured by CBA and FRET assays in acquired TTP patients enrolled in the Milan TTP Registry. We found that the two assays performed similarly and concluded that FRET, which is more rapid and easier to perform than CBA, could hence be the assay of choice for inhibitor quantification.
Secondly, we investigated whether CBA or FRET assay results reflect ADAMTS13 activity level *in vivo* in samples presenting circulating anti-ADAMTS13 antibodies and discordant results between the two assays. We performed an analysis of the multimeric pattern of VWF and measured ADAMTS13

activity using an additional assay which employs a full-length VWF substrate under flow conditions. We found that FRET assay results, despite the use of a non-physiologic peptide-based VWF substrate, more closely resemble ADAMTS13 activity level in these particular group of samples. Moreover, we showed that the presence of denaturing agents in CBA is a likely cause of the observed discrepancies. On the whole, these findings allow us to affirm that FRET rather than CBA should be considered the assay of choice for the measurement of both ADAMTS13 activity and ADAMTS13 inhibitors.

In conclusion, the research conducted during the doctorate further established the clinical utility of ADAMTS13-related assays in acquired TTP and provided methodological guidelines for the choice of such assays when assessing the severe deficiency of ADAMTS13 or the presence of anti-ADAMTS13 inhibitors.

CHAPTER VII – REFERENCES

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SUMMARY

Thrombotic thrombocytopenic purpura (TTP) is a rare but life-threatening microangiopathic disorder, due to the persistence of highly thrombogenic ultra large (UL) von Willebrand factor (VWF) multimers in the microcirculation. TTP is associated with the severe deficiency of ADAMTS13 (activity <10%), the metalloprotease responsible for the proteolytic regulation of VWF multimers size. In the acquired form of the disease, the severe deficiency of ADAMTS13 is caused by anti-ADAMTS13 antibodies that inhibit ADAMTS13 activity (neutralizing anti-ADAMTS13 antibodies or inhibitors) and/or increase its clearance from the circulation (non-neutralizing anti-ADAMTS13 antibodies). In the absence of ADAMTS13, highly adhesive ULVWF, that spontaneously aggregate platelets, accumulates promoting platelet thrombi formation and eventually leading to the clinical features typical of TTP.

In the past decade, the increasing knowledge regarding the molecular mechanisms involved in the pathogenesis of TTP, yielded to a proliferation of assays for the measurement of ADAMTS13 and anti-ADAMTS13 antibodies. Despite the numerous studies conducted to assess the clinical utility of ADAMTS13-related markers, their prognostic value is still controversial. The finding that severe ADAMTS13 deficiency and the presence of anti-ADAMTS13 antibody is associated with an increased risk of relapse is rather consistent, but studies on larger cohort of patients are needed. More uncertain is the predictive value of immunoglobulin (Ig) class subtype of anti-ADAMTS13 antibodies, whether or not inhibitory. Controversial results may be partially due to differences in the assays used to measure ADAMTS13 activity and anti-ADAMTS13 antibodies, which require further standardization.

With this background, the present research had two main objectives: to evaluate, in a large cohort of acquired TTP patients enrolled in the international Milan TTP Registry (developed and curated at the The Hemophilia and Thrombosis Centre of Milan), the clinical utility of ADAMTS13-related biomarkers and to investigate which ADAMTS13 assay may be more reliable in the management of acquired TTP.

In the first part of this study, we analyzed, during both acute and remission phase, all the biomarkers associated with ADAMTS13 (activity, antigen and class, subclass and titre of anti-ADAMTS13 autoantibodies) in acquired TTP patients referred to our centre and we correlated them to episode severity and recurrence. We found that both the Ig class and subclass are of predictive value for acute episode severity in patients with TTP. Disease recurrence seemed to be associated with the

presence of IgG antibodies during the acute phase, while alterations in several ADAMTS13-related biomarkers (ADAMTS13 activity or antigen levels <10%, presence of ADAMTS13 inhibitor or IgG) could predict recurrence risk when measured during disease remission.
The results of this study confirmed and extended previous data on the prognostic value of ADAMTS13 testing, providing valuable information for the identification of patients at higher risk requiring closer follow up.

The studies conducted in the second part of this doctorate intended to investigate the performance of two widely used methods, the collagen-binding assay (CBA) and the more recent fluorescence resonance energy transfer (FRET) assay, for the measurement of ADAMTS13 activity and anti-ADAMTS13 inhibitors. Given the prognostic value of ADAMTS13-related markers (as demonstrated in the first part of this thesis), it is essential to determine which ADAMTS13 assay is more reliable in the management of patients suffering from this rare but life-threatening disease.
CBA and FRET assay strongly differ from a methodological point of view: they use different substrates (full-length VWF VS peptide VWF), different experimental conditions (urea VS none) and different detection methods (indirect detection of cleavage products through absorbance measurement VS direct detection of cleavage products through fluorescence measurement). Several studies have been performed to evaluate the performance and utility of the two assays for the measurement of ADAMTS13 activity, but they have never been compared for the detection of anti-ADAMTS13 inhibitors in mixing studies. Moreover, despite the good correlation in the quantification of ADAMTS13 activity in normal plasmas, important discrepancies have been described in a small but relevant percentage of TTP cases.
First, we decided to compare anti-ADAMTS13 inhibitors titres measured by CBA and FRET assays in acquired TTP patients enrolled in the Milan TTP Registry. We found that the two assays performed similarly and concluded that FRET, which is more rapid and easier to perform than CBA, could hence be the assay of choice for inhibitor quantification.
Secondly, we investigated whether CBA or FRET assay results reflect ADAMTS13 activity level *in vivo* in samples presenting circulating anti-ADAMTS13 antibodies and discordant results between the two assays. We performed an analysis of the multimeric pattern of VWF and measured ADAMTS13 activity using an additional assay which employs a full-length VWF substrate under flow conditions. We found that FRET assay results, despite the use of a non-physiologic peptide-based VWF substrate, more closely resemble ADAMTS13 activity level in these particular group of samples. Moreover, we showed that the presence of denaturing agents in CBA is a likely cause of the observed discrepancies. On the whole, these findings allow us to affirm that FRET rather than CBA should be considered the assay of choice for the measurement of both ADAMTS13 activity and ADAMTS13 inhibitors.

In conclusion, the research conducted during the doctorate further established the clinical utility of ADAMTS13-related assays in acquired TTP and provided methodological guidelines for the choice of such assays when assessing the severe deficiency of ADAMTS13 or the presence of anti-ADAMTS13 inhibitors.