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DISSERTATION

Characterization of the immune response against the metalloprotease ADAMTS13 in patients with thrombotic thrombocytopenic purpura

Verfasserin

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To

The memory of my mother

Agron and Leonard

My family

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ORIGINAL PUBLICATIONS

Part of this thesis is published in the following manuscripts:

I. **Ferrari S**, Scheiflinger F, Rieger M, Mudde G, Wolf M, Coppo P, Girma JP, Azoulay E, Brun-Buisson C, Fakhouri F, Mira JP, Oksenhendler E, Poullin P, Rondeau E, Schleinitz N, Schlemmer B, Teboul JL, Vanhille P, Vernant JP, Meyer D, Veyradier A; French Clinical and Biological Network on Adult Thrombotic Microangiopathies. Prognostic value of anti-ADAMTS 13 antibody features (Ig isotype, titer, and inhibitory effect) in a cohort of 35 adult French patients undergoing a first episode of thrombotic microangiopathy with undetectable ADAMTS 13 activity.
Blood 2007; 109:2815-22.

II. **Ferrari S**, Mudde GC, Rieger M, Veyradier A, Kremer Hovinga JA, Scheiflinger F. IgG subclass distribution of anti-ADAMTS13 antibodies in patients with acquired thrombotic thrombocytopenic purpura.
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III. **Ferrari S**, Knöbl P, Kolovratova V, Varadi K, Plaimauer B, Turecek P, Rottensteiner H, Scheiflinger F. Inverse correlation of free and immune complexed-sequestred anti-ADAMTS13 antibodies in a patient with acquired TTP.
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LIST OF ABBREVIATIONS

ADAMTS13	A disintegrin-like and metalloprotease with thrombospondin type-1 repeats
ADAMTS13:Ac	ADAMTS13 activity
ADAMTS13:Ag	ADAMTS13 antigen
C1q	Complement component C1q
CD36	Cluster of differentiation 36
CUB	Complement components C1r and C1s, embryonic sea urchin protein (uEGF) and bone morphogenetic protein (Bmp1) domain
ELISA	Enzyme-linked immunosorbent assay
F	Coagulation factor
FcγR	Cell surface Fc gamma receptor
GP	Glycoprotein
HUS	Hemolytic uremic syndrome
IC(s)	Immune complex(es)
Ig	Immunoglobulin
NHP	Pooled normal human plasmas
pADAMTS13	Plasma derived ADAMTS13
PEX	Plasma exchange
rADAMTS13	Recombinant ADAMTS13
TSP1	Thrombospondin type 1 motif
TTP	Thrombotic thrombocytopenic purpura
ULVWF	Unusually large von Willebrand factor
VWD	von Willebrand disease
VWF	von Willebrand factor

ABSTRACT

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disease characterized by microangiopathic hemolytic anemia and thrombocytopenia with renal impairment or neurologic abnormalities due to the deposition of thrombi rich in platelets and von Willebrand factor (VWF) in the microcirculation. ADAMTS13 regulates the size, and therefore the thrombogenic potential, of VWF by cleaving a single peptide bond in VWF. Congenital or acquired ADAMTS13 deficiency is the major risk factor for TTP. Autoantibodies against ADAMTS13 are believed to cause ADAMTS13 deficiency in acquired idiopathic TTP. The main objective of this work was to study the pathophysiologic and prognostic value of anti-ADAMTS13 antibodies in a cohort of 76 patients with acquired TTP. To achieve this goal, enzyme-linked immunosorbent assays were established to detect and quantify IgG1-4, IgA, and IgM anti-ADAMTS13 antibodies as well as circulating ADAMTS13-specific immune complexes (ICs). The surveyed antibody profile revealed the presence of anti-ADAMTS13 antibodies of the IgG, IgA, and IgM class in 92%, 17% and 7% of the patients, respectively. IgG4 (90%) was the most prevalent IgG subclass, followed by IgG1 (52%), IgG2 (50%), and IgG3 (33%). ADAMTS13-specific immune complexes formed by IgG1-4 and IgA were found in 82 and 25% of the patients, respectively. Notably, the complexes contained antibodies of the same Ig (sub)classes as the free antibodies present in most of the samples, being IgG4-ICs the most prevalent (87%) ICs found. Anti-ADAMTS13 IgG antibodies associated with a high inhibitor titer at disease presentation were associated with persistence of undetectable ADAMTS13 activity in clinical remission. TTP patients with high IgG4 and undetectable IgG1 levels were more prone to relapse than patients with low IgG4 and detectable IgG1 levels suggesting that IgG4 could be a useful biomarker for identification of patients at risk of disease recurrence. Circulating ADAMTS13-specific immune complexes may contribute to progression and severity of the disease, because they may continuously deplete ADAMTS13 from circulation inducing organ damage due to tissue deposition. The comprehensive characterization of anti-

ADAMTS13 antibodies as well as circulating ADAMTS13-specific ICs is expected to contribute to a better understanding of the mechanisms leading to the autoimmune form of TTP.

ZUSAMMENFASSUNG

Thrombotische thrombozytopenische Purpura (TTP) ist eine lebensbedrohliche Erkrankung, die durch eine mikroangiopathische Anämie und Thrombozytopenie mit eingeschränkter Nierenfunktion oder neurologischen Auffälligkeiten durch die Ablagerung von Thromben (reich an Thrombozyten und von Willebrand Faktor (VWF)) in der Mikrozirkulation gekennzeichnet ist. ADAMTS13 regelt die Größe und damit das thrombogene Potenzial von VWF durch die Spaltung einer einzigen Peptidbindung in VWF. Angeborener oder erworbener ADAMTS13 Mangel ist der wichtigste Risikofaktor für die TTP. Autoantikörper gegen ADAMTS13 führen vermutlich zum erworbenen ADAMTS13 Mangel in der idiopathischen TTP. Das Hauptziel dieser Arbeit war es, die pathophysiologischen und prognostischen Werte von Anti-ADAMTS13 Antikörpern in einer Kohorte von 76 Patienten mit erworbener TTP zu untersuchen. Deshalb wurden Enzym-gekoppelte Immun-Assays etabliert, die IgG1-4, IgA, IgM-und Anti-ADAMTS13 Antikörper sowie die zirkulierenden ADAMTS13-spezifische Immunkomplexe (ICs) erkennen und quantifizieren können. Das untersuchte Antikörper Profil zeigte die Anwesenheit von anti-ADAMTS13 Antikörper der IgG, IgA, IgM-Klasse in 92%, 17% und 7% der Patienten. IgG4 (90%) war die häufigste IgG Unterklasse gefolgt von IgG1 (52%), IgG2 (50%) und IgG3 (33%). ADAMTS13-spezifische Immunkomplexe, bestehend aus IgG1-4-und IgA, wurden in 82 und 25% der Patienten gefunden. Es ist bemerkenswert, dass diese Immunkomplexe Antikörper der gleichen Ig (sub-) Klassen enthielten wie die in den meisten Proben gefundenen freien Antikörper, wobei IgG4-ICs am häufigsten (87%) ICS vorkamen. Anti-ADAMTS13 IgG-Antikörper, die mit einem hohen Inhibitor-Titer bei Krankheitserscheinung einhergingen, wurden mit nicht nachweisbarer ADAMTS13 Tätigkeit in klinischer Remission assoziiert. TTP Patienten mit hohen IgG4 und nicht nachweisbaren IgG1-Spiegeln waren anfälliger für Krankheitsrückfälle als Patienten mit niedrigen IgG4 und nachweisbaren IgG1-Spiegeln, was darauf hindeutet, dass IgG4 ein nützlicher Biomarker

für die Identifizierung von Patienten mit Rezidiv-Risiko sein könnte. Zirkulierende ADAMTS13-spezifische Immunkomplexe können zur Progression und Schwere der Krankheit beitragen, weil sie kontinuierlich ADAMTS13 aus der Blutzirkulation entfernen, was zu Gewebeablagerungen und nachfolgenden Organschädigungen führt. Die umfassende Charakterisierung von anti-ADAMTS13 Antikörpern sowie zirkulierenden ADAMTS13-spezifischen ICs kann zu einem besseren Verständnis der Mechanismen beitragen, die zu der Autoimmunerkrankungsform der TTP führen.

1- INTRODUCTION

1. Introduction

ADAMTS13 is the thirteenth member of the ADAMTS (A Disintegrin-like And Metalloprotease with ThromboSpondin type-1 repeats) family of metalloproteases. Its function is to regulate the size of the hemostatic protein von Willebrand factor (VWF) (Porter *et al*, 2005). VWF is released from intracellular storage compartments in the form of multimers containing a portion of very high molecular weight multimers referred to as ultra-large VWF (ULVWF) multimers (Sporn *et al*, 1986). These ULVWF multimers bear a high prothrombotic potential, but usually they are proteolyzed by ADAMTS13 immediately after secretion, thereby reducing the size and by consequence the prothrombotic properties of VWF while maintaining its hemostatic function. This function confers to ADAMTS13 an important role in the maintenance of a balanced hemostasis.

1.1 Overview of Hemostasis

Hemostasis is a protective physiological mechanism by which the body prevents loss of blood at the site of injury while maintaining normal blood flow elsewhere in the circulation. Hemostasis is a dynamic and complex system, involving cells, coagulation proteins and the vascular wall to keep the blood in a fluid state, but the same players form a barrier when trauma or pathologic conditions cause vessel damage. When the continuity of the vascular endothelium is disrupted, components of the subendothelial layers of the vessel wall are exposed to flowing blood allowing circulating platelets to adhere thereby initiating blood arrest. Activated platelets stimulate the activation of plasma coagulation factors leading to the generation of fibrin that stabilizes the platelet plug. Fibrin also provides a matrix for cell migration and wound healing. In the latter case, the platelet aggregate and fibrin clot are dissolved and removed by the fibrinolytic system which completes the process of hemostasis (Arnout *et al*, 2006).

Hemostasis is a tightly regulated process. In the absence of injury, regulatory mechanisms ensure the maintenance of blood fluidity. After tissue damage the system is immediately activated. As a consequence a thrombus is generated that remains localized to the site of injury and is proportional to the size of injury (Gale, 2011). A balance between procoagulant and anticoagulant pathways is critical to achieve a regulated hemostasis (Gale, 2011). Disturbances of the natural balance due to genetic or acquired factors may result in either bleeding (not enough clotting) or thrombosis (too much clotting).

Hemostasis can be divided into primary and secondary hemostasis. Primary hemostasis is mediated by platelets which aggregate to form a hemostatic plug at the site of injury. Secondary hemostasis is the generation of insoluble fibrin by the coagulation cascade. Both processes occur simultaneously and are mechanistically intertwined (Furie & Furie, 2008;Gale, 2011).

1.1.1 Primary Hemostasis

The intact vascular endothelium is a non-adhesive surface with which platelets are not interacting under normal blood flow. However, when the endothelium is disrupted by mechanical damage (traumatic wounds) or pathogenic stimuli (e.g. chronic vascular diseases), extracellular matrix (ECM) components become exposed to circulating blood triggering events of primary hemostasis. Platelet adhesion is the first response to vascular injury and is an important host defense mechanism to avoid bleeding. Platelet adhesion requires the synergistic and coordinated function of different plasma proteins and receptors on platelets together with ECM components (Ruggeri, 2009).

The initial platelet tethering to the vessel wall is mediated by the interaction between two platelet surface receptors (glycoprotein (GP) VI and integrin α2β1) and subendothelial collagen. Subendothelial VWF also mediates platelet adhesion by binding to the platelet receptors GPIbα (platelet membrane GPIb-IX-V complex) (Andrews *et al*, 2003) and integrin αIIbβ3 (Ruggeri & Mendolicchio, 2007). Soluble plasma VWF can also be

immobilized to ECM components via direct binding to sudendothelial collagen or self-association with other VWF multimers, thus acting as a bridge between tissue and platelets (Savage *et al*, 2002;Ruggeri, 2009).

After platelets are firmly adhered to ECM components, they can translocate on the injured vessel wall and on the surface of the developing thrombus. This step is critical for the regulation of the rate and extent of thrombus growth. Platelet translocation also generates signals that induce platelet morphological changes like rearrangement of the membrane with exposure of negatively charged phospholipids and development of tethers/pseudopodia that increase the platelet potential to establish multivalent adhesive interactions (Dahlback, 2005;Jackson, 2007). The exposition of negatively charged phospholipids (mainly phosphatidylserine) on the outer membrane layer of platelets is particularly critical for platelet procoagulant activity. Platelet activation and granule content release are responsible for activation of additional platelets (Dahlback, 2005). Platelet activation also stimulates integrin αIIbβ3 activation, enhancing its affinity for adhesive proteins such as soluble fibrinogen, VWF, fibronectin and thrombospondin (Tao *et al*, 2010). Fibrinogen physically bridges adjacent activated platelets promoting cell arrest and stable platelet aggregation. Platelet aggregation mediated by binding of soluble fibrinogen to platelet integrin αIIbβ3 is the dominant mechanism supporting platelet aggregation under low wall shear rate conditions (0 to 1000 s^{-1}). With increasing wall shear rates (1000 to 10 000 s^{-1}), the initiation of aggregation becomes more dependent on VWF and fibrinogen only supports the stabilization of the formed aggregates (Jackson, 2007). Nevertheless, fibrinogen and VWF have a synergistic role supporting platelet aggregation and both are required to ensure a stable primary plug. Primary hemostasis is coordinated with the activation of the coagulation system (secondary hemostasis) which will generate thrombin and deposition of an insoluble fibrin net stabilizing and anchoring the primary thrombus to the vessel wall.

1.1.2 Secondary Hemostasis

The original waterfall/cascade model of coagulation depicted as a Y-shaped scheme was described in 1964 independently by Davie and Ratnoff (DAVIE & RATNOFF, 1964) and Macfarlane (MACFARLANE, 1964). This model describes a complex series of sequential enzymatic reactions in which several inactive plasma coagulation zymogens are converted to active serine proteases along either an extrinsic or an intrinsic pathway that converge at a common pathway that ends with the generation of thrombin and a fibrin clot (Fig.1).

The activation of the extrinsic pathway is triggered by the exposition of tissue factor (TF) to flowing blood, which occurs after vascular damage or activation of the endothelium. Tissue factor is a transmembrane protein constitutively expressed on cells that are not in contact with blood. TF binds to activated plasma factor (F) VII (FVIIa, with the lower case "a" indicating active clotting factor), about ~1% of which circulates as active enzyme. Circulating FVIIa displays a weak catalytic activity that is enhanced only when bound to TF, calcium ions and membrane surfaces to form the catalytic TF/FVIIa tenase complex. TF/FVIIa complex activates the zymogens FIX (belonging to the intrinsic pathway) and FX to their active forms (IXa and Xa, respectively) (Butenas *et al*, 2009). At this initial stage, trace amounts of thrombin are generated by the minute quantities of FXa produced. The small amount of thrombin generated is not sufficient to induce clot formation but will partially activate platelets and the cofactors FV and FVIII to their active forms allowing the propagation and amplification of the coagulation to generate a fibrin clot (Butenas & Mann, 2002) (Fig.1).

Initiation of the intrinsic pathway is triggered by the autoactivation of plasma coagulation factor XII upon contact with negatively charged biological or artificial surfaces (e.g. platelet membrane, collagen, glass) by a process called "contact activation". In the presence of high-molecular-weight kininogen (HMWK), a platelet-derived cofactor, small amounts of FXIIa can convert prekallikrein into active kallikrein which reciprocally

activates more FXII thereby amplifying the activation signal. Factor XIIa, in the presence of HMWK, proteolytically cleaves FXI to FXIa which in turn activates FIX to FIXa (Renne & Gailani, 2007;Muller & Renne, 2008).

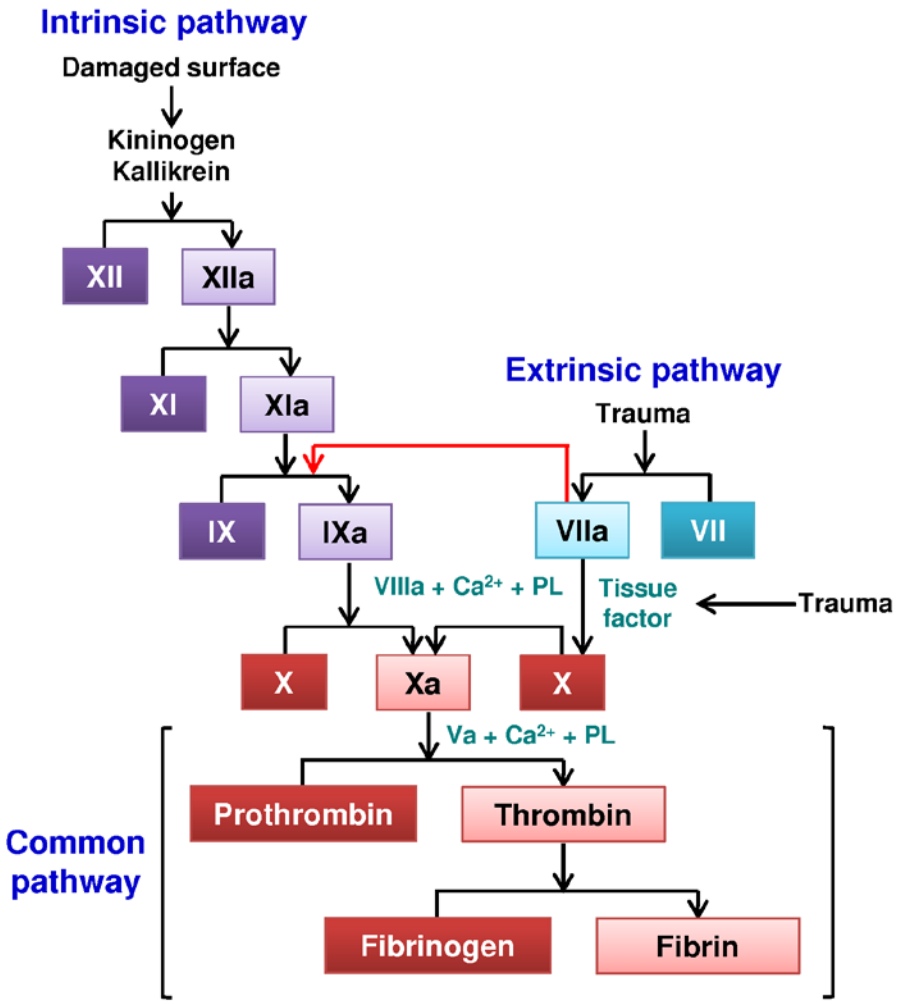

Figure 1. Schematic representation of the coagulation cascade.
The intrinsic and extrinsic pathways are depicted. Plasma coagulation zymogens are indicated by roman numbers and activated factors by a lower case "a". Non-enzymatic cofactors are indicated in green. The red line indicates a reaction that does not belong to the standard waterfall cascade described in 1964.

Factor IXa assembles with activated FVIII (FVIIIa) and calcium ions into a complex on negatively charged platelet membranes. This so called tenase complex catalyzes the activation of FX to FXa (Fig.1).

The extrinsic and intrinsic pathways converge at the step of FX activation. In the common pathway, FXa forms a complex with FVa, calcium ions and phospholipid membranes (prothrombinase complex) which activates

prothrombin to thrombin (FIIa). Thrombin converts fibrinogen to fibrin monomers that spontaneously polymerize in a soft and friable clot (Fig.1). Furthermore, thrombin activates FXIII to FXIIIa which catalyzes the formation of covalent cross-links between fibrin polymers to form a fibrin mesh thus enhancing clot stability and resistance (Lorand, 2001).

The waterfall/cascade model of coagulation supports the in vitro evaluation of coagulation function but may not explain coagulation in vivo. The catalytic reactions of blood coagulation are localized on phospholipid surfaces mainly provided by platelets, but cells also play a key role in controlling and directing the coagulation event to avoid its spreading throughout the vascular system. A modified cell-based model of coagulation has been proposed which describes the interaction of clotting factors with specific cell surfaces in an overlapping manner and not only in a sequential cascade of reactions (Hoffman, 2003;Monroe & Hoffman, 2006).

In this cell-based model, coagulation occurs in three overlapping phases: initiation, amplification and propagation (Hoffman, 2003;Monroe & Hoffman, 2006). The initiation of coagulation is driven in vivo by the extrinsic or TF-dependent pathway that includes the FVIIa/TF and the FXa/FVa complexes and operates exclusively on TF-bearing cells. During this phase, the FVIIa/TF complex activates FIX and FX. FIXa is able to migrate to other cellular surfaces whereas FXa remains on TF-bearing cells, where it activates FV to form the FXa/FVa or prothrombinase complex, which in turn produces small amounts of thrombin. These small quantities of thrombin suffice to activate platelets, FV, FVIII and FXI, thereby initiating the amplification phase. This phase occurs mainly on platelets and ends with the binding of FVa and FVIIIa to the surface of activated platelets. In the propagation phase, the tenase complex (FVIIIa/FIXa) bound to platelet membranes activates FX, which will form the prothrombinase complex (FXa/FVa) on the platelet surface. This complex generates a large amount of thrombin, which finally converts fibrinogen to fibrin and activates FXIII leading to a stabilization of the forming clot (Hoffman, 2003;Monroe & Hoffman, 2006).

On the other hand, contact activation does not play any physiological role in that process. The intrinsic pathway is only operating on platelet surfaces and is driven by FXIa and the tenase (FVIIIa/FIXa) and prothrombinase (FXa/FVa) complexes. According to this model, both pathways are required to achieve hemostasis because they work on different cell surfaces having different functional roles.

1.2 Von Willebrand Factor

The von Willebrand factor is a large plasma multimeric glycoprotein that exerts two important biological functions. In primary hemostasis, VWF supports platelet adhesion and aggregation (Ruggeri, 2009). The second relevant function of VWF relates to its role in secondary hemostasis, as it serves as a carrier protein for the procoagulant plasma factor VIII. VWF forms a complex with FVIII via non-covalent interactions increasing the plasma half-life of FVIII and conferring protection from proteolytic degradation (Terraube *et al*, 2010).

VWF is synthesized exclusively in megakaryocytes (Nachman *et al*, 1977) and endothelial cells (Jaffe *et al*, 1974). The VWF precursor protein (denoted as pre-pro-VWF) consists of a monomeric polypeptide of 2813 amino acids with a molecular weight of about 350 kDa. The structure of pre-pro-VWF is based on a 22 amino acid signal peptide, a pro-peptide comprising 741 residues and a mature subunit of 2050 amino acids. The pro-VWF is composed of four types of repeated domains (A to D) displaying considerable internal homology and linked in the order: NH2-D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK-COOH. Each domain contains different physiological binding functions. Domains D1 and D2 correspond to the VWF propeptide whereas mature VWF is composed of domains D' to CK (Wagner, 1990;Sadler, 1998).

Pro-VWF is extensively post-translationally modified to produce multimeric VWF. In the endoplasmic reticulum, pro-VWF monomers dimerize in a 'tail-to-tail' manner through disulfide bonds within the C-terminal domains. In the Golgi apparatus, pro-VWF dimers polymerize via

additional N-terminal 'head-to-head' disulfide bonds yielding high molecular weight multimeric VWF species, the sizes of which can reach molecular weights up to 20.000 kDa or even higher. Multimer assembly is promoted by the VWF-propeptide which is proteolytically cleaved from the VWF subunits inside the *trans*-Golgi. The VWF-propeptide is also required to direct and package VWF multimers into specialized storage organelles, the so-called Weibel-Palade bodies of endothelial cells and the α-granules of megakaryocytes and platelets.

In endothelial cells, VWF multimers are secreted immediately after synthesis from both cellular sides (apical and basal) via a constitutive pathway or stored in the Weibel-Palade bodies and released after induction with secretagogues via a regulated pathway (Wagner, 1990) or, probably, by an unstimulated basal pathway (Giblin *et al*, 2008). In contrast, VWF stored in megakaryocytes and platelets is secreted only upon activation via the regulated pathway (Sporn *et al*, 1985).

VWF is released from the stored compartments in the form of multimers containing a portion of very high molecular weight multimers referred to as ultra-large VWF (ULVWF) multimers (Sporn *et al*, 1986). These highest molecular weight VWF species are the hemostatically most active forms with a propensity to promote spontaneous platelet adhesion and aggregation under high shear. As this aggregation can occur even in the absence of endothelial damage ULVW multimers bear a significant prothrombotic potential (Moake *et al*, 1986).

VWF multimer size is regulated by ADAMTS13 (A Disintegrin-like And Metalloprotease with ThromboSpondin type-1 repeats), a metalloprotease that specifically cleaves the peptide bond between the amino acids Tyr1605 and Met1606 within the central A2 domain of VWF (Furlan *et al*, 1996;Tsai, 1996). This conversion of the ULVWF multimers to smaller and less active forms is thought to predominantly occur immediately after secretion of VWF.

The first association between the presence of circulating ULVWF multimers and thrombosis was made in 1982 (Moake *et al*, 1982) in a description of patients suffering of chronic relapsing thrombotic

thrombocytopenic purpura (TTP), a life-threatening thrombotic microangiopathy characterized by thrombocytopenia, microangiopathic hemolytic anemia and renal impairment or neurological abnormalities. The ULVWF multimers were present in the plasma only during the remission phase of the disease, disappearing after fresh plasma replacement or during the acute phase. This led to the speculation that ULVWF multimers are responsible for the widespread thrombus formation observed in the microvasculature of the patients and that a plasma deficiency of a VWF depolymerase (today known as ADAMTS13) could be the cause of the accumulation of ULVWF multimers.

On the other hand, deficiency of VWF can cause a bleeding disorder called von Willebrand disease (VWD). VWD is the most common autosomally inherited bleeding disorder due to quantitative (type 1 and 3) or qualitative (type 2) deficiencies of VWF (Sadler *et al*, 2006). In type 1 VWD, VWF levels are partially reduced, whereas in type 3 VWD are undetectable. Type 2 VWD is characterized by qualitative defects in VWF that lead to impaired protein function and can be divided into four different subclasses (2A, 2B, 2M and 2N) based on specific functional and structural defects. Patients with type 2A VWD have decreased VWF-dependent platelet adhesion due to a selective deficiency of high-molecular-weight VWF multimers. A VWF with increased affinity for platelet GPIb characterize type 2B VWD. Patients with type 2M VWD have decreased VWF-dependent platelet adhesion without a selective deficiency of high-molecular-weight VWF multimers. Finally, a VWF with markedly decreased binding affinity for FVIII characterize type 2N VWD (Sadler *et al*, 2006).

1.3 ADAMTS13

1.3.1 ADAMTS13 identification and cloning

Two independent groups partially purified a ~200 kDa plasma protease in 1996 that was capable of cleaving VWF at a single specific site producing the same cleavage fragments of 140 and 176 kDa as observed in normal human plasma (Furlan *et al*, 1996;Tsai, 1996). The newly identified protease cleaved VWF only after exposure to mild denaturation with

chaotropic agents such as urea (Furlan *et al*, 1996) and guanidine-HCl (Tsai, 1996) or to high fluid shear stress (Tsai, 1996). The VWF-cleaving protease (VWFCP) was found to be activated by divalent cation ions and low ionic strength, strongly inhibited by chelating agents and insensitive to serine and cysteine proteinase inhibitors. Later on, deficiency of the VWFCP was found in patients suffering from chronic relapsing TTP (Furlan *et al*, 1997) and it was shown that in some cases, an isolated IgG from plasma of TTP patients inhibited the proteolytic activity of the VWFCP, suggesting that a constitutional or acquired deficiency of the VWFCP has led to development of TTP (Furlan *et al*, 1998;Tsai & Lian, 1998).

In 2001, the VWFCP was isolated, cloned and identified as a new member of the ADAMTS (A Disintegrin-like And Metalloprotease with ThromboSpondin type-1 repeats) metalloprotease family, designed ADAMTS13 (Gerritsen *et al*, 2001;Fujikawa *et al*, 2001;Soejima *et al*, 2001;Zheng *et al*, 2001;Levy *et al*, 2001;Plaimauer *et al*, 2002).

The human *ADAMTS13* gene contains 29 exons encompassing approximately 37kb on human chromosome 9q34. The 4.7 kb transcript encoded by the *ADAMTS13* gene is predominantly expressed in the liver and a short 2.4 kb transcript was also found in placenta and skeletal muscles (Soejima *et al*, 2001;Zheng *et al*, 2001;Levy *et al*, 2001).

1.3.2 ADAMTS13 biosynthesis, secretion and catabolism

ADAMTS13 is synthesized in the hepatic stellate cells of the liver (Uemura *et al*, 2005;Zhou *et al*, 2005), platelets (Suzuki *et al*, 2004), endothelial cells (Turner *et al*, 2006) and podocytes and renal tubular epithelial cells from the kidney (Manea *et al*, 2007;Manea *et al*, 2010). In contrast to other ADAMTS proteases, ADAMTS13 is secreted into the circulation as an active enzyme (Majerus *et al*, 2003) with a plasma half-life of approximately 2-3 days (Furlan *et al*, 1999). The estimated normal plasma concentration of ADAMTS13 is ~1 µg/ml (5 nM) (Gerritsen *et al*, 2001;Rieger *et al*, 2006). A small percentage (~3%) of ADAMTS13

circulates bound to native VWF without exerting substrate proteolysis (Feys *et al*, 2009).

Full length ADAMTS13 is the major plasma circulating species, however, truncated forms have also been identified (Gerritsen *et al*, 2001;Soejima *et al*, 2006). Mature ADAMTS13 is highly posttranslationally modified with glycosylation accounting for ~20% of its molecular weight. N-glycosylation (Zhou & Tsai, 2009) and O-fucosylation of the TSP1 repeats (Ricketts *et al*, 2007) seem to be critical for ADAMTS13 secretion based on studies in cell culture with recombinantly expressed ADAMTS13. Moreover, N-glycosylation seems to be important for promoting a correct protein folding rendering ADAMTS13 proteolytically more active. N-glycans are however not required for protease activity once ADAMTS13 is correctly folded and secreted (Zhou & Tsai, 2009). Using purified plasma-derived ADAMTS13 (pADAMTS13), Hiura *et al* (Hiura *et al*, 2010) showed that ADAMTS13 contains α2-6 and α2-3-linked sialic acid residues at the non-reducing terminus and β-galactose residues on the N- and O-linked sugar chains penultimate to sialic acid.

The mechanism by which ADAMTS13 is cleared or metabolized in vivo is unknown. Recent findings indicate that all sugar chains of pADAMTS13 are capped by sialic acids with no exposure of galactose residues. This could suggest that the hepatic asialoglycoprotein receptor is involved in ADAMTS13 clearance (Hiura *et al*, 2010). It has been reported that thrombin, plasmin and factor Xa can inactivate ADAMTS13 in vitro (Crawley *et al*, 2005). Leucocyte elastase also cleaves pADAMTS13 but at sites different from those of thrombin and plasmin (Hiura *et al*, 2010). These results suggest that ADAMTS13 activity might be locally regulated by coagulation proteinases.

1.3.3 ADAMTS13 structure and domain organization

ADAMTS13 is a protein with a multidomain structure (Fig. 2). The primary ADAMTS13 sequence consists of a polypeptide with 1427 amino acid residues sharing common domains with other members of the ADAMTS

family. At its N-terminus, ADAMTS13 harbors a 33 amino acid signal peptide and a short propeptide (residues 34-74). The mature sequence consists of a reprolysin-like metalloprotease domain (residues 75-289), a disintegrin-like domain (residues 290-385), a central thrombospondin type 1 (TSP1-1) motif (residues 386-439), a cysteine-rich domain (residues 440-555), a spacer domain (residues 556-685) followed by a unique combination of seven additional TSP1 repeats (TSP1 2-8; residues 686-1131) and two CUB domains (residues 1192-1408) which were first identified in complement components C1r and C1s, embryonic sea urchin protein (uEGF) and bone morphogenetic protein (Bmp1).

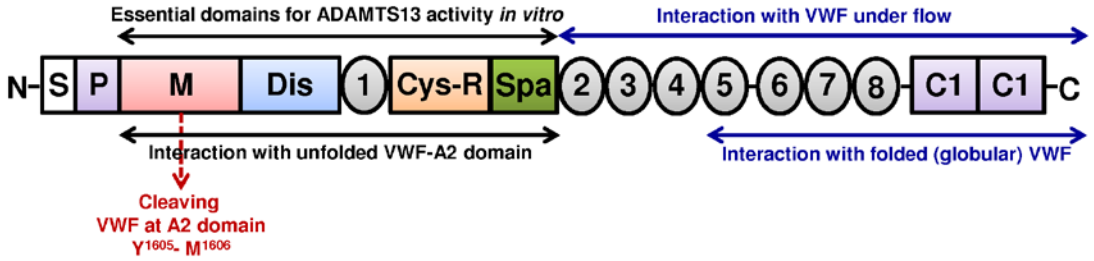

Figure 2. Schematic diagram of the ADAMTS13 domain structure and proposed VWF interaction sites.

The domains are indicated from the N-terminus as follows: signal peptide (S), propeptide (P), metalloprotease domain (M), disintegrin-like domain (Dis), the first thrombospondin type 1 motif (TSP1-1), cysteine-rich domain (Cys-R), spacer domain (Spa), the second to eighth TSP1 repeats (2-8) and two CUB domains (C1 and C2). In the metalloprotease domain resides the active site responsible for VWF cleavage. The contributions of the different domains to the binding of ADAMTS13 to folded and unfolded VWF are also depicted.

The crystal structure of full-length ADAMTS13 has not yet been resolved, but that of a fragment comprising the disintegrin-like, TSP1-1, cysteine-rich and spacer domains, all of which are relevant for ADAMTS13 activity, was published recently (Akiyama *et al*, 2009). In some of these domains peripheral loops were identified that are not conserved among the different members of the ADAMTS family. Identification of these non-conserved domains not only helped to better understand the various interactions between ADAMTS13 and VWF but also suggested that these may confer specific functions to each ADAMTS member (Akiyama *et al*, 2009).

The ADAMTS13 propeptide lacks the cysteine-switch motif present in all other ADAMTS members (Zheng *et al*, 2001). Since this motif confers enzyme latency its absence might be responsible for the secretion of

ADAMTS13 as an active enzyme (Majerus *et al*, 2003). The propeptide is also not required for protein folding and secretion, and its cleavage is not critical for proteolytic activity (Majerus *et al*, 2003).

The metalloprotease or catalytic domain of ADAMTS13 is responsible for the specific cleavage of VWF and contains the conserved active site motif ^{224}HE*XX*H*XX*G*XX*HD^{235} ("X" represent any amino acid). The active site pocket has three histidine residues coordinating a Zn^{2+} ion and a glutamic acid coordinating a water molecule being essential for ADAMTS13 catalytic activity (Bode *et al*, 1999). In addition, the active site has also a conserved Met^{249} in a proposed "Met turn" (Bode *et al*, 1999) and a predicted high-affinity Ca^{2+} -binding site adjacent to the active site cleft (Gardner *et al*, 2009).

The Cys-rich domain of ADAMTS13 has an RGD sequence that is usually used for recognition of integrins on cellular membranes and could therefore act as a potential anchor to cell surfaces, but this possibility has not yet been investigated.

The thrombospondin-like repeats are homologous to the type I repeats of thrombospondin 1 and 2. The TSPs contain six potential CD36 binding motifs and preliminary in vitro studies showed that ADAMTS13 binds to CD36 via its TSPs without affecting its proteolytic activity suggesting that TSPs may localize ADAMTS13 to the cell surface of endothelial cells (Davis *et al*, 2009).

The CUB domains are unique for ADAMTS13 (Zheng *et al*, 2001) and seem to be involved in apical sorting of ADAMTS13 in human endothelial cells and Madin-Darby canine kidney fibroblasts (a well-established cell line for polarized cell) by interacting with membrane lipid rafts (Shang *et al*, 2006). Moreover, CUB domains seem to be critical for recognition and cleavage of VWF under flow conditions (Tao *et al*, 2005a;Zhang *et al*, 2007). Four of five cysteine residues in the CUB-1 domain seem to have a critical role in ADAMTS13 secretion and stability (Zhou *et al*, 2011).

1.3.4 ADAMTS13 - VWF interactions and ADAMTS13 activity regulation

VWF is the only known substrate of ADAMTS13 thus conferring to ADAMTS13 a highly selective role. ADAMTS13 cleaves VWF at a single site between amino acids Tyr1605 and Met1606 in the central part of the A2 domain. VWF circulates in a native globular-shaped conformation where the ADAMTS13 cleavage site inside the A2 domain is buried, rendering it inaccessible for ADAMTS13 (Zhang *et al*, 2009). Partial or complete VWF A2 domain unfolding is required for ADAMTS13 to cleave VWF. In vitro, unfolding is obtained by the use of mild denaturing agents such as urea or guanidine-HCl. In vivo, unfolding is induced by flow under high shear stress as found in small arteries and capillaries. Under flow conditions, newly released ULVWF from Weibel-Palade bodies or endothelial cells remains anchored to the cellular surface via P-selectin (Padilla *et al*, 2004) or $\alpha_V\beta_3$ integrin (Huang *et al*, 2009) in the form of string-like structures that are rapidly cleaved by ADAMTS13. Recent in vitro findings suggest that this type of VWF hardly requires shear stress (Turner *et al*, 2009;Jin *et al*, 2009). Cleavage of endothelial cell-anchored ULVWF multimers by ADAMTS13 appears to be the prevalent mechanism in vivo (Dong *et al*, 2002;Dong *et al*, 2004), but a portion of VWF is likely to be also cleaved in the microcirculation.

ADAMTS13 can also act as disulfide bond reductase of VWF multimers (Yeh *et al*, 2010). This novel activity of ADAMTS13 selectively targets inter-molecular disulfide bonds formed between VWF multimers under conditions of high fluid shear stress without affecting bonds that maintain the VWF multimeric structure. This reductase activity seems to be independent of the proteolytic activity of ADAMTS13 and might prevent covalent lateral VWF multimer association, a mechanism that in the absence of ADAMTS13 may lead to an enhanced binding to platelets.

Many cofactors can positively or negatively modulate ADAMTS13 proteolytic activity against VWF by binding to the enzyme itself or to VWF. Cations like Zn^{2+} and Ca^{2+} individually and cooperatively enhance ADAMTS13 proteolytic activity in static assays (Anderson *et al*, 2006).

Under high shear stress conditions, both platelets (Shim *et al*, 2008) and coagulation FVIII (Cao *et al*, 2008) independently, or synergistically when added together (Skipwith *et al*, 2010), accelerate and enhance VWF cleavage by ADAMTS13 in vitro. On the other hand, anions (ClO_4^- > Cl^- > F^-) may negatively modulate ADAMTS13 activity by binding to VWF under both static and flow conditions (De Cristofaro *et al*, 2005;De Cristofaro *et al*, 2006).

During systemic inflammation, several reactive oxygen species are generated which cause oxidative stress that may oxidize the methionine residue at the VWF peptide bond cleavage site (Met^{1606}) rendering VWF more resistant to ADAMTS13 proteolysis (Chen *et al*, 2010;Lancellotti *et al*, 2010). This effect may generate a prothrombotic state with accumulation of ULVWF multimers in circulation without altering their interaction with platelets.

Multiple interactions between ADAMTS13 and the unfolded VWF A2 domain are required for an efficient and specific proteolysis. The unfolded VWF A2 domain exposes different exosite-binding regions (adjacent and also distant to the cleavage site) that are recognized by counterpart exosites localized within different ADAMTS13 domains favoring a precise positioning of the ADAMTS13 active site next to the target VWF peptide bond.

The contribution of the different domains to the proteolytic activity of ADAMTS13 has been investigated using C-terminally truncated ADAMTS13 fragments. The metalloprotease domain alone showed no proteolytic activity against VWF, and truncations upstream of the spacer domain cleaved full-length VWF with only very low efficacy under static (Zheng *et al*, 2003;Ai *et al*, 2005;Gao *et al*, 2006) and also under flow conditions (Tao *et al*, 2005b). Addition of the spacer region restored activity to levels similar to those of full-length ADAMTS13 (Zheng *et al*, 2003;Tao *et al*, 2005b;Ai *et al*, 2005;Gao *et al*, 2006), highlighting the functional importance of the spacer domain for ADAMTS13 activity. Addition of domains C-terminal to the spacer domain do not further

increase the proteolytic activity of ADAMTS13 against VWF (Zheng *et al*, 2003;Ai *et al*, 2005).

The minimal functional VWF substrate identified for ADAMTS13 comprises 73 amino acid residues (Asp1596 to Arg1668) of the C-terminal region of the A2 domain (abbreviated VWF73) (Kokame *et al*, 2004).

Upon unfolding of the VWF A2 domain the ADAMTS13 spacer domain is docking to residues localized at the C-terminal end of the VWF A2 domain. Amino acids Arg658, Arg660, Tyr661 and Tyr665 within the spacer domain interact with residues between Glu1660 and Arg1668 in the VWF A2 domain (Gao *et al*, 2006;Gao *et al*, 2008;Akiyama *et al*, 2009;Jin *et al*, 2010;Pos *et al*, 2010). This step seems to be critical for recognition and subsequent cleavage of VWF. Docking of the spacer domain is followed by the binding of residues within the ADAMTS13 disintegrin-like domain close to the VWF cleavage site, thus positioning the VWF cleavage site next to the ADAMTS13 active site (de Groot R. *et al*, 2009). Before proteolysis of VWF can finally occur, subsites within the metalloprotease domain of ADAMTS13 need to interact with VWF residues adjacent to the cleavage site to ensure a highly specific cleavage of the VWF peptide bond (Ai *et al*, 2005;Gao *et al*, 2008;de Groot R. *et al*, 2010). Finally, stable interactions between the metalloprotease and the disintegrin-like domain appear to be essential for ADAMTS13 activity (Akiyama *et al*, 2009). The combined observations suggest that efficient substrate recognition, binding and cleavage require multiple N-terminal domains of ADAMTS13.

A productive proteolysis additionally depends on interactions of VWF with the C-terminal domains of ADAMTS13. The first CUB domain (CUB-1) or peptides derived thereof were shown to partially inhibit the docking and cleavage of VWF by ADAMTS13 under static and flow conditions (Tao *et al*, 2005a). Moreover, removal of the distal TSP1 2-8 and CUB domains also reduced the binding and cleavage of VWF under flow conditions, suggesting a critical role of these domains in VWF recognition (Majerus *et al*, 2005;Zhang *et al*, 2007). Studies conducted with congenic mice expressing a shorter version of ADAMTS13 that lacks the TSP1 7-8 and CUB domains have demonstrated that these mice were more prone to

thrombosis than mice carrying the wild type ADAMTS13 gene, suggesting an impaired capacity of the C-terminally truncated ADAMTS13 to regulate thrombus formation (Banno *et al*, 2009). In vivo studies using ADAMTS13 knockout mice showed that injection of murine (m) ADAMTS13 variants lacking the two CUB domains abolished proteolysis of platelet-decorated VWF strings (De Maeyer *et al*, 2010). Additional injection of truncated mADAMTS13 variants lacking the TSP1 2-8 repeats restored proteolysis. These studies stressed the relevant role of the TSP repeats and CUB domains for ADAMTS13 binding and cleavage of VWF in vivo (De Maeyer *et al*, 2010).

1.4 Measurement of ADAMTS13 activity, functional inhibitor and antigen

The normal plasma ADAMTS13 activity in healthy individuals ranges from 50-150%. The most widely used laboratory assays to measure ADAMTS13 activity in plasma employ multimeric VWF (purified plasma-derived or recombinant) as substrate and measure directly (by electrophoresis) or indirectly (platelet aggregation, enzyme-linked immunosorbent assay (ELISA)) the degraded VWF products generated after incubation with ADAMTS13 (Furlan *et al*, 1996;Tsai, 1996;Gerritsen *et al*, 1999;Obert *et al*, 1999;Böhm *et al*, 2002). Overall, these assays have moderate sensitivity but they are cumbersome, time-consuming, call for expert laboratory handling and importantly, they measure ADAMTS13 activity under static and non-physiological conditions requiring long incubation times and denaturing conditions (urea or guanidine hydrochloride) to unfold VWF and to mimic the role of the physiological blood shear stress.

Assays using as substrate recombinant VWF domains or short VWF A2 domain-derived peptides have also been developed (Cruz *et al*, 2003;Whitelock *et al*, 2004). Most of these assays use the 73 amino acid sequence of VWF (VWF73) shown to be the minimal functional ADAMTS13 substrate (Kokame *et al*, 2004). The main advantage of this type of assays is that it does not require denaturing conditions. Currently, the most

widely used assay is a fluorescence resonance energy transfer (FRET)-based assay employing a fluorogenic VWF73 as substrate (FRETS-VWF73) (Kokame *et al*, 2005). The FRETS-VWF73 assay, although using a non-physiological VWF substrate, showed good agreement with ADAMTS13 activity assays employing full-length VWF (Groot *et al*, 2006;Mahdian *et al*, 2006;Kremer Hovinga *et al*, 2006). It shows high reproducibility and accuracy and requires short incubation times (~1 h).

The measurement of ADAMTS13 functional inhibitor is based on the principle of the Bethesda method originally described for anti-factor VIII antibodies (Kasper *et al*, 1975). Heat-inactivated patient and pooled normal plasmas are mixed in a 1:1 proportion and incubated for 2 hours at 37°C. After incubation, the residual ADAMTS13 activity in the mixture is assayed by any of the conventional assays described above. A patient is considered to have an ADAMTS13 functional inhibitor if the residual ADAMTS13 activity in the mixture is less than 75% of a control mixture made with pooled normal plasma and buffer.

A method for measuring ADAMTS13 activity in vitro under flow conditions uses ULVWF multimers secreted by histamine-stimulated endothelial cells as VWF source (Dong *et al*, 2002). This assay seems to be reliable in discriminating ADAMTS13 activity only at levels higher than 20% (Tripodi *et al*, 2004). Although this assay mimics the in vivo situation more closely as it takes into consideration the endothelial cells and the fluid shear stress, expert laboratory handling is required making it unsuitable for routine testing.

Recently, a vortex-based assay to quantify ADAMTS13 activity and inhibitors has been proposed (Han *et al*, 2011). According to the authors, the vortex rotation generates a shear stress similar to that found in arteries and appears to be sufficient to unfold and cleave VWF thus mimicking more closely the in vivo situation.

ELISA-based assays to quantify ADAMTS13 antigen levels have been developed using polyclonal (Rieger *et al*, 2006) or monoclonal (Feys *et al*, 2006;Yagi *et al*, 2007) anti-ADAMTS13 antibodies. When comparing the

performance of two of these assays in a multicenter study (Tripodi *et al*, 2008), a good reproducibility and linearity could be demonstrated, with a limit of detection of 10% and 5%, using monoclonal (Feys *et al*, 2006) and polyclonal (Rieger *et al*, 2006) antibodies, respectively.

1.5 Thrombotic thrombocytopenic purpura and the role of ADAMTS13

Thrombotic microangiopathies (TMA) are a group of syndromes that share the common features of thrombocytopenia and microangiopathic hemolytic anemia with erythrocyte fragmentation (schistocytes) and increased serum levels of the intracellular enzyme lactate dehydrogenase (LDH) (Moake, 2009). The classical diseases associated with TMA are thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) although TMA is also observed in a wide range of other diseases such as systemic lupus erythematosus (SLE), malignancy, disseminating intravascular coagulopathy, pre-eclampsia, and endothelial damage due to drug toxicity (Copelovitch & Kaplan, 2008;Benz & Amann, 2010).

The HUS is characterized by a triad of renal failure, thrombocytopenia and non-immune hemolytic anemia with schistocytes. The common form of HUS, called typical HUS, is triggered by infection with shiga toxin-producing bacteria and is associated with a favorable outcome. The atypical HUS is a less common form (about 10% of the cases) and is associated with poor prognosis. In 50% of the cases, the atypical HUS is associated with genetic deficiency of the complement regulator protein factors H, I, and B or of membrane cofactor proteins. In the remaining cases, the etiology is unknown (Copelovitch & Kaplan, 2008;Benz & Amann, 2010).

TTP is a rare but life-threatening TMA first described by Moschcowitz in 1924 (Moschcowitz, 1924). The clinical presentation of TTP is characterized by a pentad of signs and symptoms including fever, thrombocytopenia, microangiopathic hemolytic anemia with red cell

fragmentation and renal failure and/or fluctuating neurological abnormalities (Moake, 2002). A deficiency in plasma ADAMTS13 is the currently accepted pathophysiological mechanism of TTP (Fig. 3). ADAMTS13 deficiency leads to the accumulation of ULVWF multimers under the high shear stress of the microcirculation with subsequent spontaneous platelet adhesion and aggregation and occlusive microvascular thrombosis. The presence of widespread hyaline VWF- and platelet-rich thrombi in the microcirculation causing multiple organ ischemia are the main histopathological features found in TTP patients (Moake, 2002) (Fig. 3).

Even if the pentad of clinical symptoms was originally required as diagnostic criterion for TTP, in the current practice, only the presence of a non-immune microangiopathic hemolytic anemia and thrombocytopenia without any alternative etiology are often sufficient criteria to suggest TTP and prompt initiation of treatment to reduce fatal outcomes (Moake, 2002;George, 2006). Since the introduction of plasma exchange (PEX) with plasma replacement in 1991 (Rock *et al*, 1991) as first choice therapy, the mortality rate of TTP has been reduced from 90% to 10-20% (Fontana *et al*, 2006). The mortality is mainly due to treatment refractoriness. Relapse occurs in 30-50% of the patients who survive an initial episode of TTP, being more often during the first year after the onset (George, 2009).

TTP has an estimated annual incidence in the adult population of 4-11 cases per million (Terrell *et al*, 2005;Scully *et al*, 2008). TTP occurs more frequently in adults than in children and it affects both sexes with an increased incidence for women and black race (Terrell *et al*, 2005).

Congenital and acquired TTP are the two clinically recognized forms of TTP. Distinction at clinical presentation between these two forms is important because of differences in treatment modalities. Congenital TTP, also known as Upshaw-Schulman Syndrome, is caused by a genetic deficiency of ADAMTS13 (Levy *et al*, 2001;Kokame *et al*, 2002), whereas autoantibody-mediated ADAMTS13 deficiency is considered to be the principal cause of acquired idiopathic TTP (Furlan *et al*, 1998;Tsai & Lian,

1998) and is often associated with a severe ADAMTS13 deficiency (<10%) (Zheng *et al*, 2004;Peyvandi *et al*, 2004).

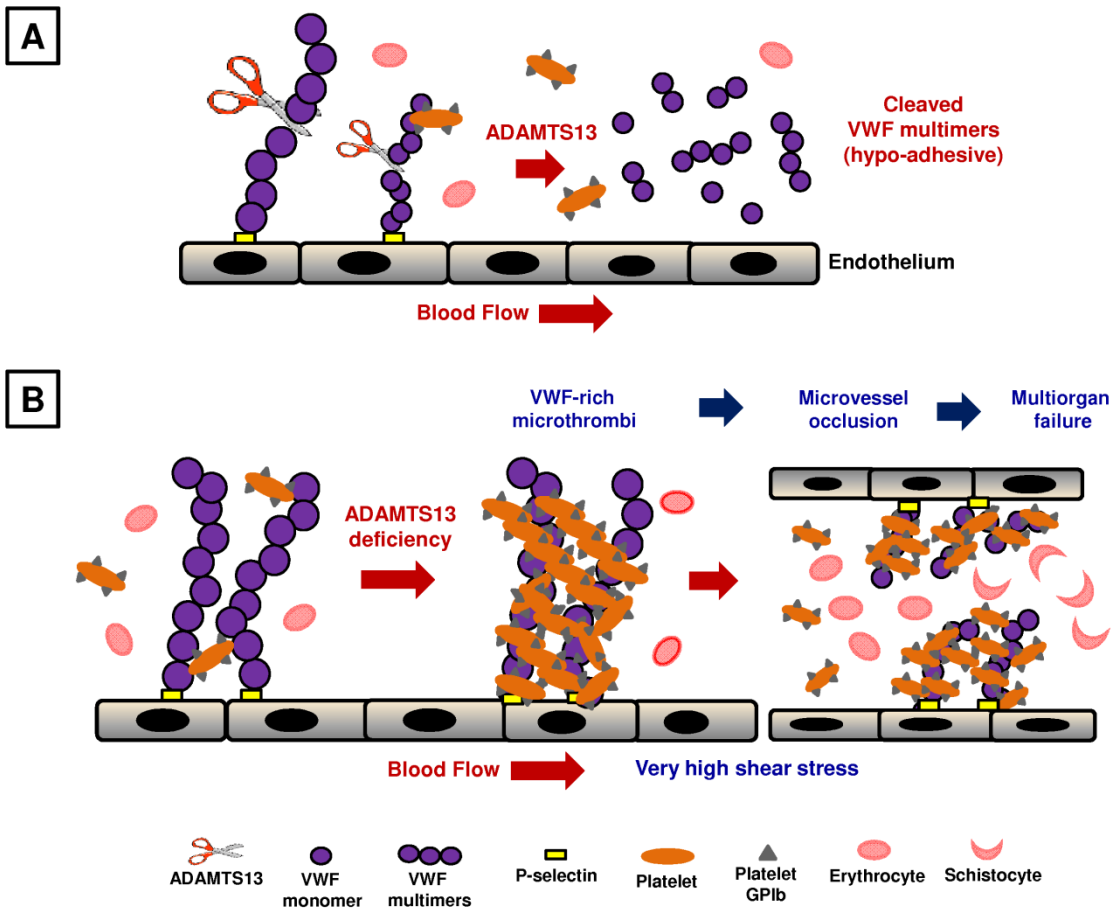

Figure 3. Role of ADAMTS13 in hemostasis

(A) Physiological role of ADAMTS13 in hemostasis. VWF multimers bind to endothelial cells (P-selectin) or to exposed extracellular matrix components. Platelets adhere to VWF through glycoprotein GPIb forming a platelet-rich thrombus the growth of which is limited by the proteolytic activity of ADAMTS13. (B) Pathogenesis of thrombotic thrombocytopenic purpura (TTP) caused by ADAMTS13 deficiency. In the absence of ADAMTS13, VWF-dependent platelet accumulation proceeds and eventually causes microvessel occlusion. This will lead to thrombosis and multiorgan failure, features characteristic of TTP.

A secondary, non-idiopathic acquired form of TTP occurs in association with pregnancy, certain drugs (e.g. ticlopidine, clopidogrel, quinine, chemotherapeutic and immunosuppressive agents), autoimmune disorders, infections, cancer and after hematopoietic stem cell transplantation (George, 2006;Zakarija *et al*, 2009). These cases are rarely associated with severe ADAMTS13 deficiency except for those associated

with pregnancy, autoimmune diseases or ticlopidine/clopidogrel treatment (Sadler, 2006).

Distinguishing TTP from atypical HUS is not always possible due to the overlap of symptoms. In the last years a differential diagnosis according to the underlying molecular defect was proposed (Tsai, 2010). In the case of atypical HUS, genetic mutations of genes regulating the complement system and for TTP, deficiency of ADAMTS13 are the proposed patho-mechanisms (Copelovitch & Kaplan, 2008;Benz & Amann, 2010).

Congenital TTP is uncommon (2-3% of TTP patients) and it has an autosomal recessive mode of inheritance. Deficiency of ADAMTS13 (levels 5-10% of normal) is due to homozygous or double heterozygous mutations in the *ADAMTS13* gene resulting in constitutional plasma deficiency or in a non-functional protein (Levy *et al*, 2001;Kokame *et al*, 2002). Until now, more than 70 mutations including missense and nonsense mutations, deletions, insertions, and splice site mutations have been reported (Lotta *et al*, 2010). Mutations are not clustered in one gene region, they are spread along the entire length of the gene encoding different protein domains (Levy *et al*, 2001). Single nucleotide polymorphisms of the ADAMTS13 gene, influencing ADAMTS13 secretion and activity have also been described in patients with congenital TTP (Kokame *et al*, 2002;Plaimauer *et al*, 2006). Congenital TTP generally presents in infancy or childhood, however some patients may develop clinical symptoms of TTP at later age (e.g. during first pregnancy, infections) (George, 2008).

Acquired idiopathic TTP is an autoimmune disease with an estimated incidence of ~4 cases/million people (Terrell *et al*, 2005), occurring mainly in previously healthy individuals. Idiopathic TTP is associated in most of the cases with severe ADAMTS13 deficiency (<10% of normal) and in 60-90% of the patients, inhibitory antibodies that block and reduce ADAMTS13 activity are identified by in vitro functional assays (Peyvandi *et al*, 2004). In a reduced number of cases, the presence of non-inhibitory antibodies was detected by ELISA (Scheiflinger *et al*, 2003;Shelat *et al*, 2006). Non-inhibitory antibodies may be involved in immune complex

formation increasing ADAMTS13 clearance or interfering with ADAMTS13 binding to cells or other plasma proteins. Antibodies against ADAMTS13 are predominantly of the IgG and IgM classes (Rieger *et al*, 2005;Tsai *et al*, 2006).

Epitope mapping of anti-ADAMTS13 antibodies in TTP patients has demonstrated that the major binding site on ADAMTS13 is located within the cysteine-rich/spacer domains (Klaus *et al*, 2004;Luken *et al*, 2005). More specifically, amino acids inside the spacer domain (Arg568, Phe592, Arg660, Tyr661 and Tyr665 on the outer surface of the spacer domain), which are directly involved in the interaction with the VWF A2 domain, are the major targeted amino acids by autoantibodies suggesting that autoantibodies may impair the binding of ADAMTS13 to VWF and by consequence inhibit its proteolysis (Jin *et al*, 2010;Pos *et al*, 2010;Pos *et al*, 2011). However, epitopes on different domains of the ADAMTS13 molecule have also been identified, mostly within the TSP1 2-8 and CUB domains (Klaus *et al*, 2004;Zheng *et al*, 2010;Pos *et al*, 2011).

Genetic factors also play a role in the etiology of acquired TTP. The presence or reduced levels of the antigens HLA-DRB1*11 and HLA-DRB1*04, respectively was identified as a risk factor for development of acquired TTP (Coppo *et al*, 2010;Scully *et al*, 2010).

1.6 Treatment of thrombotic thrombocytopenic purpura

The treatment modalities in patients with TTP differ if the patient suffers from congenital or acquired TTP. Patients with congenital TTP are treated with infusion of normal fresh frozen plasma (FFP) that contains normal levels of ADAMTS13. Potential relapses in these patients can be prevented or reduced by regular infusions of FFP every 2-3 weeks (Fontana *et al*, 2006).

The first line therapy for acquired TTP is daily PEX. During the plasmapheresis or PEX procedure, the patient´s plasma is extracorporeally separated from the blood cells followed by its replacement by a normal FFP or cryosupernatant and then re-infused together with the blood cells into the blood. The efficacy of PEX therapy has been attributed to the

combination of the infusion of normal FFP which supplies ADAMTS13 and the apheresis which removes circulating ULVWF multimers and ADAMTS13 autoantibodies when present (Fontana *et al*, 2006). Although PEX is the standard treatment for TTP, it is not safe and adverse events are reported to complicate about 5-12% of the PEX procedures (Nguyen *et al*, 2009). Immunosuppressive drugs (mainly the steroid methylprednisone) are often added as supportive therapy to improve outcomes.

More recently, the administration of rituximab, a chimeric monoclonal antibody, has been proven to be effective in the treatment of refractory (failure to respond to standard treatment) or relapsing acquired TTP mediated by autoantibodies. Rituximab is an antibody directed against CD20, a surface antigen on B-lymphocytes. Rituximab specifically depletes premature and mature B-cells thus increasing the clearance of antibody-producing cells and by consequence reducing the autoantibody titers (Elliott *et al*, 2009). Although there is still limited clinical experience and only few reports outline the advantage of rituximab in patients with TTP, its use appears to be promising and patients seems to benefit through long-lasting remission.

Splenectomy is another therapeutic option indicated in patients suffering of refractory or relapsing TTP. The diagnosis of refractory TTP is considered when incomplete, delayed or no clinical/laboratories responses are obtained with standard treatments within 7 days. Splenectomy has been used with a varying degree of success with its efficacy in preventing relapses remaining controversial (Dubois & Gray, 2010). Patients suffering from relapsing TTP seem to have more benefit from the splenectomy than those suffering of refractory TTP (Dubois & Gray, 2010). When splenectomy is performed on patients suffering from the relapsing form, it is associated with minimal morbidity and mortality and response rates greater than 80% are achieved (Dubois & Gray, 2010).

1.7 Anti-CD36 antibodies in TTP patients

CD36 (originally identified in platelets as glycoprotein VI) is an integral membrane protein of 471 amino acids that belongs to the class B scavenger receptor family. CD36 is expressed mainly in professional phagocytes, platelets, microvascular endothelium and fat and muscle cells (Silverstein & Febbraio, 2009). CD36 has multiple physiologic ligands including oxidized low-density lipoprotein (LDL), phosphatidyl serine (PS) and oxidized PS expressed on the surface of apoptotic cells, long-chain fatty acids and the TSP-1 domains on thrombospondin 1 (Silverstein & Febbraio, 2009). Based on the wide variety of ligands having diverse and complex functions CD36 could be a critical factor in different diseases such as cancer, atherosclerosis, malaria and insulin resistance (Febbraio & Silverstein, 2007).

Recent in vitro experiments using recombinant proteins showed that CD36 is able to also bind to ADAMTS13 without inhibiting its activity (Davis *et al*, 2009). The authors suggest that the binding may be mediated via its TSP-1 domains and could be responsible for localizing ADAMTS13 to the surface of endothelial cells (Davis *et al*, 2009).

Homozygous or compound heterozygous CD36 deficiency has been reported with variable incidence between different ethnic groups (Rac *et al*, 2007). In addition, a polymorphism in the blood group antigen Nak^a is associated with a selective deficiency of platelet CD36 (Yamamoto *et al*, 1990). Interestingly, no clinical symptoms appear to be associated to any type of CD36 deficiency. Patients carrying the platelet CD36 deficiency have no bleeding or thrombotic diatheses but anti-CD36 antibodies were reported in the plasma of some patients due to iso- or alloimmunization against the protein (Saw *et al*, 2010).

Interestingly, in about 70% of the patients with acquired TTP, autoantibodies directed against CD36 were detected but their pathological or clinical significance was not investigated (Tandon *et al*, 1994;Schultz *et al*, 1998;Wright *et al*, 1999). The presence of autoantibodies against CD36 has also been described in patients suffering from autoimmune diseases such as SLE (al-Shahi *et al*, 1997) and antiphospholipid syndrome (Pelegri

et al, 2003). More recently, Rock *et al* (Rock *et al*, 2005) described the concomitant presence of anti-CD36 and anti-ADAMTS13 antibodies in 16/35 patients analyzed, suggesting a possible pathogenic role of these antibodies in TTP.

2- AIM OF THE THESIS

The main goal of the thesis was to characterize the antibody response against the metalloprotease ADAMTS13 in patients suffering from acquired thrombotic thrombocytopenic purpura (TTP). Our group was the first to describe the presence of anti-ADAMTS13 IgG and IgM antibodies in TTP patients. These findings prompted a further characterization of the immune response in a cohort of adult patients with acute acquired TTP to better understand the patho-mechanism leading to acquired TTP. In addition, we attempted to investigate possible prognostic or predictive marker/s that could help to identify patients at risk of recurrence or fatal outcome.

DESIGN OF THE RESEARCH STUDY

The following aspects shall be investigated in a cohort of 76 patients diagnosed with acute acquired TTP:

- *Evaluation of ADAMTS13-specific activity, functional inhibitors and antigen levels*.

  The quantification of these parameters will be performed using standard assays.
- *Detection of total IgG, IgG subclasses, IgM, IgA and IgE anti-ADAMTS13 antibodies*.

  The detection of IgG and IgM antibodies in plasma will be performed using a pre-established in-house enzyme-linked immunosorbent assay (ELISA). For detection of IgG subclasses, IgA and IgE antibodies, specific ELISA systems will be established.
- *Detection of circulating ADAMTS13-anti-ADAMTS13 antibody immune complexes (IC)*.

  For detection of ADAMTS13-specific IC in plasma two different assays will be developed. (1) A co-immunoprecipitation of plasma ADAMTS13 with IgG, IgM, and IgA antibodies. (2) ELISA-based systems to detect and characterize the immunoglobulin type and subclass involved in the IC formation.

- *Detection of anti-CD36 antibodies*.

  For detection of anti-CD36 antibodies in plasma a specific ELISA will be established.

- *Correlation of the different parameters evaluated with the clinical outcome of the patients*

  The different parameters evaluated will be correlated with the clinical outcome of the patients using standard statistical tests and software.

3- METHODOLOGY

3.1 Patients

3.1.1 Plasma samples

Frozen plasma samples from TTP patients were obtained from three European Reference Centers: the Central Hematology Laboratory, University of Bern, Switzerland (Center 1), Service d´Hematologie biologique, Hôspital Antoine Béclère, Paris, France (Center 2) and Department of Medicine 1, Medical University of Vienna, Vienna, Austria (Center 3). Patients were enrolled after giving consent according to The Declaration of Helsinki. This study was approved by the institutional review board.

Plasma samples from healthy donors were collected at plasma collection centers of Baxter Innovations in Austria.

3.1.2 Sample collection

Before any therapeutical treatment was initiated, venous blood samples were collected into tubes containing 3.8% (w/v) sodium citrate and platelet-poor plasma was obtained by centrifugation at 2500 x g for 15 minutes. Plasma aliquots were stored at –80°C until tested. For one patient, daily plasma samples (time course samples during 2 months follow-up (death)) were collected before initiation of therapeutic PEX in which patient´ plasma is replaced by normal fresh frozen plasma.

3.1.3 Inclusion criteria

Patients had to meet the following inclusion criteria: (i) presence of microangiopathic hemolytic anemia (hemoglobin level <12 g/dL), direct antiglobulin test negative, at least 2 schistocytes per high-power field in the peripheral blood smear, LDH levels >450 IU/L and undetectable serum haptoglobin; (ii) thrombocytopenia (platelet count <150 x 10^9/L); (iii) severely reduced (<10%) plasma ADAMTS13 activity levels. Fever, neurological symptoms or renal failure were not obligatory.

3.1.4 Clinical definition

Idiopathic TTP was defined as TTP occurring in patients with no apparent preexisting disease. Patients are described as having secondary TTP if other conditions are identified that may cause thrombotic microangiopathy such as pregnancy, other autoimmune diseases, HIV infection and cancer. Remission was defined as a normal platelet count (>150 x 10^9/L) and no plasma exchange treatment for 30 consecutive days (day 1 of remission) or more. Relapse or recurrence was defined as the reappearance of clinical manifestation and/or laboratory data compatible with TTP after remission had been achieved. Refractory TTP is considered when incomplete, delayed or no clinical and laboratories responses are obtained with standard treatments within 7 days.

3.2 Measurement of the ADAMTS13 activity and inhibitor

3.2.1 Measurement of the ADAMTS13 activity

The measurements of residual ADAMTS13 activity (ADAMTS13:Ac) and anti-ADAMTS13 inhibitory activity in patients´ plasma were carried out in the participant centers at the moment when the patient was admitted to the hospital.

In Center 1, the ADAMTS13 activity and inhibitor was tested according to Studt *et al* (Studt *et al*, 2003) using a quantitative immunoblotting assay and in Center 2 according to Veyradier *et al* (Veyradier *et al*, 2001) using a two-site ELISA assay. Both methods measure ADAMTS13 activity under static conditions, employing multimeric/full-length VWF as substrate and indirect quantification of the VWF cleavage products by immunoblotting or ELISA. Both methods have similar sensitivities (Tripodi *et al*, 2004).

3.2.2 Measurement of the ADAMTS13 activity by FRETS-VWF73 assay

Samples received from Center 3 were tested in our laboratory using the FRETS-VWF73 assay performed essentially as described (Kokame *et al*, 2005) with minor modifications. Plasma samples were diluted 1 to 25 in assay buffer (5 mM Bis-Tris, 25 mM CaCl2, 0.005% v/v Tween 20, pH 6.0)

supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) in a final concentration of 2 µL/ 100 µL of reaction buffer and 2% v/v heat-inactivated pooled normal human plasma (NHP) (George King Bio-Medical, Overland Park, KS, USA) to correct for a plasma matrix effect in the dilution. The heat-inactivation of the plasmas was achieved by incubating the plasma at 56°C for 30 min followed by 15 min of centrifugation at 15000 x g. A calibration curve was made diluting a pooled NHP (George King Bio-Medical) 1 to 25 and by serial dilutions in a ratio of 3 to 4 (75%), 1 to 2 (50%), 1 to 4 (25%), 1 to 8 (12.5%), 1 to 16 (6.25) and 1 to 32 (3.12%) in supplemented assay buffer. The FRETS-VWF73 substrate (Peptide Institute Inc., Osaka, Japan) was re-suspended in 25% v/v dimethylsulfoxide, leading to 100 µM FRETS-VWF73 stock solution and subsequently diluted to a final concentration of 1.67 µM in assay buffer without supplementation. 100 µL/well of each diluted plasma sample or standard was added to a 96-well white plate (Costar, Corning Inc., NY, USA) followed by the addition of 100 µL/well FRETS-VWF73 substrate. The fluorescence was measured at 37°C every 5 min for 90 min in a SAFIRE II reader (Tecan, Zürich, Switzerland) equipped with a 340 nm excitation filter and 440 nm emission filter. The reaction rate was calculated by linear regression analysis of fluorescence over time and compared with that of a pooled NHP used as calibration curve.

3.2.3 Anti-ADAMTS13 inhibitor activity assay

The ADAMTS13 functional inhibitor titers were measured according to the Bethesda method originally described for anti-factor VIII antibodies (Kasper *et al*, 1975). Briefly, patient´ plasma and pooled NHP were heat-inactivated (as described in chapter 3.2.2) to inactivate any endogenous ADAMTS13 activity. Afterwards, a mixture of equal volumes of heat-inactivated patient´ plasma and pooled NHP as well as a control mixture made of buffer and pooled NHP were incubated for 2 hours at 37°C. After incubation, the residual ADAMTS13:Ac in both mixtures was assayed. The residual ADAMTS13:Ac is defined as the percentage between the residual ADAMTS13:Ac of the patient´ mixture compared with the control mixture.

A patient was considered to have inhibitors of ADAMTS13 if the residual ADAMTS13:Ac in the patient´ mixture was less than 75% of the control mixture. When the residual ADAMTS13:Ac of the mixture with the undiluted sample was below 25%, samples were retested by diluting until a residual ADAMTS13:Ac between 25% and 75% was obtained. The inhibitor titers are expressed in Bethesda Units per milliliter (BU/mL) and one BU is defined as the amount of inhibitor that results in 50% residual ADAMTS13:Ac. The inhibitor titer of the patient´ plasma is read from a semi-logarithmic plot representing the correlation between residual ADAMTS13:Ac (logarithmic) and the inhibitor titer in BU/mL (linear). The regression line is defined by 100% residual ADAMTS13:Ac when no inhibitor is present (0 BU/mL) and 50% residual ADAMTS13:Ac when 1 BU/mL inhibitor is present.

The procedure described above was followed by all the participant Centers with a minor modification in the incubation time done by Center 2 (30 min at room temperature (RT) instead of 2 hours at 37°C). The residual ADAMTS13:Ac was measured by the FRETS-VWF73 assay or the immunoblotting assay (Studt *et al*, 2003) (Center 1) or by ELISA (Veyradier *et al*, 2001) (Center 2).

There was a difference in the expression of inhibitor titers between Centers. Center 1 reported the exact inhibitor titers when the BU/mL were up to 2. When the residual ADAMTS13:Ac was between 11% and 25% (titers greater than 2 BU/mL), titers were reported as >2 BU/mL. When the residual ADAMTS13:Ac was equal or less than 10%, titers are reported as >>2 BU/mL. Center 2 expressed the titers semi-quantitatively; they were arbitrarily defined as high, medium or low for a residual ADAMTS13:Ac less than 10% in a 1:1, 2:1 or 3:1 volume/volume mixture of patient and pooled NHP, respectively.

Due to these differences in the expression of the ADAMTS13 functional inhibitor titers between Centers 1 and 2, a harmonization of the titer results was arbitrarily done to express them by a single measure. Inhibitor titers from Center 1 were assigned as low, medium or high when the BU were up to 2 BU/mL, >2 BU/mL and >>2 BU/mL, respectively.

3.3 Measurement of ADAMTS13 protein (antigen) levels

ADAMTS13 antigen (ADAMTS13:Ag) levels were analyzed in patients´ plasma by ELISA according to Rieger *et al* (Rieger *et al*, 2006). Microtiter plates (Nunc-Immuno Maxisorp, Roskilde, Denmark) were coated with 100 µL/well of a polyclonal rabbit anti-human ADAMTS13 IgG (2 µg/mL; Baxter Innovations, Vienna, Austria) in 0.1 M bicarbonate solution, pH 9.6 for 5 hours at RT. The non-specific binding sites were blocked with 0.5% w/v non-fat dry milk (Blotting Grade Blocker Non-Fat Dry Milk, Bio-Rad Laboratories, CA, USA) diluted into phosphate-buffered saline (PBS), pH 7.4, containing 0.1% v/v Tween-20 (Biorad) (PBS-T). Plasma and standard samples (100 µL/well) were incubated overnight at RT. Plates were washed and incubated with 100 µL/well of a horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-human ADAMTS13 IgG (Baxter) at 20 ng/mL and developed using 100 µL/well of the chromogenic 3,3',5,5'-tetra-methylbenzidine substrate (TMB; Sure Blue™ TMB Microwell Peroxidase Substrate, KPL, Maryland, USA). The color reaction was stopped by the addition of 50 µL/well of 1 N HCl and the absorbance was read at 450 nm with a reference filter of 620 nm on an iEMS microplate reader (Labsystems, Helsinki, Finland). Between each step, the plates were washed with PBS-T in an auto strip washer (Elx50 Bio-Tek instruments, Germany).

ADAMTS13:Ag levels in the samples were calculated extrapolating the corresponding OD value from a standard curve using purified recombinant ADAMTS13 (rADAMTS13, Baxter Innovations) diluted to final concentrations of 20, 10, 5, 2.5, 1.25, 0.625 and 0.5 ng/mL in ADAMTS13-depleted human plasma (Baxter Innovations). The reference interval for the ELISA (calculated with 100 individual healthy donors) was 740–1420 ng ADAMTS13/mL and the limit of quantification was 62.5 ng ADAMTS13/mL. Samples with antigen levels under the limit of quantification were expressed as <62.5 ng ADAMTS13/mL.

3.4 Detection of anti-ADAMTS13 antibodies

3.4.1 ELISA to detect total IgG, IgG subclass, IgM, IgA and IgE anti-ADAMTS13 antibodies

The presence of total IgG (IgG_{tot}) and IgG subclasses, IgM, IgA and IgE anti-ADAMTS13 antibodies in patient plasmas was tested by ELISA as described (Rieger *et al*, 2005) with minor modifications. Microtiter plates (Nunc) were coated with an anti-His tag antibody (2 µg/mL; Penta-His, Qiagen, Hilden, Germany) by overnight incubation at 4°C. The non-specific binding sites were blocked with PBS containing 2% w/v bovine serum albumin (BSA, Sigma) (PBS-BSA) and thereafter, 100µL/well recombinant His-tagged ADAMTS13 (Baxter Innovations) was added in a concentration of 2 µg/mL diluted in PBS-BSA. Diluted patients' plasma and negative controls (pooled NHP of 30 healthy donors, Baxter Innovations) were incubated overnight at 4°C. Bound antibodies were detected using an alkaline-phosphatase (AP)-conjugated goat anti-human IgG, IgM, IgA or IgE antibody (Sigma) or mouse monoclonal anti-human IgG1, IgG2, IgG3 or IgG4 antibodies (Zymed Laboratories, San Francisco, CA, USA). Finally, the enzyme substrate *p*-nitrophenylphosphate (pNPP; Sigma) was added, and the absorbance was read at 405 nm with a reference filter of 620 nm on a microplate reader (Labsystems). Between each step, the plates were washed with PBS-T in an auto strip washer (Bio-Tek instruments).

3.4.2 Cross-reactivity assay

The specificity in the detection of the AP-conjugated mouse monoclonal anti-human IgG1-IgG4 and the goat anti-human IgA and IgE antibodies used in the ELISAs was tested in an ELISA-based cross-reactivity assay. Briefly, microtiter plates (Nunc) were coated with 100 µL/well of purified human IgG1-4, IgM, IgA and IgE (Sigma) diluted in a concentration range between 4 and 0.1 µg/mL using PBS. After overnight incubation at 4°C, the plates were washed and incubated with 100 µL/well of an AP-conjugated goat anti-human IgA or IgE antibody (Sigma) or monoclonal AP-conjugated mouse anti-human IgG1, IgG2, IgG3 and IgG4 antibodies

(Zymed). The enzyme substrate pNPP (Sigma) was added, and the absorbance was read at 405 nm with a reference filter of 620 nm on a microplate reader (Labsystems). No cross-reactivity was observed at any concentration with any class or subclass of antibodies analyzed.

3.4.3 Data analysis and cut-off calculations

IgG_{tot} and IgG subclass, IgM, IgA and IgE anti-ADAMTS13 antibodies detected by the ELISA described in chapter 3.4.1 were expressed as antibody titers. For calculation of the anti-ADAMTS13 antibody titers, the ELISA read out (optical density, OD) from 100 healthy donors diluted in a range between 1 to 20 up to 1 to 6400 was used to calculate the ratio of sample OD to background OD for each sample at each dilution. As background OD the signal derived from a pooled NHP was used. To achieve an approximate normal distribution, the OD ratio values of the 100 control plasmas were transformed by the function y=log(1+log(x)). Normal distribution was assessed by quantile-quantile plots and Shapiro-Wilk tests. The means and standard deviations of the resulting 100 ratios were calculated. The thresholds for each dilution were obtained by back-transformation of the means plus two standard deviations of the transformed data. Samples with ratios below or above the cut-off levels were judged as negative or positive, respectively. When a sample was judged positive, sample dilutions were compared with the corresponding cut-off value and the last dilution above the cut–off value was taken as positive.

For the IgG subclass ELISA, the OD values were additionally normalized by the corresponding normalization factor and calculated as described below.

3.4.4 Quantification and normalization of the absorbances measured in the IgG subclass ELISA

An ELISA with subclass-specific antibodies was used to estimate the relative amount of the different IgG subclasses, first because of the absence of a true standard, and second because of the differences in the

development time between the IgG subclasses due to differences in the detection sensitivities of the secondary antibodies used. To minimize these differences, the relative OD values of the IgG subclass ELISA were compared under standardized conditions.

Each IgG subclass (purified human IgG1-IgG4, Sigma) was coated on an ELISA plate in a concentration range of 0.125 to 1 µg/mL and the time-dependent OD signal generated was analyzed. The corresponding OD values generated were recorded every 5 min up to 75 min, and plotted against time. Slopes for each concentration and subclass were calculated. The corresponding means ± standard deviations of the slopes from 4 independent experiments were plotted and analyzed by linear regression for each individual IgG subclass. The regression parameters obtained were used to calculate the OD values of each subclass at a coating concentration of 0.5 µg/mL (linear range). The OD value of IgG4 was set to "1", resulting in normalization factors for IgG1, IgG2 and IgG3 of 9, 0.7 and 0.4, as compared with IgG4. Coating efficiency was evaluated in each experiment by ELISA using an AP-conjugated goat anti-human lambda light chain antibody (Sigma) to ensure uniform IgG1-IgG4 concentrations.

The calculated normalization factors were used to normalize the OD values in patients with more than one IgG subclass of anti-ADAMTS13 antibodies. Samples were normalized by multiplying the OD values corresponding to each positive subclass by the corresponding calculated normalization factor. With this approach, an estimate of the relative contributions of the different IgG subclasses to the total anti-ADAMTS13 IgG in patient plasma was obtained. Without the normalization procedure, IgG1 levels would have been underestimated as compared to IgG2, IgG3 and IgG4 levels. Samples with normalized OD values above the linear range were excluded (six patients) from analyses.

Positive normalized OD values of each subclass were summed up (IgG_{sum}, total absorbance) and the proportion of each individual subclass was calculated as a percentage of the total absorbance (assumed to be 100%) to obtain the IgG subclass percentage distribution in a single patient.

3.4.5 Competitive inhibition of antibody binding with recombinant ADAMTS13

To ensure specificity of the anti-ADAMTS13 antibody detection, competitive inhibition experiments with rADAMTS13 in solution were carried out. Diluted patient and pooled NHP plasmas were pre-incubated with 100 µg/mL of purified rADAMTS13 at 37°C for 2 hours to allow binding of ADAMTS13-specific antibodies to rADAMTS13 in solution. Afterwards, the binding of anti-ADAMTS13 antibodies was assayed by the ELISA as described above (chapter 3.4.1). As negative control, plasma samples were pre-incubated with BSA (100 µg/mL). A sample was judged as positive when addition of rADAMTS13 decreased the antibody binding by at least 30% when compared to the decrease of the negative control.

3.5 Detection of IgG anti-ADAMTS13 antibodies by a commercial ELISA kit

The measurement of IgG anti-ADAMTS13 antibodies was also performed using a commercially available kit (TECHNOZYM ADAMTS-13 INH; Technoclone GmbH, Vienna, Austria) according to the manufacturer´s instructions. Briefly, 100 µL/well of diluted plasma samples or calibrators were added to wells coated with rADAMTS13 and incubated for 1 hour at RT. Thereafter, 100 µL/well of a HRP-conjugated anti-human IgG were added followed by 100 µL/well of the chromogenic substrate TMB. The color reaction was stopped by the addition of 1.9 M H_2SO_4 and the absorbance was measured at 450 nm with a reference filter of 620 nm on a microplate reader (Labsystems).

The antibody titers of a sample were obtained by extrapolating from the corresponding OD value of a standard curve using standards provided with the kit in concentrations ranging between 0.8-98.8 U/mL of anti-ADAMTS13 antibodies. Standards are calibrated against a plasma with a very high titer of IgG anti-ADAMST13. A 1 to 200 dilution of this reference plasma is defined to contain an antibody concentration of 100 U/mL (arbitrary units).

The positive cut-off value of the assay is 15 U/mL, samples with results between 12-15 U/mL are judged as borderline and samples with values below 12 U/mL are judged as negative.

3.6 Detection of circulating immune complexes by co-immunoprecipitation and Western blotting

Patient plasma (200 µL) was incubated with 100 µL protein G Sepharose (Protein G HP SpinTrap™ columns, GE Healthcare, Buckinghamshire, England) or anti-IgA or anti-IgM specific affinity matrixes (BAC CaptureSelect, Naarden, The Netherlands) for 30 min (IgG) and 120 min, respectively at RT on a rotator. The immunoprecipitated IgG-, IgA-, or IgM containing material was washed 6 times with binding buffer (20 mM sodium phosphate, 150 mM NaCl; pH 7.0) and the antigen-antibody complexes were eluted from the beads by adding sample loading buffer (Thermo, Rockford, IL, USA) and heating at 95°C for 10 min. The resulting supernatants were loaded on a 4–12% gradient tris-glycine SDS polyacrylamide pre-cast gel (Invitrogen, Caramillo, CA, USA) and subjected to electrophoresis under reducing conditions. The separated proteins were transferred to a polyvinyldifluoride membrane using an iBlot® Dry Blotting System (Invitrogen), and ADAMTS13 was detected with an affinity-purified polyclonal rabbit IgG anti-human ADAMTS13 antibody (1 µg/mL; Baxter Innovations) in combination with a HRP-conjugated goat anti-rabbit IgG (27 ng/mL; Jackson Immunoresearch, West Grove, PA, USA) and addition of a chemiluminescent substrate (Thermo). Immunoreactive bands were visualized with the Fusion FX7 image system (Vilber Lourmat, Germany). The ADAMTS13 luminograms were scanned and the relative amount of immune complexes (ICs; expressed in arbitrary units (AU)) was quantified by densitometry using the BIO-1D software (Vilber Lourmat) and rADAMTS13 (1 ng) as standard.

To evaluate for unspecific binding to the matrix, human serum albumin (HSA) in a final concentration of 50 µg/mL was spiked with 2 µg/mL rADAMTS13 and processed as the plasma samples. For the anti-IgA matrix, a minimal unspecific binding was observed with the negative

controls. In this case, the mean background signal generated by the pooled NHP and ADAMTS13-spiked samples was subtracted from the signal of the plasma samples.

3.7 Detection of circulating immune complexes by ELISA

Microtiter plates (Nunc) were coated with 100 µL/well of a polyclonal rabbit anti-human ADAMTS13 IgG (2 µg/mL; Baxter Innovations) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 by overnight incubation at 4°C. The non-specific binding sites were blocked with 0.5% w/v non-fat dry milk (Bio-Rad) diluted into PBS-T buffer. A 100 µL/well of diluted patient and normal plasmas (dilution range: 1 to 20, 1 to 50 and 1 to 100) were incubated overnight at 4°C. Thereafter, plates were washed and incubated with 100 µL/well of an AP-conjugated goat anti-human IgA (Sigma) or mouse monoclonal anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (Zymed Laboratories). Finally, the enzyme substrate 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (KPL) was added, and the absorbance was read at 620 nm on an iEMS microplate reader (Labsystems). Between each step, the plates were washed with PBS-T in an auto strip washer (Elx50 Bio-Tek instruments).

For cut-off calculation, 60 healthy donors diluted in the same range as the samples were used to calculate the ratio of sample OD to background OD (signal derived from a pooled NHP). Normal distribution was assessed by the Shapiro-Wilk test and the means plus three standard deviations of the resulting 60 ratios were calculated for each dilution. Samples with ratios below or above the cut-off levels were judged as negative or positive, respectively.

3.7.1 Detection of complement fixing immune complexes by C1q binding ELISA

Complement fixing immune complexes (ICs) were analyzed using a C1q binding ELISA kit (Bühlmann Laboratories, Schönenbuch, Switzerland) according to the manufacturer´s instructions. Briefly, patient plasma, calibrators and controls were incubated with human C1q adsorbed on a

microtiter plate. After incubation, AP-conjugated Protein A was added, followed by the enzyme substrate pNPP. The color reaction was stopped after 30 min of incubation by the addition of 1 N NaOH and the absorbance was measured at 450 nm with a reference filter of 620 nm on a microplate reader (Labsystems).

The concentration of ICs present in the plasma samples was determined by extrapolating the corresponding OD value to a standard curve using standards provided with the kit. Results were expressed as heat aggregated human gamma globulin equivalents per mL (µg Eq/mL). The negative cut-off value of the assay is 3.2 µg Eq/mL, samples between 3.2-5.0 µg Eq/mL are judged as borderline and samples with values above 5.0 µg Eq/mL are judged as positive.

3.7.2 Competitive inhibition of the binding of human IgG4 to rabbit anti-ADAMTS13 antibody

To confirm unspecific binding of human IgG4 to rabbit anti-ADAMTS13 antibody coated on the plate, a competitive inhibition experiment was performed. Diluted normal plasma (dilution range: 1 to 20, 1 to 50, 1 to 100, 1 to 200, 1 to 400 and 1 to 800) was pre-incubated with 11 µg/mL monoclonal mouse anti-human IgG4 antibody (Sigma) at 37°C for 30 min. After incubation, the binding of human IgG4 was assayed by ELISA as described in chapter 3.7. As negative control, diluted plasma samples were pre-incubated with 11 µg/mL of BSA diluted in PBS.

3.7.3 Binding of rabbit IgG to human antibodies

Microtiter plates (Nunc) were coated by overnight incubation at 4°C with 100 µL/well of purified human IgG1-4 (Sigma), IgM (Sigma) and IgA (Calbiochem, Darmstadt, Germany) in a concentration of 4 µg/mL diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6. The non-specific binding sites were blocked with 0.5% non-fat dry milk (Bio-Rad) diluted in PBS-T buffer. Thereafter, plates were washed and incubated with 100 µL/well of HRP-conjugated rabbit IgG (whole molecule) or rabbit IgG Fc fragment or rabbit IgG F(ab)2 fragment (Alpha Diagnostic, San Antonio, TX, USA), in a

concentration range between 1 and 4 µg/mL. After 4 hours of incubation, plates were developed with TMB substrate (KPL) and the color reaction was stopped by the addition of 1 N HCl. The absorbance was read at 450 nm with a reference filter of 620 nm on a microplate reader (Labsystems). Between each step, the plates were washed with PBS-T in an auto strip washer (Bio-Tek instruments).

3.8 Detection of anti-CD36 antibodies by ELISA

Microtiter plates (Half area, high binding; Costar, Corning Inc., NY, USA) were coated with 50 µL/well of recombinant full-length CD36 (1.5 µg/mL; Origene, Rockville, USA) diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 by overnight incubation at 4°C. The non-specific binding sites were blocked with Protein-Free blocking buffer (Thermo). Afterwards, 50 µL/well of patient and normal plasmas (diluted 1 to 50 in LowCross buffer (Candor Bioscience, Weissensberg, Germany) were added and incubated for additional 3 hours at RT. Plates were washed and incubated for 90 min at RT with 50 µL/well of biotin-conjugated goat anti-human IgG antibody (Rockland Immunochemicals, Gilbersville, PA, USA) at 10 ng/mL in LowCross buffer followed by the addition of streptavidin-peroxidase polymer conjugate (Sigma) diluted 1 to 5000 in PBS. Plates were developed by the addition of 50 µL/well of the TMB substrate (KPL) and the color reaction stopped with 50 µL/well of 1 N HCl. Absorbance was read at 450 nm with a reference filter of 620 nm on a microplate reader (Labsystems). Between each step, the plates were washed with PBS-T in an auto strip washer (Bio-Tek instruments).

As a positive ELISA control, a monoclonal rat anti-human CD36 antibody (R&D Systems, Minneapolis, MN, USA) was tested in a concentration range between 0 and 60 ng/mL and detected with HRP-conjugated rabbit anti-rat IgG (whole molecule, 100ng/mL, Sigma).

For cut-off calculation, 60 healthy donors were used to calculate the ratio of sample OD to background OD (signal derived from a pooled NHP). Normal distribution was assessed by the Shapiro-Wilk test after log-transformation of the data and the means and standard deviations of the

resulting 60 ratios were calculated. The thresholds for each dilution were obtained by back-transformation of the means plus three standard deviations of the transformed data. Samples with ratios below or above the cut-off levels were judged as negative or positive, respectively.

To ensure that the detected anti-CD36 antibodies are specific, positive samples were subjected to competitive inhibition using an excess of rCD36 (11 µg/mL) in solution. Following pre-incubation with soluble rCD36 at 37°C for 2 hours, patient and pooled NHP plasma samples were assayed for anti-CD36 antibodies by the ELISA as described above.

3.9 Statistical analysis

Statistical analyses were done with SigmaStat version 3.5 (Systat Software, San Jose, CA, USA). Demographic and clinical data are presented as the median and the interquartile range (IQR). Cut-off values for the different established ELISAs were calculated as described in the corresponding chapter. The statistical significance of the differences between OD ratios of healthy donors and TTP patients were assessed by the Mann-Whitney rank sum test. The statistical significance of the differences between medians of IgG1 and IgG4 values were assessed by the Mann-Whitney rank sum test and the correlation by the Spearman rank test. To assess the strength of the relation between IgG4 levels and recurrence, 40 patients were divided into three groups according to clinical outcome. The first group comprised 18 patients who had experienced only a single TTP event, the second comprised nine patients who relapsed during the 36 months of follow-up and the third comprised 13 patients who had had relapse(s), five of whom had had no further relapses during follow-up and eight of whom were lost to follow-up after the acute relapsing event. Deceased patients, patients lost to follow-up after the first TTP event and patients with IgG4 OD values above the linear range were excluded from this analysis (18 patients). *P*-values less than 0.05 were considered to be statistically significant.

4- RESULTS

4.1 Demographic and clinical features of the patients enrolled

Seventy-six adult patients diagnosed with acute acquired TTP were included in the study. All of them met the inclusion criteria described in chapter 3.1.3. Demographic, clinical and standard biological features of the 76 patients enrolled are summarized in Table 1. The median age was 37 year-old (range 16-75) and the sex ratio was 2F/1M (50 women and 26 men). Sixty-three had idiopathic TTP and thirteen had TTP associated with pregnancy (*n* = 6), the postpartum state (*n* = 1), psoriasis (*n* = 1), antiphospholipid syndrome (*n* = 1), SLE (*n* = 1), renal disease (*n* = 2) or cancer (*n* = 1).

Table 1. Demographic and clinical features of the patients with acute acquired thrombotic thrombocytopenic purpura (TTP) included in the study

Sex, F/M	50/26
Median age, years (range)	37 (16-75)
Median platelet count, $x10^9L^{-1}$ (range)	15 (2-96)
Median hemoglobin levels, g/dL (range)#	9.6 (4.2-14.7)
No of patients / total	
Idiopathic TTP	63/76
TTP with associated conditions	13/76
First episode	57/76
Relapse	19/76
Renal involvement*	9/68
Neurological symptoms#	52/58

* data were not available for 8 patients
\# data were not available for 17 patients

Renal involvement was considered when plasmatic creatinine values were above 120 µmol/L. Neurological involvement included: aphasia, headache, confusion, paresthesia, altered mental status and seizure.

Fifty-seven patients were analyzed during the first acute episode of TTP and 19 during relapse. Four of the 57 (7%) patients analyzed during the first episode died of acute multivisceral thrombotic microangiopathy, 10 patients were lost to follow-up and 12/43 who achieved clinical remission, relapsed during a minimum of 48-months follow-up (follow-up performed in a range of 48 to 109 months). The 19 patients studied during an acute

relapse had a documented positive history of TTP with at least one previous TTP episode. Seven of the 19 patients were included in the 48-months follow-up and they had no new relapses and 12/19 patients were lost to follow-up.

At inclusion during the acute episode, anemia (hemoglobin median levels 9.6 g/dL; range 4.2–14.7 g/dL; data available only for 59/76 patients) and thrombocytopenia (median $15X10^9$/L; range $2\text{-}96X10^9$/L) were present in the patients. Neurologic symptoms including headache, altered mental status, aphasia, paresthesia, transitory ischemic attack or convulsions were observed in 27/68 (40%) patients. Renal involvement, considered when plasmatic creatinine values were higher than 200 µmol/L, was present only in 9/68 (13%) patients whereas the mean creatinine level was 101 ± 31 µmol/L in the remaining 59 patients. For 8 patients, the neurological and renal data were not available.

4.2 ADAMTS13 activity, functional ADAMTS13 inhibitor and ADAMTS13 antigen levels in patients with acquired TTP

As a result of our inclusion criteria, all patients (n=76) had ADAMTS13:Ac values <10%. A functional inhibitor was found in 69/76 (91%) patients; with 26 patients having a low, 23 patients a medium and 20 patients a high titer. In 7 (9%) patients a functional inhibitor was not detected by the assays used at the respective reference center.

Analysis of ADAMTS13:Ag levels in TTP patients showed a variable distribution similar to earlier findings (Rieger *et al*, 2006). Eighteen patients had undetectable levels (<65 ng/mL), 10 patients had severely reduced antigen levels (65-100 ng/mL), 42 patients had reduced levels (100-740 ng/mL) and 6 patients had normal levels. There were no differences when the antigen levels between patients with single or relapsing TTP events were compared (Fig. 4). Three of the four deceased patients had undetectable antigen levels and the fourth one a borderline level (83 ng/mL) (Fig. 4).

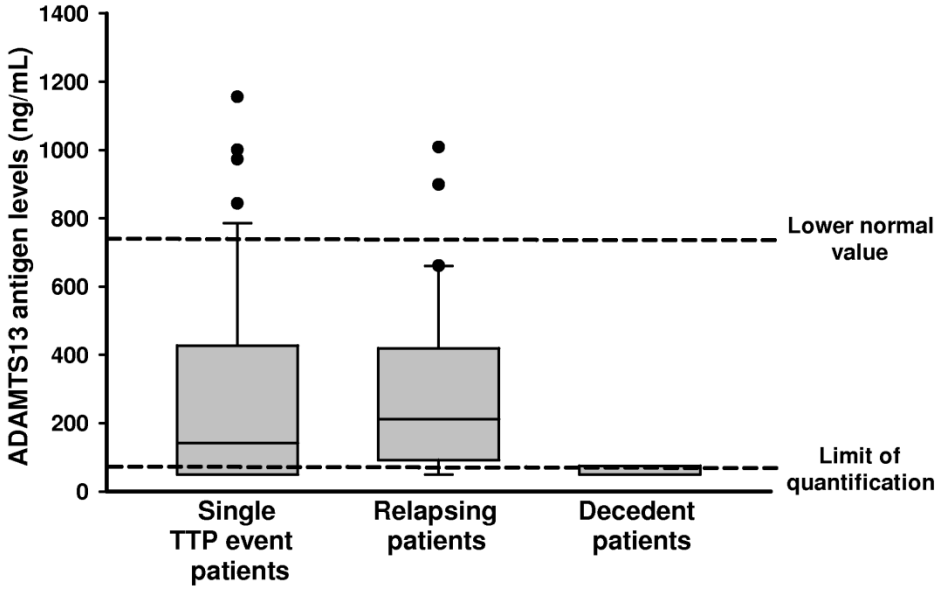

Figure 4. ADAMTS13 antigen levels in patients with acquired TTP.

Box plot of the distribution of the ADAMTS13 antigen levels in 76 patients with acute acquired TTP. Patients are divided into 3 categories according to the clinical outcome. The lower normal value (740 ng/mL) and the limit of quantification (<65 ng/mL) are indicated by dashed lines. The bottom, median and top lines of the box mark the 25th, 50th, and 75th percentiles, respectively. The vertical line shows the range of values comprised between the 5th and 95th percentiles and the dots represent outlier values.

4.3 Anti-ADAMTS13 antibody profile in patients with acquired TTP

To investigate the presence of anti-ADAMTS13 antibodies, in-house ELISA assays were employed. These ELISAs use immobilized recombinant ADAMTS13 and bound plasma antibodies are visualized by means of specific secondary, enzyme-labeled antibodies.

For detection of IgG and IgM anti-ADAMTS13 antibodies, previously established in-house ELISA assays were used (Rieger *et al*, 2005). For detection of IgA, IgE and IgG subclasses, new ELISA assays were developed introducing a single modification to the pre-established ELISA at the level of the secondary antibody.

4.3.1 Anti-ADAMTS13 antibody profile in the acute episode

Analysis of the anti-ADAMTS13 antibody profile in the acute episode showed the presence of anti-ADAMTS13 antibodies in 71/76 (93%) patients.

IgG anti-ADAMTS13 antibodies were detected by our in-house ELISA in 52/76 TTP patients. In patients whose anti-ADAMTS13 IgG titers were lower than the detection limit of our ELISA (IgG negative, 18 patients), plasma samples were retested for anti-ADAMTS13 IgG antibodies using a commercial kit with improved sensitivity due to the use of a horseradish peroxidase-conjugated secondary antibody instead of an alkaline-phosphatase-conjugated one. Using this kit, 12/18 patients tested positive for IgG anti-ADAMTS13 antibodies and 6 patients were confirmed negative.

Taking together the results of both ELISA assays (in-house and commercial), 70/76 (92%) patients with acute TTP had a specific IgG against ADAMTS13 with titers ranging from 20 to 6400 (Fig. 5).

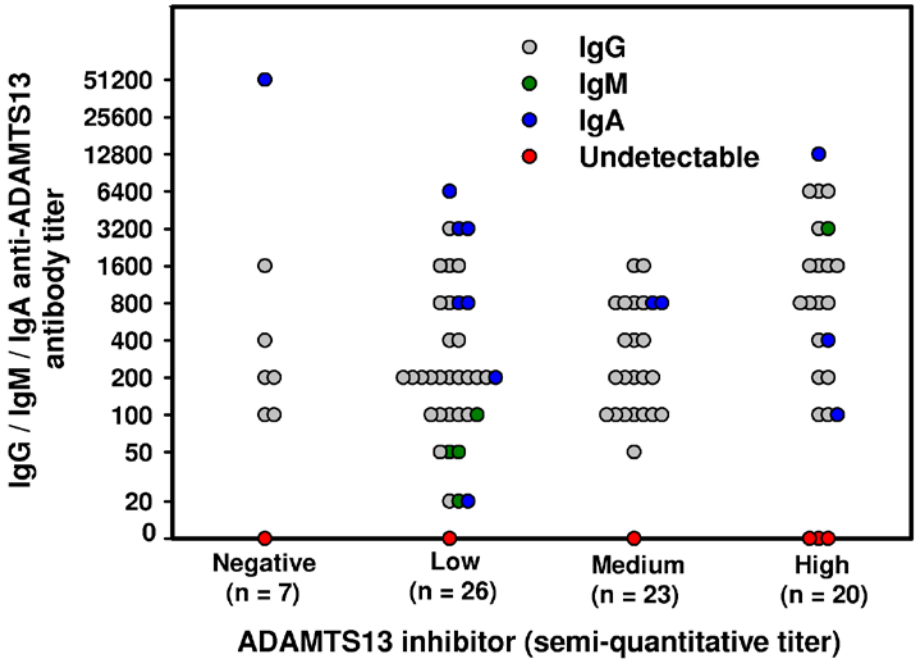

Figure 5. ADAMTS13 inhibitor and anti-ADAMTS13 IgG/IgM/IgA antibodies in patients with acquired TTP.

Seventy-six patients with acute acquired TTP were analyzed for the presence of ADAMTS13 inhibitor and the different classes of anti-ADAMTS13 antibodies. Patients are grouped in 4 categories according to their ADAMTS13 inhibitor titers (semi-quantitative): negative (7 patients), low (26 patients), medium (23 patients) and high (20 patients). Each patient is represented by a symbol. Gray circles are for patients with positive IgG, green circles for IgM, blue circles for IgA and red circles for patients with no detectable IgG/IgM/IgA (undetectable). Only positive patients are depicted for IgM and IgA antibodies.

IgM and IgA classes of anti-ADAMTS13 antibodies were investigated in addition to the IgG class. Low titers (ranging from 20-100 with exception

of one patient with 3200) of IgM anti-ADAMTS13 antibodies were detected in 5/76 (7%) patients (Fig. 5). IgA anti-ADAMTS13 antibodies were found in 13/75 (17%) patients with variable titers from 200 to 51200 (Fig. 5). In one patient, the IgA anti-ADAMTS13 antibody titer could not be determined due to the lack of a sufficient amount of plasma.
A trend toward increasing inhibiting activity with increasing anti-ADAMTS13 titers was observed, especially in those patients positive for more than one class of antibodies (Fig. 5).

Isolated IgG anti-ADAMTS13 antibodies were detected in 57 patients. IgM was not detected alone and only one patient had low titer of IgA antibodies. The combination of IgG and IgM was detected in 2 patients, the combination of IgG and IgA in 8 patients and the combination of the 3 classes of antibodies in 3 patients. Taken together, 71 out of 76 (93%) patients tested positive for anti-ADAMTS13 antibodies.

The presence of non inhibitory antibodies was detected in 6 out of 7 patients with no measurable functional inhibitor (Fig. 5). Five patients tested positive only for IgG antibodies and the sixth one for IgG in combination with high IgA antibodies. One patient tested negative for both ADAMTS13 functional inhibitor and classes (IgG, IgM or IgA) of anti-ADAMTS13 antibodies. The overall prevalence of non inhibitory antibodies in this study was 8%.

An interesting finding is that the plasma from 4 patients inhibited ADAMTS13 activity in the absence of any detectable class (IgG, IgM or IgA) of anti-ADAMTS13 antibodies.

4.3.2 Correlation between ADAMTS13 antibodies at presentation and ADAMTS13 activity at initial clinical remission

The ADAMTS13 activity was analyzed at initial clinical remission in only 32 out of the 76 patients studied. The ADAMTS13 activity showed 3 distinct courses: it either remained undetectable (<5%) in 13 patients (41%), became detectable but partially decreased (15 to 40%) in 12 patients (38%), or normal (50 to 100%) in 7 patients (21%). ADAMTS13

inhibitors in remission were detectable only in patients whose ADAMTS13 activity remained <5%, with titers either similar or decreased when compared to those of the acute episode (Fig.6).

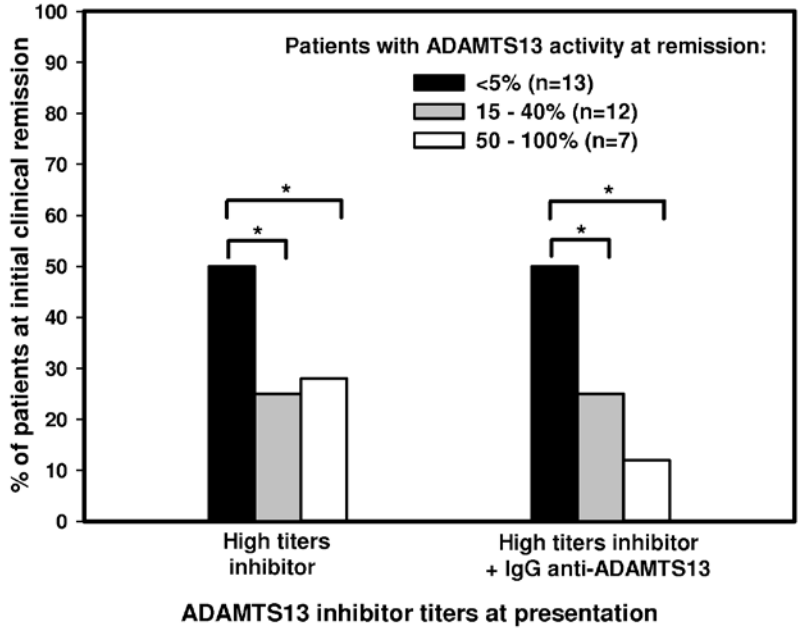

Figure 6. Correlation between ADAMTS13 inhibitor titers at presentation and ADAMTS13 activity at initial clinical remission in patients with acquired TTP. Patients are grouped in 3 categories according to the level of ADAMTS13 activity at initial clinical remission: <5% (n = 13), between 15 and 40% (n = 12) and between 50 and 100% (n =7). At presentation, the presence of high titers of ADAMTS13 inhibitor, either alone or combined with IgG anti-ADAMTS13 antibodies, was more frequent in patients having undetectable ADAMTS13 activity (below 5%) at initial clinical remission when compared to patients having detectable ADAMTS13 activity. (*): significant statistical difference ($P < 0.05$) between both groups.

When trying to correlate ADAMTS13 antibodies at presentation and ADAMTS13 activity at initial clinical remission; the presence of a high titer of ADAMTS13 inhibitor, either alone or combined with anti-ADAMTS13 antibodies of the IgG class at presentation, was associated with the persistence of an undetectable ADAMTS13 activity (<5%) in initial clinical remission (Fig.6).

4.4 Subclass distribution of IgG anti-ADAMTS13 antibodies

The biological function of IgG antibodies is determined by their specificity, affinity and subclasses. The IgG subclasses have different capacities to bind to cell surface Fcγ receptors (FcγRs) or to activate complement proteins, thus mediating different immunological effector functions

(Jefferis *et al*, 1994). Due to our findings that about 92% of the TTP patients have IgG anti-ADAMTS13 antibodies, we decided to characterize the IgG subclass distribution to elucidate a possible role of these antibodies in the patho-mechanism leading to acquired TTP.

4.4.1 Subclass distribution of IgG anti-ADAMTS13 antibodies

The subclass distribution of anti-ADAMTS13 IgG antibodies was analyzed in the 58 patients who tested positive for anti-ADAMTS13 IgG antibodies in our in-house ELISA. From the 58 patients with acquired TTP tested, 44 patients were analyzed during the first episode and 14 during a relapse. IgG4 was detected in 90% of the patients (52/58, 90%), followed by IgG1 (30/58, 52%), IgG2 (29/58, 50%), and IgG3 (19/58, 33%).

When patients were subdivided by clinical outcome, 38/44 (86%) patients analyzed during the first event had IgG4 antibodies. IgG4 was found either alone (8/38, 21%) or with other IgG subclasses (30/38, 79%). Among the 30 patients in whom IgG4 was found with other IgG subclasses, the association with all four IgG subclasses (10/30, 33%) was the most prevalent finding, followed by association with IgG2 (7/30, 23%), IgG1 (5/30, 17%) and IgG3 (1/30, 3%). IgG4 was also found with IgG1 and IgG2 (4/30, 13%) and with IgG1 and IgG3 (3/30, 10%) (Fig. 7). No IgG4 was detected in 6/44 (14%) TTP patients who had predominantly IgG1 with IgG3 (n = 2), IgG2 (n = 1) or IgG2 and IgG3 (n = 3) (Fig. 7).

The IgG-subclass distribution in 14 patients analyzed during an acute relapsing event revealed IgG4 as the predominant IgG subclass (14/14, 100%); IgG4 was found either alone (9/14, 65%) or associated with IgG2 (3/14, 21%), IgG1 (1/14, 7%) or IgG1 and IgG2 (1/14, 7%), (Fig. 7).

The IgG subclass distribution of 4/6 patients without detectable functional inhibitors showed IgG4 accompanied by IgG2 or IgG3 or IgG1 and IgG3, or all of them. The remaining two patients had no IgG4, with IgG1 being the most representative subclass.

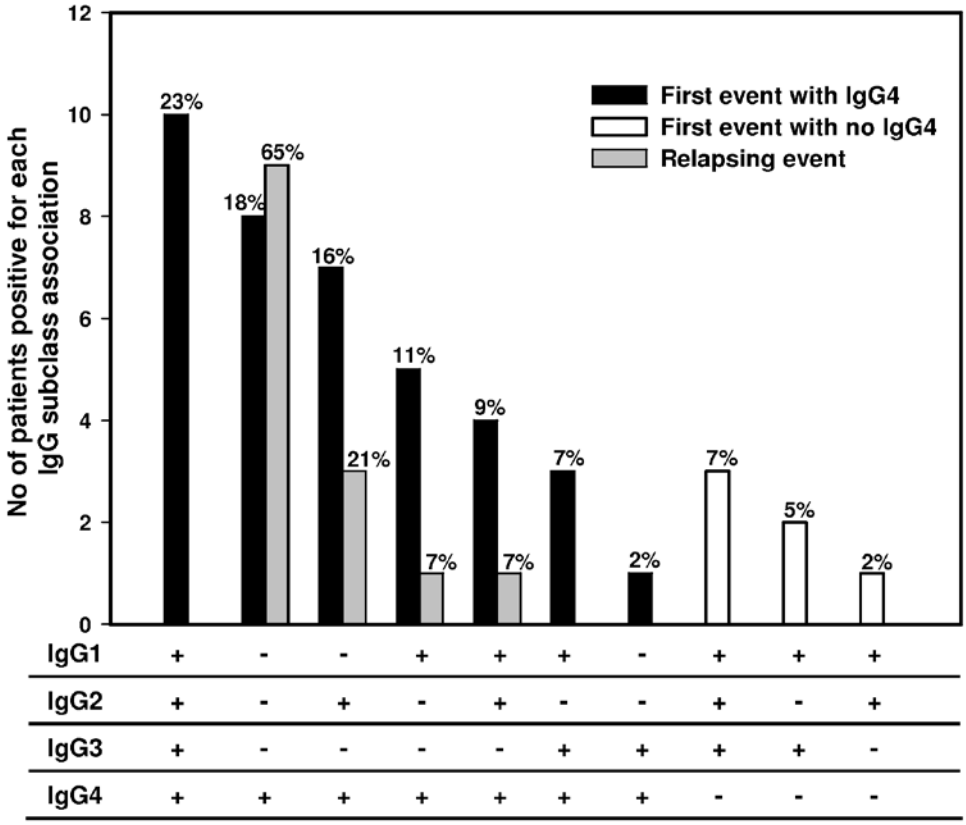

Figure 7. Subclass distribution of anti-ADAMTS13 IgG antibodies in patients with acquired TTP.

Fifty-eight patients with acute acquired TTP were analyzed for different IgG subclasses. The histograms show the frequencies of patients positive for each combination of IgG subclasses. Black and white bars represent patients analyzed during the first TTP episode (n = 44) with or without IgG4, respectively. Grey bars represent patients analyzed during a relapsing event (n = 14). Plus and minus signs denote the presence and absence of the subclass, respectively.

To assess the contribution of any subclass to total IgG (IgG_{sum}), we calculated the relative concentration of each IgG subclass in a patient. Six of 58 patients were excluded from this calculation because their IgG4 OD values were outside the linear range. IgG4 was the most abundantly produced subclass, with levels ranging from 1% to 100% (Fig. 8A). IgG1 was the second most common subclass, with levels ranging from 11% to 92%, whereas, IgG2 and IgG3 formed only a small percentage of IgG_{sum}, with levels ranging from 4% to 26% and 1% to 30% for IgG2 and IgG3, respectively (Fig. 8A). In contrast to IgG4, IgG1 was never found as a single subclass; however, in the absence of IgG4, IgG1 constituted more than 85% of IgG_{sum}. The presence and abundance of IgG4 and IgG1 were inversely correlated (r^2 = - 0.927, P < 0.01)

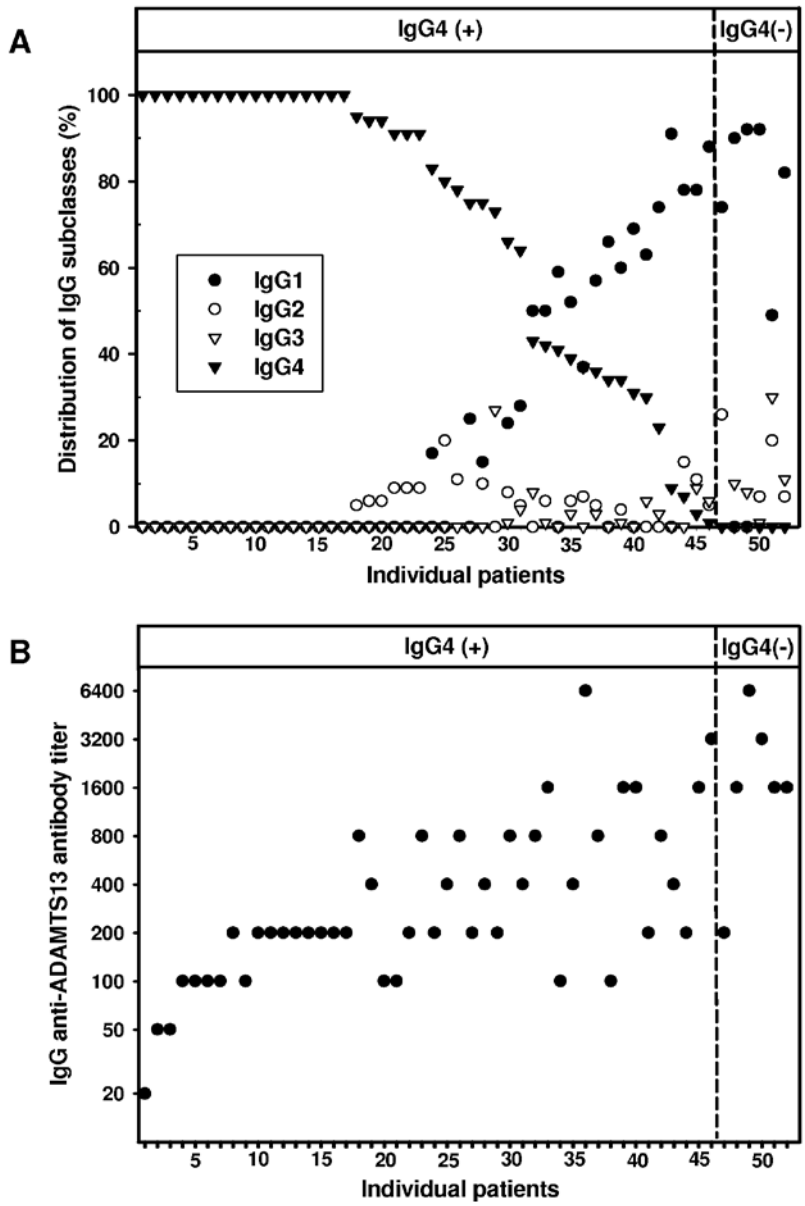

Figure 8. Relative concentrations of anti-ADAMTS13 IgG subclasses and total IgG anti-ADAMTS13 antibody titers within a patient.

(A) The relative concentration of the different IgG subclasses was calculated for plasma of 52 TTP patients. The individual IgG subclass distribution of each patient is shown. Patients are numbered 1-52 according to decreasing levels of IgG4. IgG4 and IgG1 are the dominating IgG subclasses. (B) Total IgG anti-ADAMTS13 antibody titers corresponding to each patient [numbered from 1 to 52 as in (A)]. In both (A) and (B), individual patient numbers are shown in steps of five.

Because of the dominant presence of IgG4 antibodies in our patient population, IgE anti-ADAMTS13 antibodies were also assayed, as the production of IgG4 and IgE is induced by the same cytokines [interleukin (IL)-4 and IL-13], released by T-helper 2 cells (Stavnezer, 1996). None of the 17 patients with IgG4 as the only positive subclass had IgE antibodies

against ADAMTS13, excluding a pathogenic role of IgE autoantibodies in TTP as described in, for example, bullous pemphigoid (Iwata *et al*, 2008) or SLE (Atta *et al*, 2010).

4.4.2 Relation between the IgG subclass profile and the total anti-ADAMTS13 IgG antibody titers and ADAMTS13:Ag levels

When IgG_{tot} was related to the IgG subclass profile, patients with IgG4 as the only subclass showed lower IgG_{tot} titers (range 20-200) than patients with IgG4 combined with other IgG subclasses (Fig. 8B). The combination of IgG4 with one, two or more IgG subclasses was associated with the highest IgG_{tot} titers (range 100-6400). This was even more pronounced in patients with undetectable IgG4 (range 1600-6400). Patients without detectable functional inhibitors generally had low IgG_{tot} antibody titers (100-400), except for two patients who had undetectable IgG4 (1600 for both) (Fig. 8B).

The relationship between ADAMTS13:Ag levels and the IgG subclass profile was variable, with a trend towards lower ADAMTS13:Ag levels with increasing numbers of IgG subclasses. Patients with the lowest IgG4 levels (< 10%) or highest IgG1 levels (> 85%) had significantly lower ADAMTS13:Ag levels than patients with the highest IgG4 levels or IgG4 as the only subclass present ($P < 0.01$ and $P < 0.05$, respectively; the six patients with IgG4 OD values outside the linear range were excluded from this analysis).

4.4.3 Subclass distribution of anti-ADAMTS13 IgG antibodies and clinical outcome

We observed that 4 patients who had very low (< 5%) or undetectable IgG4 with high levels of IgG1 died during the first acute TTP event (Table 2). These patients also showed high anti-ADAMTS13 IgA antibodies titers, three of them in combination with anti-ADAMTS13 IgM antibodies.

This observation prompted us to search for an association between levels of IgG4 and IgG1 and recurrence of TTP. We analyzed 40 patients who

were divided into three groups: those who had experienced only a single TTP episode [group 1; IgG4 and IgG1 median values were 44% (interquartile range (IQR) 30-85) and 50% (IQR 15-63), respectively], those who relapsed during 48 months of follow-up [group 2; IgG4 and IgG1 median values were 91% (IQR 72-95.5) and 0% (IQR 0-15), respectively] and those who had had relapse(s) before [group 3; IgG4 and IgG1 median values were 100% (IQR 91.2-100) and 0% (IQR 0-0), respectively] (Fig. 9) (for inclusion criteria, see Methodology, chapter 3.9). High levels of IgG4 were associated with relapse (P = 0.03 and P = 0.001 for comparison between groups 1 and 2 and groups 1 and 3, respectively).

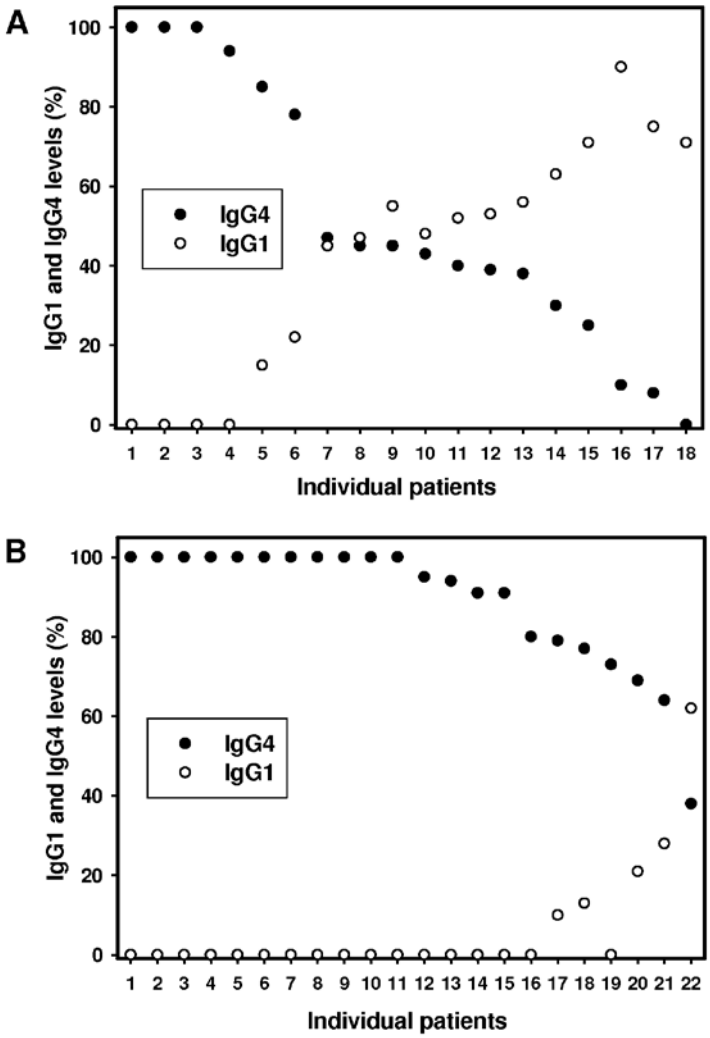

Figure 9. Levels of IgG1 and IgG4 anti-ADAMTS13 antibodies in patients with acute acquired TTP.

(A) Patients who had experienced only a single TTP event. (B) Patients who relapsed during the 48 months of follow-up or had had relapse(s) including patients without further relapses during follow-up and patients lost to follow-up after the acute relapsing event.

Table 2. ADAMTS13 laboratory findings and clinical outcome in patients with very low (< 5%) or undetectable levels of IgG4 and high levels of IgG1

Patient N°*	ADAMTS13:Ac (U/mL)	ADAMTS13 inhibitor	ADAMTS13:Ag (ng/mL)	IgG anti-ADAMTS13 (titer)	IgM anti-ADAMTS13 (titer)	IgA anti-ADAMTS13 (titer)	IgG1 (%)	IgG4 (%)	Outcome
49	< 0.05	High	< 65	6400	3200	12800	92	0	Death
50	< 0.05	Low	< 65	3200	0	0	92	0	Remission
48	< 0.05	Neg.	< 65	1600	0	51200	91	0	Death
52	< 0.05	Neg.	< 65	1600	50	800	85	0	Death
46	< 0.05	Low	< 65	3200	0	3200	88	1	Remission
45	< 0.05	Low	83	1600	20	3200	78	3	Death
47	< 0.05	Low	560	200	0	0	71	0	Remission

ADAMTS13:Ac, ADAMTS13 activity; ADAMTS13:Ag, ADAMTS13 antigen; Neg., Negative. *Patient' numbers correspond to the individual numbers in Fig. 4. All patients had idiopathic thrombotic thrombocytopenic purpura

Likewise, patients with undetectable or very low IgG1 levels experienced more TTP events than patients with detectable (moderate or low) levels (P = 0.005 and $P < 0.001$ for comparison between groups 1 and 2 and groups 1 and 3, respectively).

Taken together, our findings suggest that patients with high levels of IgG4 and undetectable IgG1 are more prone to relapse than patients with low levels of IgG4 and detectable IgG1.

4.5 Detection of circulating ADAMTS13-anti-ADAMTS13 antibody immune complexes in patients with acquired TTP

During the establishment of our in-house ELISA to quantify ADAMTS13 antigen levels (Rieger *et al*, 2006), we observed that removal from plasma of anti-ADAMTS13 IgG antibodies by Protein G also completely removed any measurable residual ADAMTS13 antigen, indicating that all ADAMTS13 was bound to IgG antibodies. These findings were the first evidence for the presence of soluble ADAMTS13-specific immune complexes (ICs) in plasma from patients with acquired TTP.

These observations prompted us to establish two types of assays to experimentally detect circulating ADAMTS13-specific ICs: one based on co-immunoprecipitation of ADAMTS13 with IgG antibodies using Protein G and the second one based on ELISA methodology.

4.5.1 Detection of circulating ADAMTS13-anti-ADAMTS13 antibody immune complexes by co-immunoprecipitation

For co-immunoprecipitation (Co-IP) experiments, plasma samples from all those patients with acute acquired TTP (n=16) were included where sufficient amounts of plasma were still available. As negative controls, plasmas from healthy individuals, different batches of pooled NHP and human serum albumin (HSA, 50 µg/mL) spiked with recombinant ADAMTS13 (rADAMTS13, 2 µg/mL) were also analyzed.

All samples were subjected to immunoprecipitation with Protein G and co-isolated ADAMTS13 was detected by Western blot analysis using an affinity-purified polyclonal rabbit anti-human ADAMTS13 antibody. The

sensitivity of this antibody to detect rADAMTS13 in a blot is approximately 0.5 ng.

ADAMTS13 was not detectable in the protein G-bound total IgG derived from healthy individual plasmas, pooled NHP or HSA spiked with rADAMTS13 (Fig. 10A). ADAMTS13 was however detectable in most of the 16 TTP-derived samples (Fig. 10B) suggesting the specific detection of circulating ADAMTS13-IgG ICs.

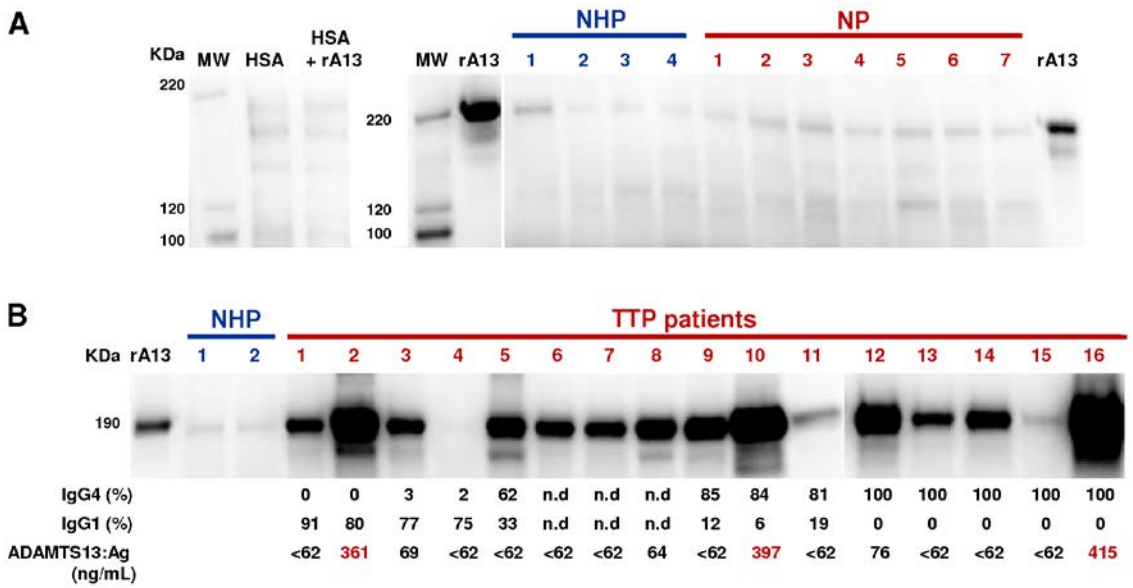

Figure 10. Detection of circulating ADAMTS13-anti-ADAMTS13 antibody immune complexes (ICs) in TTP patients by co-immunoprecipitation.

Circulating ADAMTS13-specific ICs were analyzed by co-immunoprecipitation of ADAMTS13 with Protein G-bound IgG followed by Western blot analysis to detect ADAMTS13. The position of ADAMTS13 in the gels was determined by loading 1 ng of recombinant ADAMTS13 (rA13). (A) Healthy individuals (NP), different batches of pooled normal human plasma (NHP), human serum albumin (HSA; 50 µg/mL) and HSA spiked with 2 µg/mL of recombinant ADAMTS13 (HSA + rA13). (B) TTP patients. Two batches of NHP served as negative control. The relative percentage of IgG1 and IgG4 antibodies and ADAMTS13:Ag values of the samples are also shown. Each lane contains equal amounts of total IgG.

Except for one patient (Fig. 10B; TTP 4), circulating ADAMTS13-IgG ICs were detected in all plasma samples (n=9) where free ADAMTS13:Ag levels were not detectable (Fig. 10B). An interesting observation is that 3 patients who had measurable residual ADAMTS13:Ag levels (levels between 361 – 415 ng/mL) showed also higher IC levels compared to those patients with no detectable protein (Fig. 10B; TTP 2, TTP 10 and TTP 16), suggesting that the ELISA employed to quantify ADAMTS13:Ag levels is

also detecting circulating ADAMTS13-specific ICs at least to some extent, in line with a previous report (Rieger *et al*, 2006).

No differences could be observed when the presence and relative amounts of circulating ADAMTS13-IgG ICs were compared with those of the subclasses of the free anti-ADAMTS13 IgG antibodies. Patients having mainly IgG1 antibodies (Fig. 10B; TTP 1 to 5) had similar amount of ICs as patients having mainly IgG4 antibodies (Fig. 10B; TTP 9 to 16). These observations may suggest that the formation and clearance of ADAMTS13-IgG ICs is independent of the IgG subclass involved in IC formation.
Patients with low levels of free IgG anti-ADAMTS13 antibodies (detectable only with a commercial ELISA kit) also contained ADAMTS13-IgG ICs (Fig. 10B; TTP 6 to 8).

4.5.2 Detection of circulating ADAMTS13-anti-ADAMTS13 antibody immune complexes by ELISA

The positive findings obtained with the Co-IP experiments and considering our previous observation that an ADAMTS13:Ag ELISA was able to indirectly detect ADAMTS13-specific ICs, we aimed to establish an ELISA-based assay that should detect and characterize the immunoglobulin fraction of the circulating ADAMTS13-specific ICs.
For this, the basic set-up of the in-house ELISA to quantify ADAMTS13:Ag levels was modified at the detection step to visualize the antibody fraction of the immune complex. Briefly, a polyclonal rabbit anti-ADAMTS13 antibody is used to capture any ADAMTS13-anti-ADAMTS13 antibody ICs present in the plasma and the immunoglobulin fraction of the IC is detected by means of enzymatically-labeled anti-human IgG1-4 or IgA antibodies. The major limitation of this ELISA is that only ICs bearing an ADAMTS13 with free available epitopes will be detected.

The presence of circulating ADAMTS13-specific ICs was analyzed in 60 healthy donor plasmas to establish assay cut-off values. Circulating ADAMTS13-specific ICs were also tested in a total of 57 out of 76 TTP patients for which plasma samples were available. The levels of

ADAMTS13-specific ICs were expressed as ratio between the OD values obtained with the tested plasma and OD values of a pooled NHP.

Circulating IgG- and IgA-ADAMTS13 ICs were detected in 47/57 (82%) and 13/53 (25%) of the TTP patients, respectively. When the IgG subclass distribution of ADAMTS13-IgG-specific ICs were analyzed; 41/47 (87%) of the TTP patients showed an IgG4-IC, 19/47 (40%) an IgG2-IC, 15/47 (32%) an IgG1-IC and only 7/47 (15%) of the patients an IgG3-IC (Fig. 11). Comparing the levels of ADAMTS13-specific ICs between TTP patients and healthy donors revealed a statistically significant difference for IgG1, IgG2, IgG4 and IgA ICs ($P < 0.001$ for IgG1, IgG2, IgG4 and IgA, respectively), but not for IgG3 ($P = 0.456$).

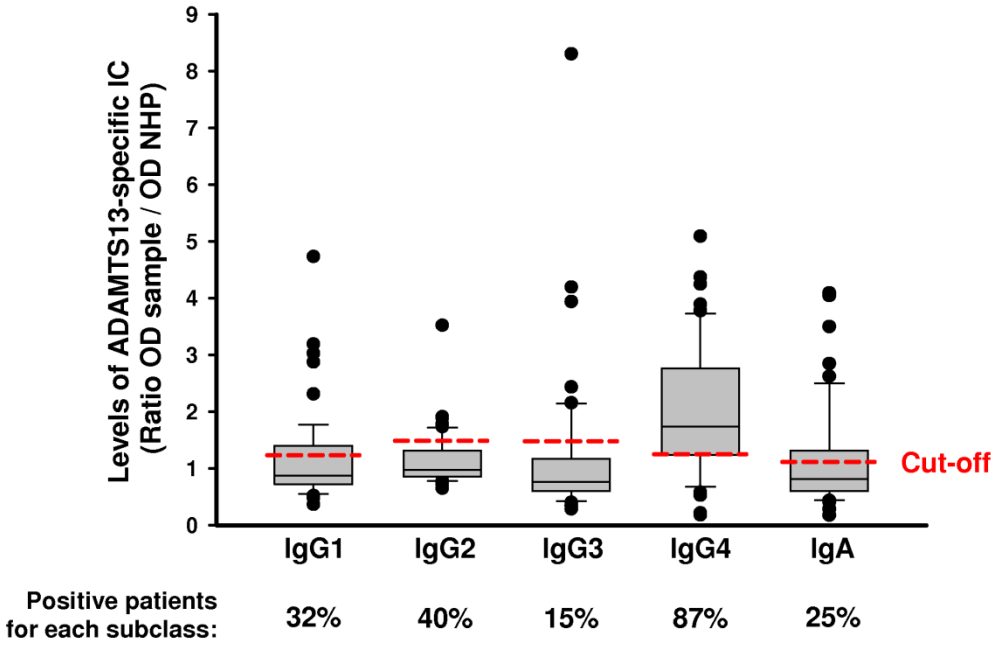

Figure 11. Detection of circulating ADAMTS13-anti-ADAMTS13 antibody ICs in patients with acute acquired TTP by ELISA.

Box plot of the distribution of circulating IgG1 to IgG4- and IgA-ADAMTS13 specific ICs in plasma from 57 TTP patients. The IC levels are presented as ratio between the OD of the sample and the OD of a pooled NHP (dilution 1 to 20). The dashed lines represent the normal cut-off value for each ELISA. The bottom, median and top lines of the box mark the 25th, 50th, and 75th percentiles, respectively. The vertical line shows the range of values comprised between the 5th and 95th percentiles and the dots represent outlier values. The percentage of positive TTP patients for each subclass of ADAMTS13-specific IC is shown at the bottom.

In 68% of the patients tested positive for IgG-ICs a correlation between the subclasses of the free IgG anti-ADAMTS13 antibodies found in the plasma

and the subclasses of the IgG-containing ICs was observed. Importantly, all patients that were tested negative for free IgG4 antibodies also tested negative for IgG4-containing ICs. In 8/12 (67%) of the TTP patients tested positive for free IgA anti-ADAMTS13 antibodies also tested positive for IgA-containing ICs.

Interestingly, in 2 out of 6 and 5 out of 41 patients tested negative for IgG and IgA anti-ADAMTS13 antibodies, respectively, circulating ADAMTS13-specific ICs (involving IgG2, IgG4 or IgA) were detected by ELISA. This indicated that a negative finding of free anti-ADAMTS13 antibodies in plasma may be due to the sequestration of these antibodies in ICs.
In only 6 patients circulating ADAMTS13-IgG-specific ICs were not detected although free IgG anti-ADAMTS13 antibodies and practically no ADAMTS13:Ag were present.
Eight out of 9 patients who tested positive for free IgG anti-ADAMTS13 antibodies only by the commercial ELISA were tested positive for IgG4-IC suggesting that our in-house ELISA is not detecting low levels of free IgG4 antibodies. These observations confirm previous findings that the goat anti-human IgG antibody used to visualize IgG in the in-house ELISA detected IgG4 with ~50% lower sensitivity than IgG1-3 antibodies.
Four patients tested negative for anti-ADAMTS13 antibodies also tested negative for all classes and subclasses of ICs (IgG1-4 and IgA).

4.5.3 Detection of complement fixing immune complexes by binding to immobilized C1q

The complement subcomponent C1q binds to the Fc region of complexed IgG or IgM thereby triggering the activation of the classical complement pathway. Based on these properties, we used a commercially available C1q binding ELISA to evaluate if ADAMTS13-containing ICs are able to activate complement. Plasmas derived from 37 TTP patients included in the current study and from 3 healthy individuals were tested. In 6 out of 37 (16%) patients circulating ICs bound to immobilized C1q were detected (Fig. 12). Borderline and negative values were obtained for 6/37 (16%) and 25/37 (68%) of the patients, respectively (Fig. 12). All 3 healthy

individuals tested also negative. Since C1q is efficient in binding ICs only when containing IgG subclasses 1 to 3, the results are in accordance with the observed high prevalence (87%) of circulating ADAMS13-IgG4-specific ICs.

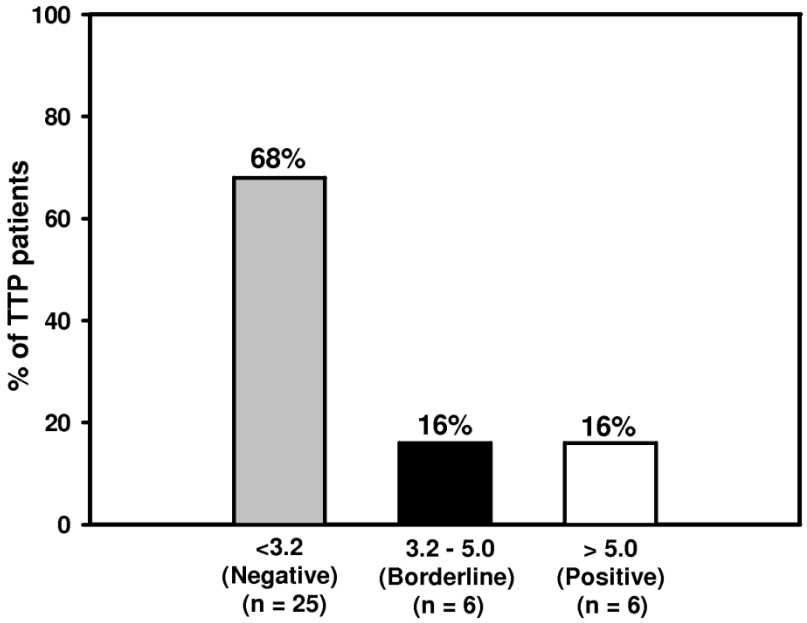

Figure 12. Detection of complement fixing ICs by binding to immobilized C1q
The levels of circulating ICs (µg EQ/mL) binding to immobilized C1q was evaluated in 37 TTP plasma samples by ELISA. The negative cut-off value of the assay was 3.2 µg Eq/mL, samples with 3.2-5.0 µg Eq/mL were judged as borderline and samples with values above 5.0 µg Eq/mL were judged as positive.

4.6 Binding of human IgG4 to rabbit anti-ADAMTS13 antibody

During the establishment of the ELISA to detect circulating ADAMS13-IgG4-specific ICs, undesired high background signals were reached when testing either pooled NHP or individual healthy donor plasmas. These findings might have been caused by the potential presence of naturally occurring circulating ADAMS13-IgG4-specific ICs in the healthy population or by a propensity of human IgG4 present in normal plasma to bind rabbit IgG antibodies.

The first possibility was explored comparing pooled NHP with an ADAMTS13-depleted pooled NHP (depleted-NHP) prepared as previously described (Rieger *et al*, 2006), and the second by using a commercially available purified human IgG4 (hIgG4) preparation in a concentration range comparable to the final amount of IgG4 present at each tested

dilution of pooled NHP. Interestingly, similar results were obtained with both set-ups (Fig. 13), indicating that hIgG4 is able to bind to coated rabbit IgG in a dose-dependent manner, thereby causing the high background signals in the ELISA.

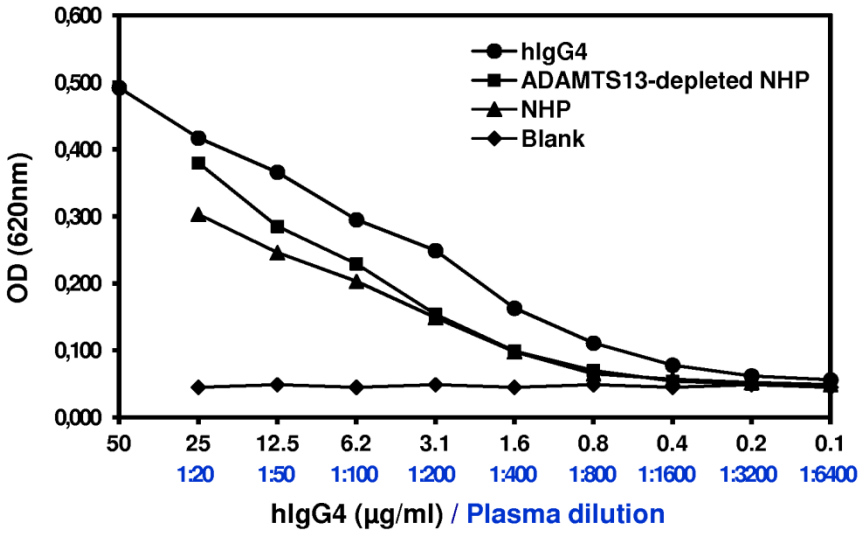

Figure 13. Binding of human IgG4 to coated rabbit IgG antibodies.
Serial dilutions of pooled NHP and ADAMTS13-depleted NHP (indicated in blue color) as well as purified human IgG4 (hIgG4) at concentrations comparable to the final amount of IgG4 present at each tested dilution of pooled NHP (indicated in black color) were allowed to bind to coated rabbit IgG. As negative control (blank), blocking buffer was used as a sample.

Similar results were obtained when rabbit IgG antibodies in solution were allowed to bind to coated hIgG4. Moreover, hIgG4 in solution was capable of competing with rADAMTS13 for binding to coated rabbit anti-ADAMTS13 IgG (data not shown).

This conclusion was further corroborated by pre-incubating pooled NHP, ADAMTS13-depleted-NHP and hIgG4 with a monoclonal anti-human IgG4 (Fc-specific) antibody in a final concentration of 11 µg/mL. The binding of IgG4 to coated rabbit IgG was equally inhibited in all three samples but not in control samples pre-incubated with BSA (differences approximately 5-fold; data not shown). These preliminary results suggested that hIgG4 is able to bind to coated rabbit IgG via its Fc fragment because the Fc-specific anti-human IgG4 antibody was able to abrogate the binding.

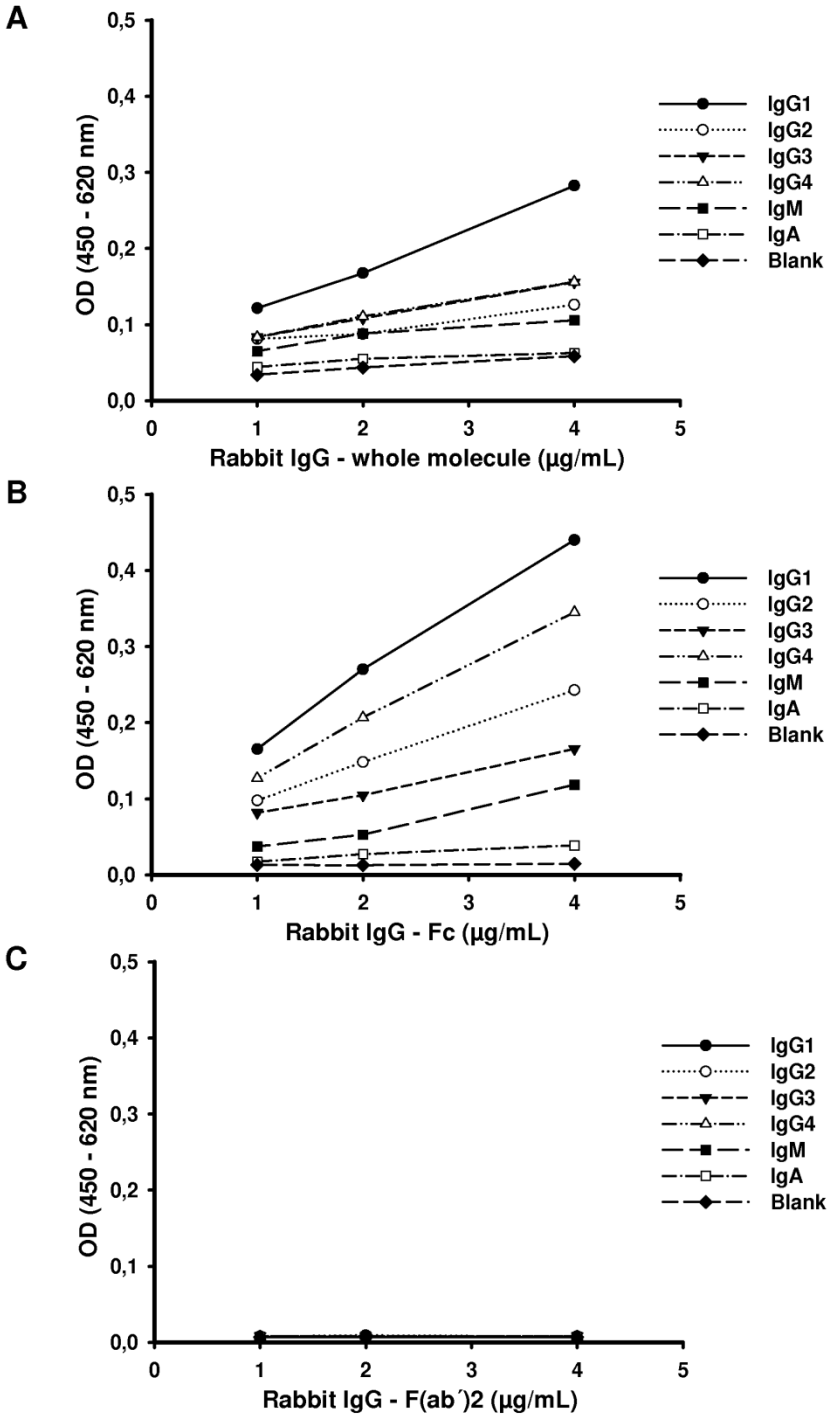

Figure 14. Binding of rabbit IgG antibodies to coated human immunoglobulins.

Horseradish peroxidase (HRP)-labeled whole rabbit IgG, IgG (Fab´)2 or IgG Fc antibodies were allowed to bind to coated purified human IgG1-4, IgM or IgA antibodies. (A) Binding of whole rabbit IgG to Igs. (B) Binding of rabbit IgG Fc to Igs. (C) Binding of rabbit IgG (Fab´)2 to Igs.

We next explored the nature of the hIgG4-rabbit IgG interaction and also the potential binding of other human immunoglobulins (IgM, IgA or IgG1-

3) to rabbit IgG. For this, HRP-labeled whole rabbit IgG, HRP-labelled IgG (Fab´)2 fragment and HRP-labelled IgG Fc fragment were allowed to bind to coated purified human IgG1-4, IgM or IgA antibodies. The results confirmed that the binding of rabbit IgG antibodies to human IgG4 is mediated via its Fc rather than its F(ab´)2 portion (Fig. 14). Interestingly, we also observed an increased dose-dependent binding of rabbit IgG (Fc fragment) to coated human IgG1 and to a lesser extent to IgG2, IgG3 and IgM. No binding was observed to IgA (Fig. 14).

Because of these findings, we assayed for binding of human IgG1-3 to rabbit IgG antibodies. Different concentration of human IgG1-3 (comparable to those of each tested dilution of pooled NHP) and diluted pooled NHP were allowed to bind to coated rabbit IgG antibodies. Neither IgG1, IgG2 or IgG3 had the ability to bind to rabbit IgG under the conditions used (data not shown).

4.7 Detection of anti-ADAMTS13 antibodies and circulating ADAMTS13-immune complexes in a follow-up of a patient with refractory TTP

The dynamic course of anti-ADAMTS13 antibodies and circulating ADAMTS13-IC during treatment was investigated in a patient suffering from refractory TTP (Scheiflinger *et al*, 2003). Throughout the follow-up until the decease of the patient at day 64, plasma samples were collected daily before treatment and ADAMTS13-related parameters were determined. ADAMTS13:Ac and ADAMTS13:Ag were undetectable at most time points. High inhibitor titers were measured during the first two weeks and declined to low levels thereafter. Anti-ADAMTS13 antibodies of the IgG, IgM and IgA class were detected concomitantly (Fig. 15). For IgG and IgA antibodies, high to moderate titers were found practically throughout the entire observation time.

By contrast, IgM antibodies were detected only at the beginning and with low titers. The anti-ADAMTS13 IgG subclass profile showed prevalence of IgG1 (82%) and very low levels of IgG2 (7%) and IgG3 (11%) antibodies,

while IgG4 was undetectable. This antibody profile did not change during the course of the disease.

The presence of ADAMTS13-specific IgG, IgM and IgA ICs was investigated by Co-IP and ELISA. The former method detected ADAMTS13-specific ICs for all the 3 classes of antibodies. By determining the relative amounts of circulating ICs through scanning of the luminograms (Fig. 16A-D), it appeared that the plasma concentration of the IgG-ICs is higher than that of the IgM- and IgA-ICs. Interestingly, ADAMTS13-specific ICs followed an inverse kinetics to that of the measured free antibody titers. ICs were detectable most of the time but became apparently undetectable when the titers of the free antibodies rose (Fig. 16A-D). Notably, low amounts of IgM-ICs were detected at most time points although no free IgM antibodies were detected after day 12 (Fig. 16A-D).

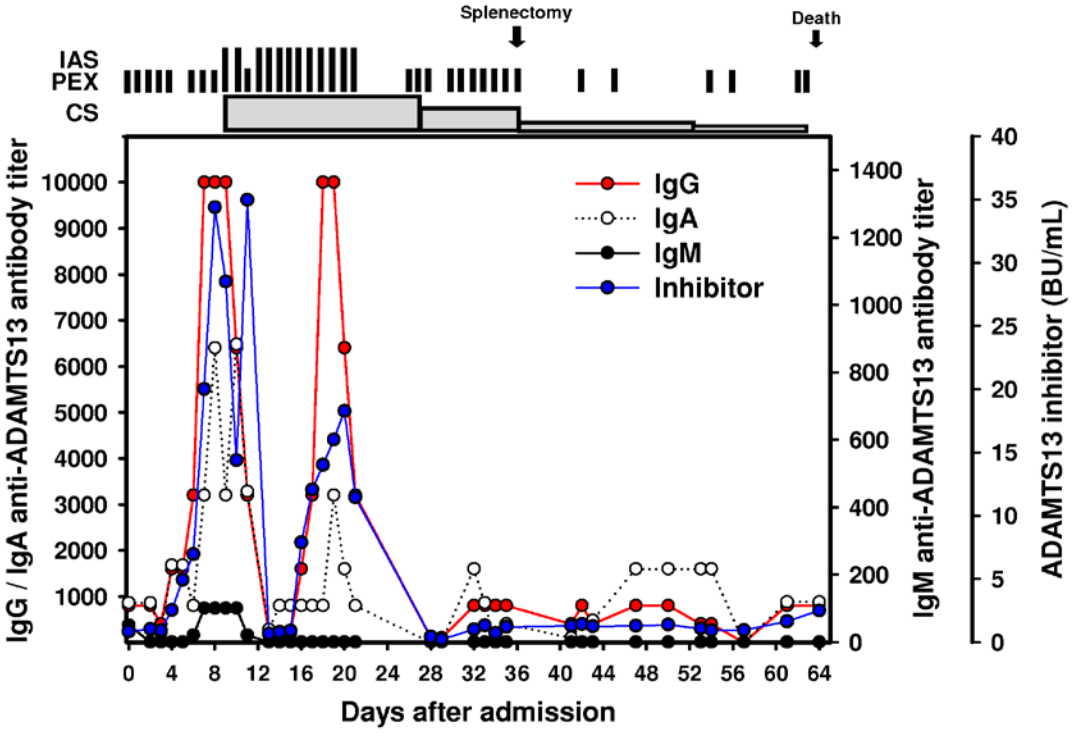

Figure 15. Dynamic course of anti-ADAMTS13 antibodies in a patient with refractory TTP.

Dynamic course of anti-ADAMTS13 antibody titers (IgG, IgM, IgA and functional inhibitor (BU/mL)) during the two months follow-up. The treatment regimen including plasma exchange (PEX), immunoadsorption (IAS), corticosteroids (CS), and splenectomy is shown above the diagram.

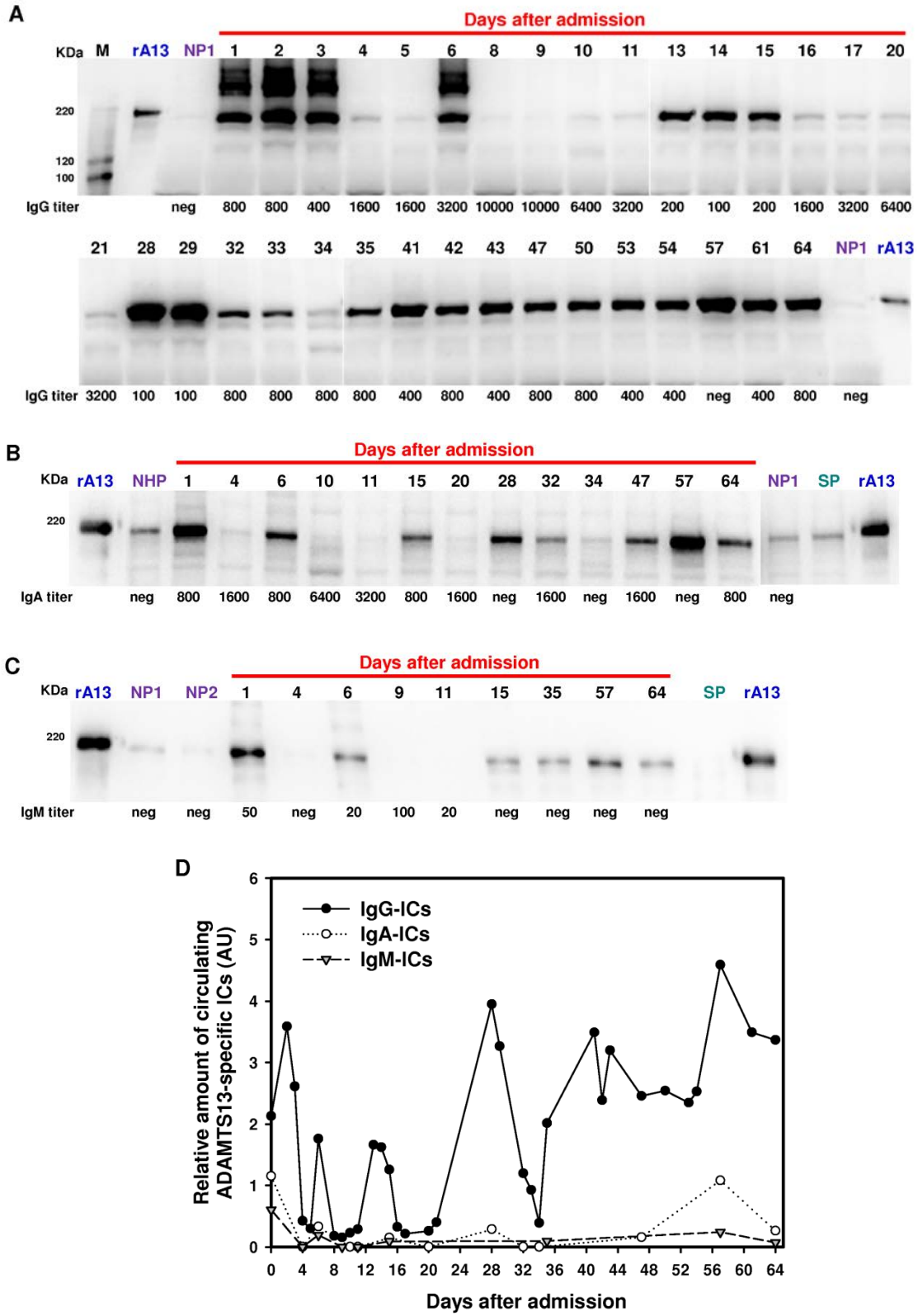

Figure 16. Dynamic course of circulating ADAMTS13-specific ICs in a patient with refractory TTP.

Immunoblots showing the course of circulating (A) ADAMTS13-IgG ICs, (B) ADAMTS13-IgA ICs and (C) ADAMTS13-IgM ICs. Recombinant ADAMTS13 (rA13, 1ng/well) was used as negative control. Pooled NHP (NHP), a single healthy donors (NP) and human serum albumin (HSA; 50 µg/mL) spiked with 2 µg/mL of rA13 (SP) were used as negative controls. M denotes the molecular weight marker. Each

lane contains equal amounts of total IgG. The corresponding anti-ADAMTS13 antibody titers are also shown in the lower panel of each blot. (D) Relative amount of circulating ADAMTS13-specific ICs after densitometric quantification using rA13 (1 ng) as standard. AU denotes arbitrary units.

The presence of ICs was also analyzed by ELISA in the sample collected at day 1. Circulating IgG1-ICs, IgG3-ICs and IgA-ICs were detected. IgM-ICs were not investigated by ELISA. A correlation could again be observed when the classes and subclasses of free ADAMTS13 antibodies were compared with those of the immunoglobulin forming the ICs.

4.8 Detection of anti-CD36 antibodies in TTP patients

Previous studies have shown that approximately 70% of the TTP patients have autoantibodies against the membrane antigen CD36 (Tandon *et al*, 1994;Schultz *et al*, 1998;Wright *et al*, 1999;Rock *et al*, 2005). In these studies, platelet lysates were used as source of human CD36 and the presence of anti-CD36 antibodies was evaluated by either Western blot with patient´s plasma as first antibody or by immunoprecipitation of patient´s plasma antibodies with platelet lysates-derived CD36. Alternatively, a Dot blot using purified platelet-derived CD36 as protein source was performed.

The published high incidence of anti-CD36 antibodies in TTP patients prompted us to develop a more sensitive assay based on ELISA technique to detect these antibodies in the plasma from TTP patients. The source of human CD36 used to coat the ELISA plates was a commercially available full-length recombinant human CD36 (rCD36) expressed in human embryonic kidney cells (HEK 293).

The presence of anti-CD36 antibodies was investigated in 57 of the 76 TTP patients included in the current study and in 36 additional TTP patients whose plasma samples became available at a later point in time.

A total of 63 healthy donor samples were analyzed to establish assay cut-off values. In the absence of any human-derived positive control, a monoclonal rat anti-human CD36 antibody directed against the extracellular part of human CD36 was used as positive control to allow following the assay performance.

Circulating anti-CD36 antibodies were detected in only 7/93 (8%) of the TTP patients and in 2/63 (3%) of the healthy donors (Fig.17). From the 57 patients included in the study described in previous chapters, only 3 patients tested positive. There was no statistically significant difference ($P < 0.197$) between healthy donors and TTP patients when the levels of anti-CD36 antibodies (expressed as ratio between OD of the tested sample and the OD of a pooled NHP) were compared (Fig.17).

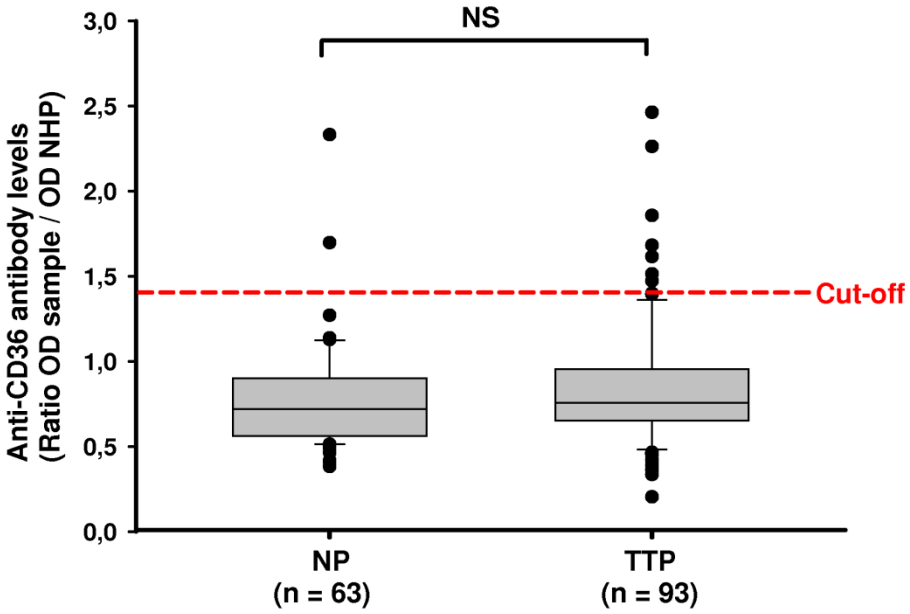

Figure 17. Detection of anti-CD36 antibodies in patients with acquired TTP.
Box plot of the distribution of anti-CD36 antibodies in plasma from 63 healthy donors (NP) and 93 patients with acquired TTP. The anti-CD36 antibodies levels are presented as ratio between the OD of the sample and the OD of a pooled NHP. The dashed line represents the normal ELISA cut-off value. The bottom, median and top lines of the box mark the 25th, 50th, and 75th percentiles, respectively. The vertical line shows the range of values comprised between the 5th and 95th percentiles and the dots represent outlier values. NS= non-significant statistical difference between both groups.

For 3 out of the 7 TTP plasma samples tested positive for anti-CD36 antibodies competitive inhibition experiments between immobilized rCD36 and rCD36 in solution could be performed. Specificity of the detected anti-CD36 antibody could be shown in 2 samples, whereas the third sample displayed inhibition also with different protein sources used as negative control (rADAMTS13 and BSA).

In summary, we confirmed the detection of anti-CD36 antibodies in 2 out 3 TTP patients by our in-house ELISA but not with the commercial kit.

The positivity of 4 TTP patients could not be confirmed with any additional assay due to unavailability of plasma sample.

5- DISCUSSION

The pathogenesis of acquired idiopathic TTP is characterized by severe ADAMTS13 deficiency due to the presence of circulating autoantibodies directed against ADAMTS13 that may or may not inhibit its functional activity (Scheiflinger *et al*, 2003;Rieger *et al*, 2005;Shelat *et al*, 2006). The main goal of the present study was to characterize the immune response against ADAMTS13 in a well-defined cohort of 76 adult TTP patients who underwent a first acute episode (n=57) or who had an acute relapsing event (n=19).

Characterization of the free anti-ADAMTS13 antibodies

Anti-ADAMTS13 antibodies were identified in 71 out of the 76 (93%) TTP patients. IgG, IgM and IgA antibodies were detected in 92%, 7% and 17% of the patients, respectively (Fig. 5). The IgG and IgM findings are in agreement with a previous study of our group in which IgG and IgM antibodies were detected in 97% and 11% of the TTP patients, respectively (Rieger *et al*, 2005). Subsequent studies reported a similar prevalence of IgG (Tsai *et al*, 2006;Shelat *et al*, 2006;Scully *et al*, 2008;Pos *et al*, 2011) and IgM (Pos *et al*, 2011) antibodies in TTP patients.

This is the first study on IgA antibodies against ADAMTS13 in TTP patients. The frequency rate of 17% for IgA antibodies found here (Ferrari *et al*, 2007) is very similar to the 19% of a more recent study conducted by Pos *et al* (Pos *et al*, 2011). Interestingly, one patient from our cohort with a positive functional inhibitor had detectable antibodies only of the IgA class, suggesting that IgA antibodies can also have inhibiting activity against ADAMTS13. High levels of IgA antibodies were recently found to be associated with mortality (Bettoni *et al*, 2011). In line with this observation, we observed that high levels of IgA antibodies were present in 3 out of 4 death cases. Pathogenic IgA autoantibodies are described in patients suffering of SLE and antiphospholipid syndrome and they are considered a risk factor for development of thrombosis (Wilson *et al*, 1998;Shen *et al*, 2008). The clinical significance and pathogenic potential

of IgA antibodies in acquired TTP is however unknown and further studies are needed to assess their contribution to the pathogenesis of TTP.

Inhibitory antibodies were found in 92% of the patients while the rate of non-inhibitory antibodies in this study was only 8%. These data indicate that a majority of the anti-ADAMTS13 antibodies is inhibitory in vitro and, since most of them target domains that are required for ADAMTS13 proteolytic activity (Klaus *et al*, 2004;Zheng *et al*, 2010), these antibodies are probably also inhibitory in vivo. On the other hand, the detection of non-inhibitory antibodies in samples from patients with acquired TTP may be assay dependent and their frequency of occurrence remains controversial. Depending on the study, the prevalence of non-inhibitory antibodies lies between 7% and 29% (Tsai *et al*, 2006;Shelat *et al*, 2006;Scully *et al*, 2008). Since most of the functional assays used for functional inhibitor detection suffer from a lack of sensitivity, antibodies with a low affinity and/or concentration may be missed, thereby overestimating the rate of acquired TTP patients with non-inhibitory antibodies.

Another novel finding of this study was the characterization of the subclasses of anti-ADAMTS13 IgG antibodies. The IgG subclass distribution of anti-ADAMTS13 IgG antibodies was investigated in 58 out of 76 TTP patients included in this cohort. In a remarkable number of patients (17/58), IgG4 was the only subtype that could be detected, implying that IgG4 plays a dominant role in the pathogenesis of autoimmune TTP. The second most frequent subtype was IgG1, while IgG2 and IgG3 were less represented (Fig. 7). A similar prevalence of IgG subclasses in a cohort of 48 acquired TTP patients was recently reported (Pos *et al*, 2011). Importantly, IgG4 antibodies were found in all relapsed patients suggesting a chronic stimulation of the immune system since IgG4 is mainly produced under conditions of chronic antigen exposure driven by a T-helper 2 immune response (Aalberse *et al*, 2009). There was also an inverse correlation between the frequency and abundance of IgG4 and IgG1 antibodies ($P < 0.01$) and we consider this inverse relation to be

an important factor in the pathophysiology of acquired TTP due to the distinct biological functions exerted by these two subclasses. IgG1 is known to bind to both complement C1q and FcγRs thus leading to activation of the classical complement pathway and immune and endothelial cells, whereas IgG4 has poor ability to induce both complement and cell activation (Jefferis *et al*, 1994).

The ELISAs used in this thesis to detect anti-ADAMTS13 antibodies were developed in-house and utilized immobilized recombinant ADAMTS13 to bind the various antibodies. Compared to the method published previously by our group for the detection of anti-ADAMTS13 IgG and IgM antibodies (Rieger *et al*, 2005), we have introduced a slight modification to the basic set-up so as to allow the additional detection of the different IgG subclasses, IgA and IgE anti-ADAMTS13 antibodies. The method was able to detect both inhibitory and non-inhibitory autoantibodies. Thus, it appears suitable for treatment monitoring because it is easy to perform, the results can be available within a few hours, and seems to have a much greater sensitivity than the classical inhibitor assay due to the detection also of non-inhibitory antibodies. In the future, the sensitivity of the ELISA could be further improved by replacing the alkaline-phosphatase-conjugated secondary antibodies by a horseradish peroxidase-conjugated one.

Taken together, our findings indicate that the autoimmune response against ADAMTS13 is polyclonal and heterogeneous with different classes and subclasses of autoantibodies playing key roles in the patho-mechanism of acquired TTP. Clearly, further studies are needed to investigate the specific pathogenic role of each antibody type.

Anti-CD36 antibody incidence is not correlated with acquired TTP

Based on reports on a high incidence of anti-CD36 antibodies in acquired TTP patients (Tandon *et al*, 1994;Schultz *et al*, 1998;Wright *et al*, 1999), we developed an ELISA assay to analyze our own cohort for presence of these autoantibodies. Anti-CD36 antibodies were found in only 8% of the TTP patients analyzed and in 3% of the healthy donor control group. This

result was in contrast to the frequency of about 70% observed previously by other authors (Tandon *et al*, 1994;Schultz *et al*, 1998;Wright *et al*, 1999).

This gross discrepancy in the frequency of detected anti-CD36 antibodies is likely due to differences in the methods employed for antibody detection. We performed an ELISA using recombinant CD36 as protein source and the biotin-streptavidin system for detection, which is well-known to confer a high sensitivity to the ELISA detection. In the past, Western blot analysis was employed, with platelet extracts as source for CD36 and patient´s plasma as a first antibody. This may well lead to a high incidence of false positives due to binding of antibodies to unrelated proteins. Furthermore, we have tested a total of 93 TTP plasma samples whereas in previous studies a range between 10 to 35 patients had been used (Tandon *et al*, 1994;Schultz *et al*, 1998;Wright *et al*, 1999;Rock *et al*, 2005). Such differences in population number could also introduce a bias in the observed high frequency of positive samples. We are therefore confident that our data show that acquired TTP patients do not exhibit an increased incidence of anti-CD36 antibodies.

Presence of ADAMTS13-specific circulating ICs in acquired TTP patients

Elevated levels of soluble circulating autoantibody-antigen immune complexes are a hallmark of certain autoimmune diseases such as SLE, rheumathoid arthritis, antiphospholipid syndrome, vasculitis, and glomerulonephritis (Mayadas *et al*, 2009). Also acquired TTP has been thought to be an IC-mediated disease ever since the isolation of ADAMTS13-inhibiting IgG antibodies from plasma of TTP patients (Furlan *et al*, 1998). Early reports attributed the benefit of PEX therapy in TTP patients to the removal of potential circulating ICs (Bayer *et al*, 1977;Bukowski *et al*, 1977) but the presence of circulating ICs in TTP patients remained controversial (Celada & Perrin, 1978;Neame & Hirsh, 1978).

A previous study by our group showed that removal of anti-ADAMTS13 IgG antibodies from plasma of TTP patients also removed any measurable residual ADAMTS13 antigen suggesting for the first time that the ELISA employed to quantify ADAMTS13 antigen levels is also detecting, at least to some extent, ADAMTS13-specific ICs (Rieger *et al*, 2006). Similar observations were recently reported by Yang *et al* (Yang *et al*, 2011) using a commercially available kit to quantify ADAMTS13 antigen levels. Based on these observations, we decided to investigate the presence of circulating ADAMTS13-specific ICs in more detail using two different approaches: one based on co-immunoprecipitation of ADAMTS13 with IgG antibodies using Protein G and the second one based on ELISA methodology.

Co-immunoprecipitation experiments showed the presence of circulating ADAMTS13-IgG ICs in plasma of patients with TTP but not in healthy donors (Fig. 10). IgG-ICs were detected in patients with or without detectable free ADAMTS13 antigen, indicating that the absence of measurable ADAMTS13 antigen might be due to sequestration in ICs. These findings also suggest that our in-house ADAMTS13 antigen ELISA is detecting ICs only in those patients whose complexed ADAMTS13 still conserves available epitopes for binding to both the capture and detection anti-ADAMTS13 antibodies. The availability of free epitopes might indicate the presence of small-sized ICs. Moreover, IgG-ICs were detected in patients having either IgG4 or IgG1 anti-ADAMTS13 antibodies as predominant IgG subclasses, suggesting an apparently equal avidity of some IgG subclasses of antibodies for ADAMTS13. These experiments confirmed our previous findings and suggested that formation of circulating ADAMTS13-IgG-specific ICs is a common feature of acquired TTP.

We found IgG4 as the predominant subclass of free autoantibodies in TTP patients. Since this IgG subclass is the least effective in terms of final effector functions under normal conditions, we reasoned that the lack of detection of other free IgG subclasses might be due to their sequestration

in ICs. Such circulating ICs (containing IgG1-3 or IgA antibodies) would also better explain the pathogenesis of TTP than the exclusive presence of rather harmless IgG4 antibodies. For their detection, we developed ELISA-based assays where a polyclonal anti-ADAMTS13 antibody was used to capture the ICs through ADAMTS13 and the immunoglobulin constituents (IgG1-4 and IgA) of the ICs were detected by enzyme-labeled antibodies specific for the Ig classes and subclasses. The ELISA set-up used was clearly suitable and sufficiently sensitive for the detection of ICs. A limitation of this ELISA setup is however its underestimation of the real concentration of circulating ICs in case of large-sized ICs harboring more than one ADAMTS13 molecule and ICs with no free ADAMTS13 epitopes available.

Characterization of circulating ICs by ELISA revealed the presence of IgG- and IgA-containing ICs in 81% and 23% of the 57 patients with TTP investigated, respectively. Moreover, in the patients tested positive for IgG-ICs, a substantial overlap between free and IC-sequestered IgG subclasses was observed. IgG4-ICs were most frequent (72%), followed by IgG2-ICs (37%) and IgG1-ICs (26%) (Fig. 11). These findings suggested a correlation between free and complexed-IgG subclasses of anti-ADAMTS13 antibodies and did not support our hypothesis of a formation of ICs with a preference for certain IgG subclasses.

Due to the high prevalence of free and complexed IgG4 autoantibodies, we investigated the ability of the ADAMTS13-specific ICs to fix complement. This was evaluated using an ELISA that takes advantage of the specific binding of the complement subcomponent C1q to the Fc region of complexed IgG1-3. Circulating ICs binding to C1q were detected only in 6 out of 37 (16%) patients with TTP suggesting that the majority of the types of ICs detected in patients with TTP are not fixing complement. It remains possible though, that complement-fixing ICs did form but could not be detected because of their rapid deposition in tissues.

Inverse correlation of free and IC-sequestered anti-ADAMTS13 antibodies in a patient with acquired TTP

We also analyzed the anti-ADAMTS13 antibody response and the concomitant appearance of circulating ICs during treatment of a patient with acquired TTP who died at day 64 after admission due to treatment refractoriness. This patient tested positive for the 3 classes of anti-ADAMTS13 antibodies with IgG1 as the main IgG subtype. By Co-IP, we detected circulating IgG-, IgA- and IgM-ICs despite daily PEX therapy. Interestingly, the ICs followed an inverse kinetics to the measured free antibody titers, becoming apparently undetectable when the titers of the free antibodies and inhibitor activity rose. These data strongly suggest that samples from acquired TTP patients need to be analyzed not only for free but also for complexed anti-ADAMTS13 antibodies to obtain a full picture of the status of the disease.

The IC ELISA revealed that for this patient the classes and subclasses of the immunoglobulin fraction forming the ICs correlated with those of the detected free antibodies. The patient had an IgG1-predominant immune response with detectable free and complexed-IgG1 antibodies and no detectable IgG4 (free or complexed) antibodies. These findings may be responsible, at least in part, for the treatment refractoriness and fatal outcome for this patient.

Interestingly, free ADAMTS13 antigen was not detected in most samples by our in-house ELISA whereas circulating ICs could be demonstrated by Co-IP and ELISA, indicating that ADAMTS13, either newly synthesized or exogenously administrated by plasma infusion during PEX, was quantitatively sequestered by IC-formation. This mechanism may play an important role in the final response to treatment and should be particularly considered in patients refractory to treatment. The combined data of this study strongly suggest that samples from acquired TTP patients need to be analyzed not only for free but also for complexed anti-ADAMTS13 antibodies to obtain a full picture of the status of the disease.

Interaction of human IgG4 with rabbit IgG

A sequential set of experiments, aimed initially to assess the origin of undesired background signals in the ADAMS13-IgG4-specific IC ELISA, revealed the unexpected fact that human IgG4 (but not IgG1-3) antibodies specifically bind to the Fc region of rabbit IgG antibodies. Publications describing the binding of human IgG4 to IgG antibodies of different animal species (including rabbit) came out after our experiments were performed (Ito *et al*, 2010;Rispens *et al*, 2011). Ito *et al* (Ito *et al*, 2010) showed that human IgG4 antibodies strongly bind to mouse, rabbit, guinea pig, bovine and goat IgG antibodies, but do not bind to sheep, horse and rat IgG. Interactions between human IgG4 and animal IgGs should therefore be taken into account when a new assay is designed because they are a potential source of high background and interferences.

Potential roles of anti-ADAMTS13 antibodies of the IgG4 and IgG1 subtypes and ADAMTS13-specific ICs in the pathogenesis of acquired TTP

The relatively high incidence of IgG4 anti-ADAMTS13 antibodies and IgG4-containing ADAMTS13-specific ICs is a novel and rather unexpected finding, as association of IgG4 antibodies with direct pathologic effects are described in only a few diseases including pemphigus (Mihai *et al*, 2007), sclerosing autoimmune pancreatitis and IgG4-related diseases (Okazaki *et al*, 2011). IgG4 antibodies are thought to be functionally monovalent and non-cross-linking antibodies (Aalberse *et al*, 2009) through a process described as “Fab-arm exchange of IgG4 half-molecules”, in which a heavy-light chain pair of one IgG4 molecule joins with another, unrelated, IgG4 half molecule, leading to a newly combined IgG4 molecule with two different antigen-binding sites (van der Neut *et al*, 2007). Such bi-specific molecules are unable to crosslink antigen and, consequently, only form small and relatively harmless ICs with a low potential for induction of immune activation. Furthermore, IgG4 fails to activate potentially dangerous effector mechanisms; it has low affinity for complement C1q, which is required for IC-binding to endothelial cells and the initiation of the classical complement cascade (Stokol *et al*, 2004), and binds to

cellular FcγRs with less efficiency than the other IgG subclasses (Jefferis *et al*, 1994).

Moreover, the Fc region of human IgG4 antibodies can interact with its respective counterpart in other human IgG4 molecules (Rispens *et al*, 2009). This phenomenon may contribute in vivo to an enhancement of the antigen binding capacity (avidity) of IgG4, thereby optimizing IgG4-derived responses and maybe, in the case of autoantibodies, to a pathogenic effect.

A reduced number of patients presented with a predominant IgG1 response. In these patients the formation of high levels of IgG1-ICs may mediate more severe effector functions. IgG1 is known to have the potential to bind complement C1q and to activate cellular FcγRs (Jefferis *et al*, 1994). Circulating IgG1-ICs can lead to endothelial cell activation, promoting inflammation and a pro-adhesive state, thus probably carrying a higher pathogenic potential when compared to IgG4-ICs. Furthermore, when present, IgA antibodies and IgA-ICs may also contribute to severity of the pathogenesis, because they have been shown to activate complement via the mannan-binding lectin pathway (Roos *et al*, 2001) and immune cells when binding to FcαRs (Monteiro, 2010), favoring an enhancement of complement-mediated inflammation and tissue damage.

Although anti-ADAMTS13 IgG4 antibodies have yet to be shown to be functionally monovalent and to interfere with complement activation *in vivo*, it is tempting to speculate that IgG4 autoantibodies, to some extent, act as "protective" antibodies in TTP patients, inducing a milder and treatable form of TTP, whereas IgG1 (and IgA) anti-ADAMTS13 antibodies have a higher pathogenic potential due to IC-related effector functions.

Even though our study is limited by the number of patients, and further studies are needed, we observed that patients are less likely to survive their first TTP event (four of seven patients died) if they have IgG1 and very low or undetectable IgG4 levels plus higher titers of other classes of anti-ADAMTS13 antibodies (particularly IgA antibodies), maybe due to formation of harmful ICs by these subclasses of antibodies. Similar

observations were reported recently (Bettoni *et al*, 2011), where the presence of IgG1, IgG3 and IgA anti-ADAMTS13 antibodies in acute phase were associated with clinical severity and in the particular case of IgA antibodies, also with mortality.

The presence of increased levels of circulating ADAMTS13-specific ICs is likely to contribute to the pathogenic mechanisms leading to the onset of acquired TTP. Elevated levels of ICs are the main reason for systemic inflammatory manifestations associated with autoimmune diseases. Under normal conditions, ICs are quickly cleared by the reticuloendothelial system, but their continuous formation exceeds the clearance capacity of the system and the ICs build up in the circulation leading to endothelial cell activation and inflammation. The continuous presence of excessive amounts of circulating ICs might perpetuate a pro-inflammatory state promoting thrombosis and predisposing to relapse.
Furthermore, as described for SLE (Chiang *et al*, 2011), ICs can also bind to B-cell receptors of auto-reactive B-cells resulting in signals leading to B-cell activation, proliferation and differentiation into autoantibody-producing cells. This activating cycle might cause formation of more autoantibodies and thus more ICs leading to progression of a deleterious effect.

While the amounts of circulating ICs should correlate with disease severity and activity, their quantification in plasma samples needs to consider several points: (i) the detected types of ICs may be irrelevant because harmful ICs could have already been deposited in tissues; (ii) the concentration of antigen, antibody and as a consequence ICs can change continuously during a chronic process, thus pathogenic ICs can form and deposit in a short period of time; (iii) the formation and clearance of ICs may not be in a steady state; and (iv) the setup of the assays does not allow discriminating between harmful and harmless ICs. Nonetheless, we believe that the detection and characterization of circulating ICs in patients with TTP will seed light onto the possible patho-mechanisms

underlying TTP development. Whether such data are also of use for clinical monitoring, however, remains to be seen.

Prognostic marker for acquired TTP

Beyond the characterization and elucidation of the role of anti-ADAMTS13 antibodies in the pathogenesis of TTP, the identification of prognostic factors during the first acute episode would be of crucial importance for making decisions in the clinic. TTP is a severe disease and its acute events may be life-threatening in the absence of fast and appropriate treatment. In addition, relapse occurs in 30-50% of the patients who survive an initial episode of TTP, being more often during the first year after the onset (Willis & Bandarenko, 2005;George, 2009). However, identifying prognostic factors for both short-term and long-term outcome still remains very difficult considering the broad heterogeneity of patients with TTP. This heterogeneity includes the clinical background (idiopathic versus disease-associated TTP, sporadic versus recurrent TTP) and the ADAMTS13-specific parameters (undetectable versus detectable ADAMTS13 activity or antigen, presence versus absence of ADAMTS13 inhibitor).

Several studies have investigated the significance of ADAMTS13 activity and inhibitors levels to predict outcomes. Most concluded that severe ADAMTS13 deficiency in combination with the presence of antibodies against ADAMTS13 either at presentation and/or during clinical remission are associated with a higher risk of disease recurrence (Vesely *et al*, 2003;Raife *et al*, 2004;Zheng *et al*, 2004;Peyvandi *et al*, 2004;Jin *et al*, 2008;Peyvandi *et al*, 2008;Hovinga *et al*, 2010).

We also have sought for prognostic factors that may help identifying patients at risk. We observed that anti-ADAMTS13 antibodies of the IgG class together with a high inhibitor titer at presentation were associated with persistence of undetectable ADAMTS13 activity in clinical remission (Fig. 6). Moreover, we were the first to investigate the possibility of an association between IgG subclasses and relapse, and found that high levels of IgG4 with undetectable IgG1 were statistically significantly

associated with a trend towards recurrence of TTP. This suggests that high levels of IgG4 could help to identify patients who are at risk of recurrence and could be used, in association with ADAMTS13 activity and inhibitors, as a prognostic marker to predict possible relapse.

6- CONCLUSIONS

The pathophysiology of acute acquired TTP is characterized by severe deficiency of ADAMTS13 (activity and antigen) due to the presence of different classes (IgG, IgM and IgA) and subclasses (mainly IgG4 and IgG1) of inhibiting anti-ADAMTS13 antibodies. An immune response characterized by high levels of IgG4 predicts, at least partially, a more treatable form of TTP than if IgG1 antibodies are present. Antibodies against ADAMTS13 are also involved in immune-complex formation (mainly IgG4-containing immune complexes). Immune complexes might contribute to ADAMTS13 depletion from the circulation and probably to organ damage due to tissue deposition.

Measurement of inhibitory activity; free anti-ADAMTS13 antibodies as well as ADAMTS13-specific circulating ICs should be performed in patients presenting with acute acquired TTP. This information will help to better understand the course of the disease, especially in cases of refractory patients which may benefit of a more aggressive therapy.

Levels of IgG4 autoantibodies could be a recurrent predicting biomarker, although prospective trials with well-characterized patients are needed to substantiate our preliminary observations. The contribution made by IgG4 antibodies and IgG4-ICs to the overall pathologic mechanism in acquired TTP needs further investigation. We believe that the results of this study contributed to a better understanding of the pathogenesis leading to acquired TTP and the prognostic markers here identified might help to recognize patients at risk of disease recurrence or having a poor prognosis.

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Oral Presentations

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APENDIX PUBLICATIONS

I. **Ferrari S**, Scheiflinger F, Rieger M, Mudde G, Wolf M, Coppo P, Girma JP, Azoulay E, Brun-Buisson C, Fakhouri F, Mira JP, Oksenhendler E, Poullin P, Rondeau E, Schleinitz N, Schlemmer B, Teboul JL, Vanhille P, Vernant JP, Meyer D, Veyradier A; French Clinical and Biological Network on Adult Thrombotic Microangiopathies. Prognostic value of anti-ADAMTS 13 antibody features (Ig isotype, titer, and inhibitory effect) in a cohort of 35 adult French patients undergoing a first episode of thrombotic microangiopathy with undetectable ADAMTS 13 activity.
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II. **Ferrari S**, Mudde GC, Rieger M, Veyradier A, Kremer Hovinga JA, Scheiflinger F. IgG subclass distribution of anti-ADAMTS13 antibodies in patients with acquired thrombotic thrombocytopenic purpura.
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III. **Ferrari S**, Knöbl P, Kolovratova V, Varadi K, Plaimauer B, Turecek P, Rottensteiner H, Scheiflinger F. Inverse correlation of free and immune complexed-sequestred anti-ADAMTS13 antibodies in a patient with acquired TTP.
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Prognostic value of anti-ADAMTS13 antibody features (Ig isotype, titer, and inhibitory effect) in a cohort of 35 adult French patients undergoing a first episode of thrombotic microangiopathy with undetectable ADAMTS13 activity

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To study both the pathophysiologic and the prognostic value of ADAMTS13 in thrombotic microangiopathies (TMAs), we enrolled a cohort of 35 adult patients combining a first acute episode of TMA, an undetectable (below 5%) ADAMTS13 activity in plasma, and no clinical background such as sepsis, cancer, HIV, and transplantation. All patients were treated by steroids and plasma exchange, and an 18-month follow-up was scheduled. Remission was obtained in 32 patients (91.4%), and 3 patients died (8.6%) after the first attack. At presentation, ADAMTS13 antigen was decreased in 32 patients (91.4%), an ADAMTS13 inhibitor was detectable in 31 patients (89%), and an anti-ADAMTS13 IgG/IgM/IgA was present in 33 patients (94%). The 3 decedent patients were characterized by the association of several anti-ADAMTS13 Ig isotypes, including very high IgA titers, while mortality was independent of the ADAMTS13 inhibitor titer. In survivors, ADAMTS13 activity in remission increased to levels above 15% in 19 patients (59%) but remained undetectable in 13 patients (41%). Six patients relapsed either once or twice (19%) during the follow-up. High levels of inhibitory anti-ADAMTS13 IgG at presentation were associated with the persistence of an undetectable ADAMTS13 activity in remission, the latter being predictive for relapses within an 18-month delay. (Blood. 2007;109:2815-2822)

Introduction

Thrombotic microangiopathies (TMAs) are defined by the association of acute mechanical hemolytic anemia, thrombocytopenia, and visceral ischemic manifestations related to the formation of platelet thrombi in the microcirculation.[1] Clinically, TMA includes mainly the thrombotic thrombocytopenic purpura (TTP) and the hemolytic uremic syndrome (HUS) characterized by a multivisceral ischemia and a renal ischemia, respectively.[2] Although mechanisms for HUS remain very heterogeneous, pathophysiology for most forms of TTP is related to a severe deficiency of a plasma metalloprotease, ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats).[3-6] Physiologically, ADAMTS13 is the specific cleaving protease for von Willebrand factor (VWF), a large multimeric glycoprotein crucial for both platelet adhesion and aggregation in the high stress–associated hemodynamic conditions of the microcirculation.[7] A severe enzymatic deficiency of ADAMTS13 causes highly adhesive unusually large multimers of VWF to accumulate in plasma, which may spontaneously bind to platelets and thus induce the formation of platelet thrombi in the microcirculation. In rare cases, clinically relevant ADAMTS13 severe deficiency is related to compound heterozygous or homozygous mutations of the ADAMTS13 gene (Upshaw-Schulman syndrome).[8-10] In most cases, severe ADAMTS13 deficiency is secondary to the development of anti-ADAMTS13 autoantibodies (auto-Abs).[11-12] Anti-ADAMTS13 auto-Abs can be detected in vitro either functionally because of their inhibitory effect on ADAMTS13 enzymatic activity[13-14] or, more recently, physically as immunoglobulin G (IgG) or IgM by enzyme-linked immunosorbent assay (ELISA).[15-16] In more than 80% of acquired TTP, anti-ADAMTS13 antibodies are inhibitory IgG.[11-12] In some cases, the mechanisms for acquired TTP may be different, involving either anti-ADAMTS13 noninhibitory IgG or IgM[15-16] or circulating inhibitors not related to an IgG or an IgM.[17] However, although most undetectable ADAMTS13-associated TMA overlaps with the clinical entity named TTP, some exceptions remain, consisting of either clinical TTP with detectable ADAMTS13 activity or clinical HUS with undetectable ADAMTS13 activity.[18,19] This observation is currently motivating

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experts in the field to reconsider a new classification of TMA more focused on pathophysiological mechanisms instead of clinical symptoms.[3,20]

Beyond the elucidation of pathophysiological mechanisms, a second step crucial for TMA characterization is the identification of prognostic factors. Indeed, TMAs are severe diseases with acute events that may be life-threatening in the absence of fast and appropriate treatment (plasmatherapy) and with a global mortality rate that still remains close to 10%.[21,22] In addition, in patients who achieved remission after an acute event, the risk of relapse is estimated to be at least 11%, with extreme rates reported at 73% as a function of the clinical background.[2,3] However, identifying prognostic factors for both short-term and long-term outcome still remains difficult considering the broad heterogeneity of TMA patients. This heterogeneity including both the clinical background (idiopathic versus disease-associated TMA, sporadic versus recurrent TMA) and the ADAMTS13 plasmatic features (undetectable versus detectable ADAMTS13 activity, presence versus absence of an ADAMTS13 inhibitor) is well emphasized as a limit to determine clear prognostic factors in the recent literature.[3,23-27]

More specifically, the *first* acute TMA episode, classically described as a "storm in a blue sky," remains a crucial challenge for physicians in terms of both pathophysiologic and prognostic issues. The first TMA attack is always a trauma whether it is idiopathic and then a first step within an autoimmune disease or whether it is associated with a preexisting clinical context and then a crucial turn in the course of the latter. More pathophysiologic explanations and prognostic markers are obviously needed. Undetectable ADAMTS13 activity–associated TMA, in addition to being a homogeneous entity, has the advantage of offering an exciting biologic parameter on which to focus, especially thanks to the recent advances in both ADAMTS13 antigen (ADAMTS13Ag) and auto-Ab characterization.[16] Thus, in the current study, we chose to focus on both a pathophysiologic and a prognostic analysis (initial outcome and 18-month follow-up) of a homogeneous cohort of adult patients characterized by 3 criteria: (1) a first episode of acute TMA; (2) an undetectable ADAMTS13 activity; and (3) no severe clinical background that could, in itself, influence the prognosis (ie, severe sepsis, cancer, HIV infection, or organ transplantation). Standard clinical and biologic features as well as specific VWF/ADAMTS13-related parameters (VWF antigen [VWFAg], ADAMTS13 activity and antigen, ADAMTS13 inhibitors, and anti-ADAMTS13 IgG/IgM/IgA) were evaluated at presentation in all patients. In patients who achieved remission, ADAMTS13 activity was reevaluated during the follow-up. Thus, we evaluated the correlation of ADAMTS13-related parameters at presentation with the initial clinical and biologic outcome (mortality versus remission, recovery versus nonrecovery of ADAMTS13 activity in remission) as well as with the relapse rate during the follow-up.

Patients, materials, and methods

Patients

All adult patients (more than 18 years old) admitted in the 10 participating French centers with a clinical diagnosis of acute TMA between January 2003 and December 2004 were eligible and tested for ADAMTS13 activity in plasma. Diagnostic criteria for acute TMA were as follows: (1) microangiopathic hemolytic anemia (hemoglobin level below 120 g/L [12 g/dL]), negative direct antiglobulin test, at least 2 schistocytes per high-power field in the peripheral blood smear, lactate dehydrogenase above 450 U/L, and undetectable serum haptoglobin; (2) thrombocytopenia (platelet count below 150×10^9/L); (3) no requirement for fever, neurologic symptoms, or renal failure. For each patient, a questionnaire was completed, allowing initial clinical and laboratory evaluation. Inclusion criteria combined both a first acute event of TMA and an ADAMTS13 activity in plasma below 5%. Exclusion criteria were severe sepsis, cancer, HIV infection, organ or hematopoeitic stem cell transplantation, and disseminated intravascular coagulation. As a first intention treatment, all enrolled patients received both steroids (1 mg prednisone per kilogram of body weight daily) and plasma exchange (exchanging 1.0 to 1.5 times the predicted plasma volume of the patient) at the discretion of the attending physician. Evaluation for the initial outcome (achievement of remission or death) and an 18-month follow-up were performed. Patients were enrolled after appropriate consent was obtained in agreement with the institutional review board of Assistance Publique-Hôpitaux de Paris (Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale, Ile-de-France–Paris–Saint Antoine) and the Declaration of Helsinki.

Clinical definition

Idiopathic TMA was defined as TMA occurring in patients with no apparent preexisting disease. Remission was defined by a normal platelet count (above 150×10^9/L) and no plasma exchange treatment for 30 days (day 1 starting on remission) or more.

Sample collection

Before any treatment was initiated, venous blood was collected into 1:10 final volume of 3.8% sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2500*g* for 20 minutes, and aliquoted samples were stored at −80°C before being tested. Plasma samples were collected both at presentation and in remission (between day 1 and day 7 of remission according to the previous definition).

VWFAg

VWF antigen (VWFAg) levels in plasma were measured using an enzyme-linked immunosorbent assay (ELISA Asserachrom VWF; Diagnostica Stago, Asnières, France).

ADAMTS13 activity ELISA

Measurement of ADAMTS13 activity in plasma was performed as previously described[18] with minor modifications. Briefly, the method relies on the hydrolysis of a constant amount of wild-type recombinant VWF used as substrate by serial dilutions of tested plasma used as ADAMTS13 provider. Plasma samples treated with Pefabloc (Roche Applied Science, Indianapolis, IN) (2 mM final) for 10 minutes were serially diluted from 1:10 to 1:640 in 5 mM Tris-HCl, pH 8, 1.5 M urea. Aliquots of 90 μL were preincubated with 10 μL of 100 mM $BaCl_2$ for 5 minutes at room temperature. This mixture (60 μL) was added into wells of microtitration plates to 40 μL wild-type recombinant VWF (0.05 IU) previously dialyzed against 5 mM Tris-HCl, pH8, 1.5 M urea. Incubation was performed for 48 hours at 37°C. The proteolysis was stopped by addition of 5 μL of 200 mM EDTA in water. The residual VWFAg contained in the hydrolysate was thus estimated by a 2-site ELISA using the anti–C-terminal VWF MoAb 453 for coating and a pool of immunoperoxidase-conjugated anti–N-terminal VWF MoAbs for staining.[28] Normal pooled plasma (NPP) was arbitrarily defined as containing 100% of ADAMTS13 activity and used as an internal control.

ADAMTS13 inhibitor assay

Patient plasma was heat treated at 56°C for 30 minutes to inactivate any endogeneous ADAMTS13 activity. Circulating inhibitor for ADAMTS13 was assayed by measuring the residual ADAMTS13 activity in mixtures of TMA patient plasma and NPP at 3 distinct volume-volume ratios: 1:1, 2:1, and 3:1 as previously described.[18] Titer was semiquantitatively defined as high, medium, or low for a residual ADAMTS13 activity below 10% in 1:1, 2:1, and 3:1 mixtures, respectively.

ADAMTS13 inhibitor assay was systematically performed in addition to the ADAMTS13 activity assay in all patients.

Anti-ADAMTS13 antibody ELISA

Anti-ADAMTS13 IgG, IgM, and IgA were tested in all patients at presentation. Anti-ADAMTS13 IgG and IgM antibodies were detected as published by Rieger et al.[16] Briefly, microtiter plates were coated with an anti-His tag antibody (overnight incubation), nonspecific binding sites were blocked for 2 hours at room temperature with PBS-BSA, and recombinant His-tagged ADAMTS13 was added and incubated for 3 hours at room temperature. Serial dilutions of plasma samples were incubated overnight at +4°C. After washing, plates were incubated with an alkaline phosphatase–conjugated goat anti–human IgG, IgM, or IgA antibody (Sigma, Saint Louis, MO) for 2 hours at room temperature. After washing, the enzyme substrate p-nitrophenyl phosphate (PNPP) was added and incubated 45 minutes. Absorbance was read at 405 nm. Anti-ADAMTS13 IgG/IgM/IgA antibodies titers were calculated according to Rieger et al.[16] In patients whose anti-ADAMTS13 IgG titer was lower than the detection limit of our developed ELISA (IgG negative), anti-ADAMTS13 IgGs were retested using the TECHNOZYME ADAMTS13-INH commercial kit (Technoclone, Vienna, Austria) to improve sensitivity in the detection (IgG positive if above 15 U/mL). The IgG anti-ADAMTS13 ELISA using the commercial kit was performed according the manufacturer's instructions.

ADAMTS13 Ag ELISA test

ADAMTS13 Ag was measured using a recently developed ADAMTS13 Ag ELISA.[29] Briefly, microtiter plates were coated with a polyclonal rabbit IgG against human ADAMTS13. Dilutions of plasma samples were incubated overnight at room temperature. After washing, plates were incubated with a horseradish peroxidase–conjugated polyclonal rabbit IgG against human ADAMTS13 for 2 hours at room temperature. After washing, a chromogenic substrate (Sure Blue TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added. The color reaction was stopped by 1N HCl solution, and absorbance was read at 450 nm. The reference interval for the ELISA is 740 to 1420 ng ADAMTS13 per milliliter, and the limit of quantification is 62.5 ng ADAMTS13 per milliliter. Samples with antigen levels under the limit of quantification are expressed as less than 62.5 ng ADAMTS13 per milliliter.

Statistical analysis

Statistics were performed using the computer-assisted program Statview (Abacus Concepts, Berkeley, CA). Means and standard deviations (SD) were calculated for each continuous variable. Comparisons were performed using an independent sample *t* test for continuous parametric variables and a χ^2 test for categoric variables. Relationships between ADAMTS13-related parameters and other variables were studied using univariate and multivariate analysis with both linear and logistic regression analysis.

Results

Patients

Over a 24-month period, 755 adult patients with a clinically suspected acute TMA were enrolled in our national registry and screened for ADAMTS13 activity. A total of 658 patients exhibited a normal or partially decreased ADAMTS13 activity, and 97 patients had an undetectable ADAMTS13 activity. Among the latter, 47 patients had previously been included in our registry because of 1 or several acute TMA episodes, while 50 patients had a first TMA episode. Among the latter, 15 had exclusion criteria such as cancer, HIV, severe sepsis, organ transplantation. Thus, finally, a cohort of 35 consecutive patients with a first acute episode of TMA associated with an undetectable ADAMTS13 activity (below 5%) in plasma was enrolled. Demographic, clinical, and standard biologic features of each patient are presented in Table 1. Mean age was 36 years (SD = 12.7), and the sex ratio was 2.5:1 (25 women and 10 men). Twenty-two patients exhibited an idiopathic TMA, while 13 patients had a TMA associated either with pregnancy (n = 6), the postpartum state (n = 1), oral contraceptives (n = 1), Crohn disease (n = 1), psoriasis (n = 1), chronic hepatitis C (n = 1), diabetes (n = 1), or hypertension (n = 1). At inclusion during the acute TMA, fever was present in 5 patients (14.3%), and neurologic symptoms including either headache, altered mental status, aphasia, or convulsions were observed in 18 patients (51.4%); anemia (mean hemoglobin level ± SD, 71 ± 18 g/L and thrombocytopenia (mean platelet count ± SD, 17 × 10^9/L ± 13 × 10^9/L) were present in all patients. The mean creatinine level was 111 ± 52 μM, and only 3 patients exhibited a creatinine level higher than 200 μM. Three patients (8.6%) died of acute multivisceral TMA involvement with acute renal failure within a delay of 10 days of extensive plasma exchanges. Remission was obtained in 32 patients (91.4%) with the classical "steroids plus plasma exchanges" protocol described in "Patients" within a mean delay of 4 ± 1 weeks; no survivor patient exhibited a flare-up episode. The 18-month follow-up showed that 6 patients relapsed.

VWF- and ADAMTS13-related parameters in patients at presentation

Results of both VWFAg and ADAMTS13Ag are presented in Figure 1. VWFAg levels were higher than 100 IU/dL in 32 patients (91.4%), including 11 patients with levels higher than 200 IU/dL (31.4% of total). ADAMTS13Ag levels were decreased in 32 patients (91.4%) (including 7 patients with undetectable levels) and normal in 3 patients (8.6%). However, statistical analysis showed no correlation between VWFAg and ADAMTS13Ag levels.

Titrations for both ADAMTS13 inhibitor and anti-ADAMTS13 IgG, IgM, and IgA are presented in Figure 2. An ADAMTS13 inhibitor was detected in 31 patients (89%), while an anti-ADAMTS13 IgG (titer ranging from 25 to 6400) and/or IgM (titer from 20 to 3200) and/or IgA (titer from 200 to more than 10 000 arbitrary units) were detected in 33 patients (94%) (Figure 2). A mild correlation between ADAMTS13 inhibitor titer and anti-ADAMTS13 Ig titer was present with an overlapping between both parameters in 29 of 35 patients (inhibitory anti-ADAMTS13 Ig, 83%). In contrast, in the absence of ADAMTS13 inhibitor, an anti-ADAMTS13 Ig was detected in 4 of 35 patients (noninhibitory anti-ADAMTS13 Ig, 11.5%). Plasma of 2 patients inhibited ADAMTS13 activity despite the absence of anti-ADAMTS13 IgG/IgM/IgA (Figure 2). Statistical analysis showed no correlation between ADAMTS13Ag levels and either ADAMTS13 inhibitor or anti-ADAMTS13 IgG/IgM/IgA titers, respectively.

Analysis of decedent patients

Three patients died after the first acute TMA episode (patients 15, 17, and 22; Table 1). Table 1 indicates their specific clinical characteristics, and Table 2 indicates their main VWF/ADAMTS13 features. All patients had an idiopathic TMA, none of them had fever at presentation, while 2 of them exhibited neurologic symptoms (altered mental status and aphasia). In all of them, the platelet count was lower than 15 × 10^9/L and the hemoglobin level lower than 65 g/L. Interestingly, all of them also had a significant alteration of renal function (creatinine level higher than 175 μM), and 2 of them were older than 60 years. (Table 1). Analysis of the VWF/ADAMTS13 features (Table 2) shows a remarkably increased VWFAg level in 2 patients (350 and 540 IU/dL in patients 15 and 22, respectively) and a remarkably decreased ADAMTS13Ag in 2 patients (less than 62.5 ng/mL in patients 15 and 17). An ADAMTS13 inhibitor was either detectable in 2 patients (low or high titer) or not detectable in 1 patient. Interestingly, all patients were characterized by the association of several anti-ADAMTS13

Table 1. Demographic, clinical, and standard biologic features of each TMA patient (n = 35)

Pt no.	Age, y	Sex	Context	Fever	CNS symptoms	Platelet count, × 10^9/L	Hb level, g/L	Creatinine level, μM	Initial outcome	No. of relapses*
1	21	M	Idiopathic	No	None	10	63	116	R	0
2	19	F	Idiopathic	No	None	3	44	88	R	0
3	27	M	Idiopathic	Yes	None	10	69	85	R	0
4	35	F	Idiopathic	Yes	None	21	42	90	R	0
5	57	F	Idiopathic	No	Aphasia	28	51	92	R	0
6	41	F	Idiopathic	No	Aphasia	27	81	83	R	1
7	53	F	Idiopathic	Yes	Aphasia	10	50	77	R	0
8	35	F	Idiopathic	No	None	9	62	81	R	0
9	39	M	Idiopathic	No	None	10	91	85	R	0
10	50	F	Idiopathic	No	None	15	80	87	R	0
11	29	F	Idiopathic	No	None	30	70	90	R	0
12	34	F	Idiopathic	No	AMS	15	80	83	R	0
13	40	M	Idiopathic	Yes	None	15	57	100	R	1
14	31	M	Idiopathic	No	Headache	23	110	159	R	2
15	61	M	Idiopathic	No	AMS	4	62	240	D	—
16	38	F	Idiopathic	No	None	5	81	100	R	2
17	45	M	Idiopathic	No	None	9	60	175	D	—
18	28	M	Idiopathic	Yes	AMS	15	72	88	R	0
19	39	F	Idiopathic	No	Aphasia	17	85	105	R	0
20	16	F	Idiopathic	No	None	10	70	100	R	0
21	26	F	Idiopathic	No	None	12	68	81	R	1
22	70	F	Idiopathic	No	Aphasia	13	52	195	D	—
23	30	F	Pregnancy	No	None	11	83	82	R	0
24	29	F	Pregnancy	No	Aphasia	7	67	139	R	0
25	27	F	Pregnancy	No	None	20	100	300	R	0
26	24	F	Pregnancy	No	Headache	35	81	79	R	0
27	34	F	Pregnancy	No	None	10	71	82	R	1
28	33	F	Pregnancy	No	AMS	36	67	85	R	0
29	23	F	Postpartum	No	Headache	11	64	81	R	0
30	43	F	Contraceptives	No	AMS	11	72	76	R	0
31	23	F	Crohn	No	AMS	27	44	78	R	0
32	45	M	Psoriasis	No	None	17	116	90	R	0
33	59	F	HCV	No	Convulsions	14	70	95	R	0
34	29	F	Diabetes	No	Headache	20	73	97	R	0
35	31	M	Hypertension	No	AMS	80	106	210	R	0

Patients 15, 17, and 22 are dead.

Pt indicates patient; CNS, central nervous system; Hb, hemoglobin; M, male; R, remission; F, female; AMS, altered mental status; D, death; —, not applicable; HCV, hepatitis C virus.

*During an 18-month follow-up.

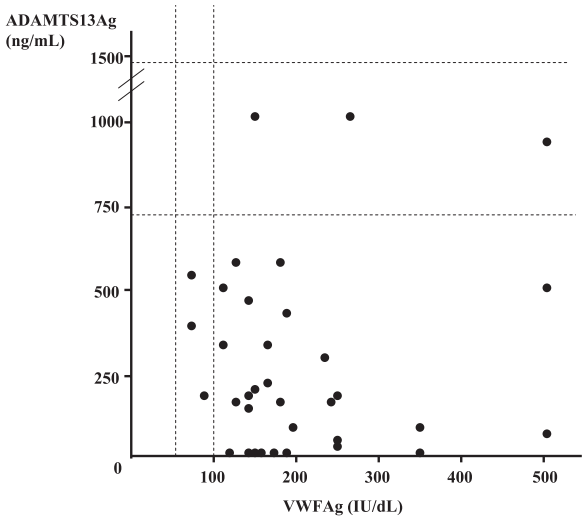

Figure 1. von Willebrand factor antigen (VWFAg) and ADAMTS13 antigen (ADAMTS13Ag) levels in plasma in a cohort of 35 patients with acute TMA. Normal ranges are indicated by dashed lines (50 to 100 UI/dL for VWFAg and 740 to 1420 ng/mL for ADAMTS13Ag). Each TMA patient is represented by a circle. All but 3 patients exhibited a VWFAg level higher than 100 IU/dL, while all but 3 patients had a decreased ADAMTS13Ag level. Statistical analysis showed no correlation between VWFAg and ADAMTS13Ag levels.

Ig isotypes (2 patients with IgG, IgM, and IgA and 1 patient with both IgG and IgA) with very high Ig titers, especially for IgA (Table 2).

Analysis of ADAMTS13 in survivors at initial clinical remission

ADAMTS13 activity measured at initial clinical remission showed 3 distinct courses: It either remained undetectable (below 5%) in 13 patients (41%) or became detectable but partially decreased (15% to 40%) in 12 patients (38%) or normal (50% to 100%) in 7 patients (21%). ADAMTS13 inhibitors in remission were still detectable only in patients whose ADAMTS13 activity remained below 5%, with titers either similar or decreased when compared with those of the acute episode (Figure 3).

Correlation between ADAMTS13 parameters at presentation and ADAMTS13 activity at initial clinical remission is presented in Figure 4. Neither ADAMTS13 Ag levels nor the presence of an ADAMTS13 inhibitor (independently of the titer) nor the presence of anti-ADAMTS13 IgG, IgM, or IgA at presentation were predictive of the course of ADAMTS13 activity in remission. In contrast, the presence of a high titer of ADAMTS13 inhibitor, either alone or combined with an anti-ADAMTS13 IgG, was associated with the persistence of an undetectable ADAMTS13 activity (below 5%) in remission (Figure 4).

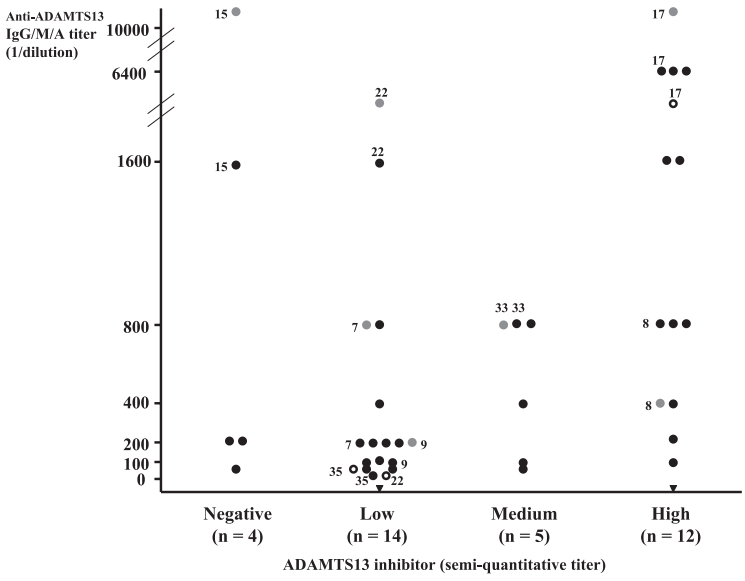

Figure 2. ADAMTS13 inhibitor and anti-ADAMTS13 IgG/IgM/IgA in a cohort of 35 patients with acute TMA. Each TMA patient is represented by a symbol. Black circles are for patients with IgG, gray circles for patients with IgA, white circles for patients with IgM, and black triangles for patients with no detectable IgG/IgM/IgA. IgG/IgM/IgA titers are expressed as the reciprocal of patient plasma dilution. An isolated IgG was detected in 26 patients, the combination IgG and IgM in 1 patient, the combination IgG and IgA in 4 patients, and the combination of IgG, IgM, and IgA in 2 patients (indicated by numbers referring to patients in Table 1). Two patients had neither an IgG, IgM, nor IgA. ADAMTS13 inhibitors are expressed as semiquantitative titers: 4 patients had no detectable ADAMTS13 inhibitor, 14 patients a low titer, 5 patients a medium titer, and 12 patients a high titer. Twenty-nine patients had both an ADAMTS13 inhibitor and an anti-ADAMTS13 IgG/IgM/IgA (inhibitory anti-ADAMTS13 antibodies), and 4 patients had an anti-ADAMTS13 IgG/IgA with no ADAMTS13 inhibitor (noninhibitory anti-ADAMTS13 antibodies). Two patients had no IgG/IgM/IgA but an ADAMTS13 inhibitor.

Follow-up of the survivors

Of the 32 patients who achieved clinical remission after the first acute TMA episode, 6 patients relapsed either once (patients 6, 13, 21, and 27; Table 1) or twice (patients 14 and 16; Table 1) during the 18-month follow-up. Delay for relapses ranged from 5 to 17 months after the first acute TMA episode, and 50% of relapses occurred as soon as the first year. Interestingly, all relapses were associated with an undetectable ADAMTS13 activity and an ADAMTS13 inhibitor titer similar to that at first diagnosis (data not shown).

Both the mean age (36 years old) and the sex ratio (2:1 female-male) of relapsing patients were similar to those of the cohort; all but 1 patient whose acute TMA occurred during pregnancy (patient 27) had an idiopathic TMA, and both their clinical and standard biologic parameters at presentation were similar to the ones of nonrelapsing patients (Table 1). VWF/ADAMTS13-related parameters of the 6 relapsing patients are presented in Table 3. At presentation of the initial acute TMA episode, VWFAg levels were either normal or moderately increased (70 to 250 IU/dL), ADAMTS13Ag levels were either decreased or normal (75 to 1010 ng/mL), an ADAMTS13 inhibitor was detected in 5 of 6 patients, while an anti-ADAMTS13 IgG was present in all patients. Interestingly, at initial remission, ADAMTS13 activity was still undetectable (below 5%) in 5 patients together with ADAMTS13 inhibitor titers either identical or decreased when compared with inhibitor titers of the initial acute phase (Table 3).

The relapse rate was significantly higher in patients whose ADAMTS13 activity at initial remission was still undetectable (below 5%) (5 of 13; 38.5%) when compared with patients whose ADAMTS13 activity at initial remission was detectable (above 15%) (1 of 19; 5%).

Discussion

In adult patients, an autoimmunization against ADAMTS13 inducing a severe ADAMTS13 functional deficiency in plasma has been described as a key mechanism for most undetectable ADAMTS13-associated TMA.[3]

The first goal of the current study was to explore the pathophysiology for ADAMTS13 severe deficiency in a cohort of adult patients who underwent a first acute episode of TMA. Because none of these patients had ever received plasma infusions before, any potential alloimmunization against ADAMTS13 and variations in anti-ADAMTS13 auto-Abs titers after plasmatherapy[30] could be excluded. Interestingly, this study is the first one to show anti-ADAMTS13 IgA in TMA patients. In all cases, however, these IgAs were associated with IgG. In our cohort, a circulating inhibitor against ADAMTS13 was detected with a lower frequency (89%) than an anti-ADAMTS13 IgG/IgM/IgA (94%) as previously reported by Rieger at al,[16] who recently demonstrated a higher sensitivity of IgG/IgM detection (97%) versus inhibitor detection (83%) in 59 patients with an undetectable ADAMTS13-associated acute TMA. This discrepancy between inhibitors and Ig rates is, however, not surprising considering the broad range (50% to almost 90%) of circulating ADAMTS13 inhibitors reported in TMA patients[3,11-13,18,31,32] and the established variability of sensitivity of the miscellaneous methods used for the detection of these inhibitors.[14]

In our cohort, the overlapping rate between ADAMTS13 inhibitors and anti-ADAMTS13 IgG/IgM/IgA was 83%, corresponding to inhibitory IgG/IgM/IgA, while the rate for noninhibitory IgG/IgM/IgA was 11.5%. These proportions are in agreement with the literature[15,16,33] and confirm that most anti-ADAMTS13 auto-

Table 2. Main VWF/ADAMTS13 features of the 3 decedent patients

Patient no.	VWFAg, IU/dL	ADAMTS13Ag, ng/mL	ADAMTS13 inhibitor titer	Anti-ADAMTS13 IgG titer*	Anti-ADAMTS13 IgM titer*	Anti-ADAMTS13 IgA titer*
15	350	< 62.5	−	1600	0	> 10 000
17	150	< 62.5	+++	6400	3200	> 10 000
22	540	85	+	1600	20	3 200

− indicates negative; +++, high; +, low.
*1/dilution.

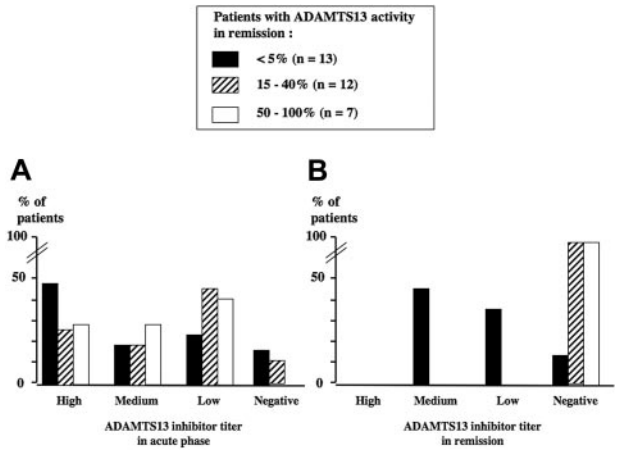

Figure 3. ADAMTS13 inhibitor titer. Titer is shown in the acute phase (A) and in remission (B) in 32 surviving TMA patients. The level of ADAMTS13 activity in survivors at initial clinical remission defines 3 groups of TMA patients: below 5% (group 1, n = 13, black histograms), 15% to 40% (group 2, n = 12, dashed histograms), and 50% to 100% (group 3, n = 7, white histograms). The percentage of patients with either high, medium, low, or negative ADAMTS13 inhibitor titers within each group is presented both in the acute phase (A) and in remission (B). In group 1, ADAMTS13 inhibitor titers remained identical or decreased in remission when compared with the acute phase. In groups 2 and 3, all ADAMTS13 inhibitors, when present in the acute phase, disappeared in remission.

Abs are functional (inhibitory) in vitro and potentially in vivo. However, the decrease of ADAMTS13Ag plasma levels found in 91.4% of patients in our cohort suggests that the formation of immune complexes that may be rapidly cleared in vivo also strongly participates in ADAMTS13 deficiency whatever the inhibitory or noninhibitory capacity of ADAMTS13 auto-Abs, as previously reported.[34,35] Also, in addition to the inhibitory effect on ADAMTS13 function, the residual levels of both ADAMTS13 activity and ADAMTS13Ag in plasma may be influenced by several other parameters—either Ig related (ie, the affinity for ADAMTS13, the epitope(s) on ADAMTS13,[36-38] a proteolytic degradation of ADAMTS13[39]) or non-Ig related (ie, a decreased liver synthesis,[40] an inactivation by thrombin or plasmin,[41] or an in vitro inhibition by hemoglobin,[17] the latter being excluded in our patients). Finally and interestingly, we found that anti-ADAMTS13 IgG associated with a high inhibitor titer at presentation was predictive of a nonrecovery of ADAMTS13 activity in clinical remission (Figure 4). The pathophysiology for this discrepancy between clinical remission and ADAMTS13-related biologic remission in some patients remains, however, unclear.

The second goal of our study was to search for prognostic factors among ADAMTS13-related parameters at presentation. The originality and strength of the current study was to specifically include patients with a first acute TMA episode associated with an undetectable ADAMTS13 activity and to exclude patients whose clinical background in itself could have influenced the prognosis (cancer, HIV infection, organ transplantation, severe sepsis).

These exclusion criteria may therefore explain the mortality rate of 8.6%, which is lower than most rates reported in the literature.[3,21,22] Interestingly, a detailed analysis of the 3 decedent patients reveals a specific association between mortality and the combination of several Ig isotypes (IgG, IgM, and IgA) with the presence of very high IgA titers in all patients (Table 2). The association of both an IgG and an IgM has already been described in a 70-year-old patient with a fatal TTP.[15] The presence of anti-ADAMTS13 IgA has never been described in any patient to date, and both their etiology and their potential toxicity remain unknown. However, IgAs are known to be associated with a bad prognosis with several pathological conditions such as nephropathies,[42] myeloma,[43] celiac disease,[44] and heparin-induced thrombocytopenia.[45] In our patients, the role of anti-ADAMTS13 IgA toxicity on renal tissue may be questionable, because all of them had a severe alteration of renal function as soon as at presentation. Also, in our 3 decedent patients, the inhibitory function of the anti-ADAMTS13 IgG/IgM/IgA does not appear closely related to mortality although ADAMTS13Ag levels were severely decreased. This last point underlines that an immune-depletion mechanism leading to a severe ADAMTS13 quantitative deficiency is the most important mechanism for the severe ADAMTS13 enzymatic deficiency identified in the decedent patients. The high titers of the anti-ADAMTS13 IgG/IgM/IgAs are likely to be strongly involved in the formation of immune complexes, but a particularly high affinity of these IgG/IgM/IgAs to ADAMTS13 may also be hypothesized. Hence, both the high titer and the potential high affinity of the anti-ADAMTS13 IgG/IgM may also explain the lack of response to the first intention treatment (steroids and plasma exchanges) that led to a fatal outcome with a dramatic multivisceral involvement in our 3 patients. Retrospectively, in these patients, the indication of a first-line immunosuppressive treatment associated with plasmatherapy is debatable.

In our cohort, however, 32 patients of 35 (91.4%) achieved remission within 3 to 5 weeks with a treatment associating steroids and plasma exchanges. No patient required an additive treatment (ie, splenectomy or immunosuppressive agents) to induce remission. This good response to plasmatherapy alone may be explained by both the exclusion of TMA associated with sepsis, cancer, organ transplantation, HIV, and enrollment during a first acute TMA episode. Indeed, TMA refractory to plasmatherapy has been described mainly either in the previous clinical contexts or, independently of these specific clinical backgrounds, preferentially in patients with a recurrent disease.[3]

Using an 18-month follow-up of the 32 patients who achieved remission, we found a global relapse rate of 19%. Although relapse rates previously reported are very heterogeneous and look very dependent on the clinical background,[3,23] the relapse rate of our cohort is, however, in agreement with data from the literature: In

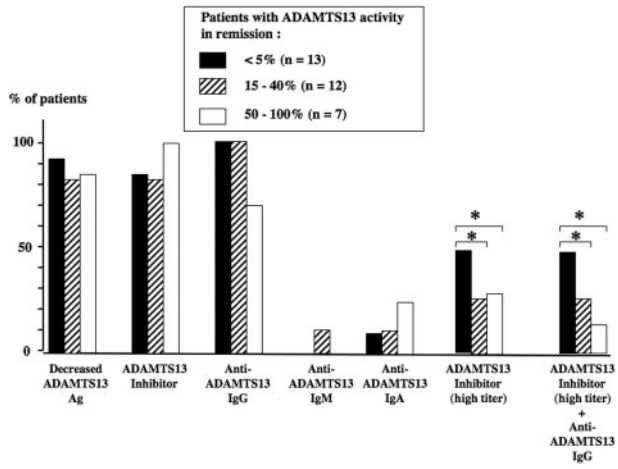

Figure 4. ADAMTS13-related parameters at presentation in 32 surviving TMA patients. The level of ADAMTS13 activity in survivors at initial clinical remission defines 3 groups of TMA patients: below 5% (group 1, n = 13, black histograms), 15% to 40% (group 2, n = 12, dashed histograms), and 50% to 100% (group 3, n = 7, white histograms). At presentation, the percentage of patients with either a decreased ADAMTS13Ag level, an ADAMTS13 inhibitor (independently of the titer), or the presence of an anti-ADAMTS13 IgG, IgM, or IgA was not significantly different between the 3 groups. In contrast, at presentation, the presence of either a high titer of ADAMTS13 inhibitor or a highly inhibitory anti-ADAMTS13 IgG was significantly (*$P < .05$) more frequent in group 1 when compared with group 2 and group 3, respectively.

Table 3. Main VWF/ADAMTS13 features at presentation and at initial remission of the first TMA attack of the 6 relapsing patients during the 18-month follow-up

Patient no.	VWFAg, IU/dL	ADAMTS13 Ag, ng/mL	ADAMTS13 inhibitor titer	Anti-ADAMTS13 IgG titer, 1/dilution	ADAMTS13 activity, %; inhibitor in remission
6	140	450	+++	200	< 5; ++
13	180	145	+++	800	< 5; ++
14	245	75	+	400	< 5; +
16	140	1010	+++	800	< 5; ++
21	70	300	+++	1600	15; −
27	140	160	−	200	< 5; −

Anti-ADAMTS13 IgM titer and anti-ADAMTS13 IgA titer equal 0 for all patients listed in this table.
+++ indicates high; ++, medium; +, low; −, negative.

adult idiopathic TMA, data from the Oklahoma TTP-HUS registry report a relapse rate of about 20%[2]; in adult TMA occurring concomitantly with another clinical condition (especially pregnancy and autoimmune diseases), the risk of relapse is reported to be higher than 25%.[46,47] Also in agreement with these data, of our 6 relapsing patients, 5 had an idiopathic TMA while 1 had a pregnancy-associated TMA, and 50% of relapses occurred within the first year.

Interestingly, the relapse rate was significantly higher in patients whose ADAMTS13 activity in remission was still undetectable (38.5%) when compared with patients who recovered a detectable ADAMTS13 activity in remission (5%). This observation is in agreement with a previous report from our group showing that most patients with multirelapsing TTP becoming progressively refractory to plasmatherapy were characterized by a persistent undetectable ADAMTS13 activity during remission.[48] However, the predictive value for relapse of an undetectable ADAMTS13 activity in remission remains debatable: Indeed, in our cohort, 1 patient (patient 21) who recovered a detectable ADAMTS13 activity in remission (15%) relapsed 16 months after the initial episode, while 8 patients with a still undetectable ADAMTS13 activity in remission did not relapse during the 18-month follow-up. On one hand, in our study, the positive predictive value for relapsing in a delay of 18 months of an undetectable ADAMTS13 activity in remission (number of relapsing patients with an undetectable ADAMTS13 activity in remission/total number of patients with an undetectable ADAMTS13 activity in remission) is only 38.5%. These data are not surprising considering that even patients with an inherited, and thus persistent, severe ADAMTS13 deficiency (Upshaw-Schulman syndrome) exhibit greatly heterogeneous clinical phenotypes in terms of both severity and frequency of relapses.[49,50] Indeed, similar to the incomplete penetrance and variable expressivity related to gene modifiers observed in most thrombotic diseases,[51] both specific environmental and genetic backgrounds are likely to associate synergistically with persistent acquired ADAMTS13 severe deficiency in the trigger of TMA relapses. On the other hand, the negative predictive value for relapse of a detectable ADAMTS13 activity in remission (number of nonrelapsing patients with a detectable ADAMTS13 activity in remission/total number of patients with a detectable ADAMTS13 activity in remission) is 94.7%, which means that recovering a detectable ADAMTS13 in remission may be considered as a good prognostic factor (lower risk of relapse) in patients after a first acute TMA episode.

In conclusion, this study emphasizes that the pathophysiology for a first TMA attack related to an undetectable ADAMTS13 activity mainly involves anti-ADAMTS13 IgG combining both an inhibitory and an immune complex–depletion mechanism. Among ADAMTS13-related parameters at presentation, the combination of several anti-ADAMTS13 Ig isotypes (IgG, IgM, and IgA), including very high IgA titers, is linked to a higher risk of mortality. In survivors, highly inhibitory anti-ADAMTS13 IgGs at presentation are associated with the persistence of an undetectable ADAMTS13 activity in remission, the latter being predictive for relapses within 18 months. Similarly to other autoimmune diseases,[52] the role of anti-ADAMTS13 auto-Abs in acquired TMA as prognostic markers needs to be further addressed using longitudinal studies involving large numbers of patients, ideally as soon as the first TMA attack.

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Authorship

Contribution: A.V., F.S., and S.F. designed the study, analyzed the results, and wrote the paper; S.F. and G.M. did all the antibody assay work; the study protocol was discussed with P.C., E.A., C.B.-B., F.F., J.-P.M., E.O., E.R., B.S., and J.-P.V., who were also in charge of patients; P.P., N.S., J.-L.T., and P.V. were in charge of patients; and A.V., D.M., J.-P.G., M.W., F.S., M.R., G.M., and S.F. were responsible for the VWF and ADAMTS13 assays.

Conflict-of-interest disclosure: S.F., M.R., G.M., and F.S. are employees of Baxter Healthcare Inc. They declare that they have no commercial interest in the assay systems developed to detect anti-ADAMTS13 antibodies.

A complete list of the members of the French Clinical and Biological Network on Adult Thrombotic Microangiopathies appears as a data supplement to the online version of this article (Document S1, available on the *Blood* website; see the Supplemental Material link at the top of the online article).

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ORIGINAL ARTICLE

IgG subclass distribution of anti-ADAMTS13 antibodies in patients with acquired thrombotic thrombocytopenic purpura

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Summary. *Background*: ADAMTS13-neutralizing IgG autoantibodies are the major cause of acquired thrombotic thrombocytopenic purpura (TTP). *Objective*: To analyze the IgG subclass distribution of anti-ADAMTS13 antibodies and a potential relationship between subclass distribution and disease prognosis. *Methodology*: An enzyme-linked immunosorbent assay-based method was used to quantify the relative amounts of IgG subclasses of anti-ADAMTS13 antibodies in acquired TTP plasma. *Results*: IgG_4 (52/58, 90%) was the most prevalent IgG subclass in patients with acquired TTP, followed by IgG_1 (52%), IgG_2 (50%), and IgG_3 (33%). IgG_4 was found either alone (17/52) or with other IgG subclasses (35/52). IgG_4 was not detected in 10% of the patients. There was an inverse correlation between the frequency and abundance of IgG_4 and IgG_1 antibodies ($P < 0.01$). Patients with high IgG_4 levels and undetectable IgG_1 are more prone to relapse than patients with low IgG_4 levels and detectable IgG_1. *Conclusions*: All IgG subclasses of anti-ADAMTS13 antibodies were detected in patients with acquired TTP, with IgG_4, followed by IgG_1, antibodies dominating the anti-ADAMTS13 immune response. Levels of IgG_4 could be useful for the identification of patients at risk of disease recurrence.

Keywords: ADAMTS13, anti-ADAMTS13 antibodies, IgG subtypes, thrombotic thrombocytopenic purpura.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening thrombotic microangiopathy (TMA) characterized by microangiopathic hemolytic anemia, thrombocytopenia with renal impairment or neurologic abnormalities due to the deposition of platelet, and von Willebrand factor (VWF)-rich thrombi in the microcirculation [1]. ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif) regulates the size, and therefore the thrombogenic potential, of VWF by cleaving the peptide bond between Tyr1605 and Met1606 in the A2 domain of VWF subunits. Congenital or acquired ADAMTS13 deficiency is the major risk factor for TTP [2,3].

Autoantibodies against ADAMTS13 are believed to cause ADAMTS13 deficiency in acquired idiopathic TTP [4,5]. The antibody response to ADAMTS13 includes neutralizing or non-neutralizing antibodies [4–6]. Neutralizing antibodies block the proteolytic activity of ADAMTS13 towards VWF and are detected with *in vitro* functional assays. Non-neutralizing antibodies can only be identified by enzyme-linked immunosorbent assay (ELISA) [7]. Such antibodies may contribute to increasing ADAMTS13 clearance from the circulation or may interfere with ADAMTS13 interaction with cells or other plasma proteins [6]. Principally, the ELISA assay detects neutralizing or non-neutralizing antibodies against ADAMTS13. Such antibodies are predominantly of the IgG class and, to a lesser extent, of the IgM class [7,8]. We have also identified IgA anti-ADAMTS13 antibodies in some patients with acquired TTP [8], but their clinical significance is unknown.

The biological function of IgG antibodies is determined by their specificity, affinity, and subclass. IgG subclasses have different capacities to bind to cell surface Fcγ receptors (FcγRs) or to activate complement proteins [9], thus mediating different immunologic effector functions. We characterized the ADAMTS13-specific IgG antibody subclass distribution to elucidate the role of IgG anti-ADAMTS13 autoantibodies in the pathologic mechanism leading to acquired TTP.

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Materials and methods

Patients

Fifty-eight patients diagnosed with acute acquired TTP were analyzed. Fifty-three had idiopathic TTP, and five had TTP associated with pregnancy ($n = 2$), the postpartum state ($n = 1$), systemic lupus erythematosus (SLE) ($n = 1$), or cancer ($n = 1$).

Forty-four patients were analyzed during the first episode of TTP, and 14 during relapse. Four of the 44 patients analyzed during the first episode died, eight were lost to follow-up, and 11/32 who achieved clinical remission relapsed during the 36-month follow-up (follow-up, 36–77 months). The 14 patients studied during an acute relapse had a documented positive history of TTP with at least one previous TTP episode. Five of 14 patients who were included in the 36-month follow-up had no new relapses, and nine were lost to follow-up.

Inclusion criteria Patients had to meet the following inclusion criteria: (i) presence of microangiopathic hemolytic anemia (hemoglobin level < 12 g dL^{-1}), direct antiglobulin test-negative, at least two schistocytes per high-power field in the peripheral blood smear, lactate dehydrogenase levels > 450 IU L^{-1}, and undetectable serum haptoglobin; (ii) thrombocytopenia (platelet count $< 150 \times 10^9$ L^{-1}); and (iii) severely reduced ($< 10\%$) plasma ADAMTS13 activity levels. Fever, neurologic symptoms or renal failure were not obligatory.

Clinical and laboratory data The patients' demographic and clinical features and ADAMTS13-related laboratory findings are summarized in Table 1. The clinical characteristics and laboratory findings of some of these patients have been described previously [7,8].

Table 1 Demographic and clinical features and ADAMTS13-related laboratory findings of patients with acute acquired thrombotic thrombocytopenic purpura (TTP)

Sex, F/M	41/17
Median age, years (range)	38 (16–75)
Median platelet count, $\times 10^9$ L^{-1} (range)	12 (3–82)
No. of patients/total	
Idiopathic TTP	53/58
TTP with associated conditions	5/58
ADAMTS13:Ac ($< 10\%$)/*n*	58/58
ADAMTS13 functional inhibitor/*n*	52/58
ADAMTS13:Ag (ng mL^{-1})	
Undetectable (< 65)	14
65–100	10
101–740	32
Normal levels (740–1420)	2
IgG anti-ADAMTS13/*n*	58/58
IgM anti-ADAMTS13/*n*	4/58
IgA anti-ADAMTS13/*n*	10/47

n, number of patients tested; ADAMTS13:Ac, ADAMTS13 activity; ADAMTS13:Ag, ADAMTS13 antigen.

Remission was defined as a normal platelet count ($> 150 \times 10^9$ L^{-1}) and no plasma exchange treatment for 30 consecutive days (day 1 of remission) or more. Relapse or recurrence was defined as the reappearance of clinical manifestations and/or laboratory data compatible with TTP after remission had been achieved.

Plasma samples Frozen plasma samples from TTP patients were obtained from the Central Haematology Laboratory, University of Bern, Switzerland (Center 1) and Service d'Hematologie biologique, Hôspital Antoine Béclère, Paris, France (Center 2). Patients were enrolled after giving consent according to the Declaration of Helsinki. This study was approved by the institutional review board.

Determination of the activity, inhibitors and antigen of ADAMTS13

ADAMTS13 activity (ADAMTS13:Ac) and functional ADAMTS13 inhibitors were measured in patients' plasma at Centers 1 and 2 as previously described [10,11]. ADAMTS13 antigen (ADAMTS13:Ag) levels were analyzed in patients' plasma by ELISA [12].

Determination of total IgG, IgG subclass, IgM, IgA and IgE anti-ADAMTS13 antibodies by ELISA

Total IgG (IgG_{tot}) and IgG subclass, IgM, IgA and IgE anti-ADAMTS13 antibodies were analyzed by ELISA as previously described [7], with minor modifications. Briefly, microtiter plates (Nunc-Immuno Maxisorp, Roskilde, Denmark) were coated with anti-His tag antibody (2 μg mL^{-1}) (Penta-His; Qiagen, Hilden, Germany). The non-specific binding sites were blocked. Recombinant His-tagged ADAMTS13 (2 μg mL^{-1}) was added. Diluted patients' plasma and negative controls (pooled normal human plasma; Baxter BioScience, Vienna, Austria) were incubated overnight at 4 °C. Bound antibodies were detected using an alkaline phosphatase (AP)-conjugated goat anti-human IgG, IgM, IgA or IgE antibody (Sigma, St Louis, MO, USA) or mouse monoclonal anti-human IgG_1, IgG_2, IgG_3 and IgG_4 antibodies (Zymed Laboratories, San Francisco, CA, USA). Finally, the enzyme substrate *p*-nitrophenylphosphate (Sigma) was added, and the absorbance was read at 405 nm with a reference filter of 620 nm on an iEMS microplate reader (Labsystems, Helsinki, Finland). Between each step, the plates were washed with phosphate-buffered saline containing 0.1% (v/v) Tween-20 (pH 7.4).

The specificity of the AP-conjugated mouse monoclonal anti-human IgG_1–IgG_4 and goat anti-human IgE antibody detection was checked. No cross-reactivity was observed at any concentration with any class or subclass of antibodies.

Data analysis and statistical calculations

IgG_{tot} and IgG subclass, IgM, IgA and IgE anti-ADAMTS13 antibodies were expressed as antibody titers, and the cut-off

levels were calculated as previously described [7]. Samples above the cut-off level were judged to be positive. The IgG subclass optical density (OD) values were normalized by the corresponding normalization factor, calculated as described below. To ensure specificity of the anti-ADAMTS13 antibody detection, the positivity of samples was confirmed by competitive inhibition experiments, using purified recombinant ADAMTS13.

Quantification and normalization of the absorbances measured in IgG subclass ELISA

We used ELISA with subclass-specific antibodies to estimate the relative amounts of the different IgG subclasses, first because of the absence of a true standard, and second because of the differences in the development time between the IgG subclasses due to differences in the detection sensitivities of the secondary antibodies used. To minimize these differences, the relative OD values of the IgG subclass ELISA were compared under standardized conditions.

The time-dependent OD signal generated from each IgG subclass (purified human IgG_1–IgG_4, lambda chain; Sigma) coated on an ELISA plate in a concentration range of 0.125–1 μg mL^{-1} was analyzed. The corresponding OD values generated were recorded every 5 min up to 75 min, and plotted against time. Slopes for each concentration and subclass were calculated. The corresponding means ± standard deviations of the slopes from four independent experiments were plotted and analyzed by linear regression for each individual IgG subclass. The regression parameters obtained were used to calculate the OD values of each subclass at a coating concentration of 0.5 μg mL^{-1} (linear range). The OD value of IgG_4 was set to '1', resulting in normalization factors for IgG_1, IgG_2 and IgG_3 of 9, 0.7 and 0.4, as compared with IgG_4. Coating efficiency was evaluated in each experiment by ELISA using an AP-conjugated goat anti-human lambda light chain antibody (Sigma) to ensure uniform IgG_1–IgG_4 concentrations.

The calculated normalization factors were used to normalize the OD values in patients with more than one IgG subclass of anti-ADAMTS13 antibodies. Samples were normalized by multiplying the OD values corresponding to each positive subclass by the corresponding calculated normalization factor. With this approach, an estimate was obtained of the relative contributions of the different IgG subclasses to the total anti-ADAMTS13 IgG in patient plasma. Without the normalization procedure, IgG_1 levels would have been underestimated as compared with IgG_2–IgG_4 levels, as inferred from the normalization factor values. Samples with normalized OD values above the linear range were excluded (six patients) from analyses.

Positive normalized OD values of each subclass were summed (IgG_{sum}, total absorbance), and the proportion of each individual subclass was calculated as a percentage of the total absorbance (assumed to be 100%) to obtain the IgG subclass percentage distribution in a single patient.

Statistical analysis

Statistical analyses were performed with SigmaStat version 3.5 (Systat Software, San Jose, CA, USA). Data are presented as the median and the interquartile range (IQR). The statistical significance of the differences between medians of IgG_1 and IgG_4 values were assessed by the Mann–Whitney rank sum test, and the correlation by the Spearman rank test. To assess the strength of the relationship between IgG_4 levels and recurrence, 40 patients were divided into three groups according to clinical outcome. The first group comprised 18 patients who had experienced only a single TTP event, the second comprised nine patients who relapsed during the 36 months of follow-up, and the third comprised 13 patients who had had relapse(s), five of whom had had no further relapses during follow-up, and eight of whom were lost to follow-up after the acute relapsing event. Deceased patients, patients lost to follow-up after the first TTP event and patients with IgG_4 OD values above the linear range were excluded from this analysis (18 patients). *P*-values < 0.05 were considered to be statistically significant.

Results

ADAMTS13:Ac, functional ADAMTS13 inhibitor and ADAMTS13:Ag levels

All patients ($n = 58$) had ADAMTS13:Ac values < 10%. The corresponding ADAMTS13:Ag levels showed a variable distribution, from undetectable to normal levels (Table 1), similar to earlier findings [12]. A functional inhibitor was found in 52 patients (89%; Table 1).

Anti-ADAMTS13 antibody profiles

Analysis of the anti-ADAMTS13 antibody profile in acquired TTP patients revealed anti-ADAMTS13 IgG antibodies in all of the patients, including the six with severe ADAMTS13 deficiency without functional inhibitors. Titers ranged from 20 to 6400. Additionally, low titers of anti-ADAMTS13 IgM antibodies were detected in four patients (7%) and of anti-ADAMTS13 IgA antibodies in 10/47 patients (21%), with titers ranging from 200 to > 10 000 (Table 1). The anti-ADAMTS13 IgA antibody titer was not determined in 11 patients, owing to insufficient plasma.

Subclass distribution of IgG anti-ADAMTS13 antibodies

The subclass distribution of anti-ADAMTS13 IgG antibodies was analyzed in 58 patients with acquired TTP: 44 during the first episode, and 14 during relapse. IgG_4 was detected in 90% of the patients (52/58, 90%), followed by IgG_1 (30/58, 52%), IgG_2 (29/58, 50%), and IgG_3 (19/58, 33%) (Fig. 1A).

When patients were subdivided by clinical outcome, 38/44 (86%) patients analyzed during the first event had IgG_4 antibodies. IgG_4 was found either alone (8/38, 21%) or with

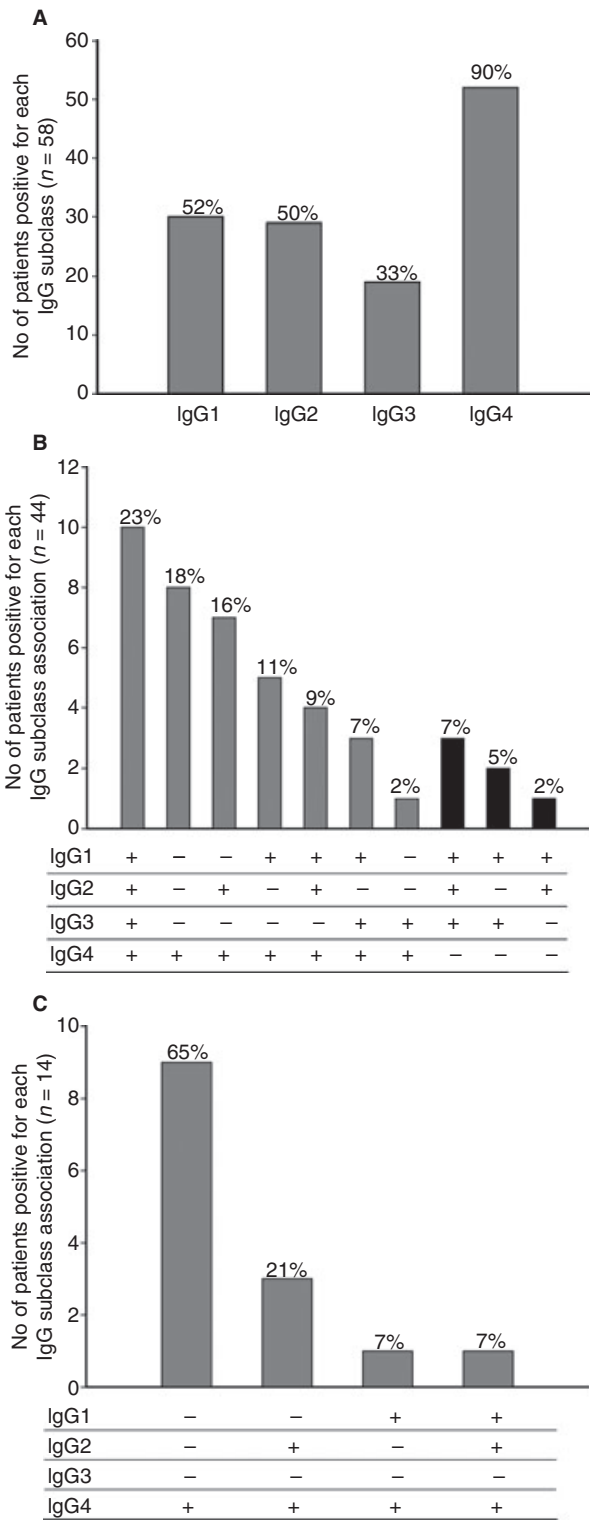

Fig. 1. Subclass distribution of anti-ADAMTS13 IgG antibodies in patients with acquired thrombotic thrombocytopenic purpura. Fifty-eight patients with acute acquired TTP were analyzed for different IgG subclasses. (A) The histogram represents the overall frequencies of the four anti-ADAMTS13 IgG subclasses. (B) Patients analyzed during the first TTP episode ($n = 44$). (C) Patients analyzed during a relapsing event ($n = 14$). (B, C) Histograms showing the frequencies of patients positive for each IgG subclass combination. Plus signs denote the presence of the subclass; minus signs denote the absence of the subclass.

other IgG subclasses (30/38, 79%). Among the 30 patients in whom IgG_4 was found with other IgG subclasses, the association with all four IgG subclasses (10/30, 33%) was the most prevalent finding, followed by associations with IgG_2 (7/30, 23%), IgG_1 (5/30, 17%), and IgG_3 (1/30, 3%). IgG_4 was also found with IgG_1 and IgG_2 (4/30, 13%) and with IgG_1 and IgG_3 (3/30, 10%) (Fig. 1B). No IgG_4 was detected in 6/44 (14%) TTP patients who had predominantly IgG_1 with IgG_3 ($n = 2$), IgG_2 ($n = 1$), or IgG_2 and IgG_3 ($n = 3$) (Fig. 1B).

The IgG subclass distribution in 14 patients analyzed during an acute relapsing event revealed IgG_4 as the predominant IgG subclass (14/14, 100%); IgG_4 was found either alone (9/14, 65%) or associated with IgG_2 (3/14, 21%), IgG_1 (1/14, 7%), or IgG_1 and IgG_2 (1/14, 7%) (Fig. 1C).

Patients having TTP associated with pregnancy ($n = 2$) and the postpartum state ($n = 1$) had IgG_4 accompanied by IgG_2, IgG_1, or IgG_3. The patient with TTP associated with SLE had all four IgG subclasses, whereas the patient with TTP associated with cancer had no IgG_4.

The IgG subclass distribution of 4/6 patients without detectable functional inhibitors showed IgG_4 accompanied by IgG_2, IgG_3, or IgG_1 and IgG_3, or all of them. The remaining two patients had no IgG_4, with IgG_1 being the most representative subclass (Fig. 2A; individual patient numbers 21, 29, 31, 41, 48, and 52).

To assess the contribution of any subclass to IgG_{sum}, we calculated the relative concentration of each IgG subclass in a patient. Six of 58 patients were excluded from this calculation because their IgG_4 OD values were outside the linear range. IgG_4 was the most abundantly produced subclass, with levels ranging from 1% to 100% (Fig. 2A). IgG_1 was the second most common, with levels ranging from 11% to 92 %, whereas IgG_2 and IgG_3 formed only a small percentage of IgG_{sum} (levels ranging from 4% to 26% and 1% to 30% for IgG_2 and IgG_3, respectively) (Fig. 2A). In contrast to IgG_4, IgG_1 was never found as a single subclass; however, in the absence of IgG_4, IgG_1 constituted more than 85% of IgG_{sum}. The presence and abundance of IgG_4 and IgG_1 were inversely correlated ($r^2 = -0.927$, $P < 0.01$) (Fig. 2A). Patient numbers 1–3, 6–8, 11, 13, 16, 18, 25, 26 and 38 represent those patients analyzed during an acute relapsing event (Fig. 2A,B).

IgE anti-ADAMTS13 antibodies were also assayed, because production of IgG_4 and IgE is induced by the same cytokines [interleukin (IL)-4 and IL-13], released by T-helper 2 (Th2) cells [13]. None of the 17 patients with IgG_4 as the only positive subclass had IgE antibodies against ADAMTS13, excluding a pathogenic role of IgE autoantibodies in TTP as described in, for example, bullous pemphigoid [14].

Relationship between the IgG subclass profile and the total anti-ADAMTS13 IgG antibody titers and ADAMTS13:Ag levels

When IgG_{tot} was related to the IgG subclass profile, patients with IgG_4 as the only subclass showed lower IgG_{tot} titers (range 20–200) than patients with IgG_4 combined with other

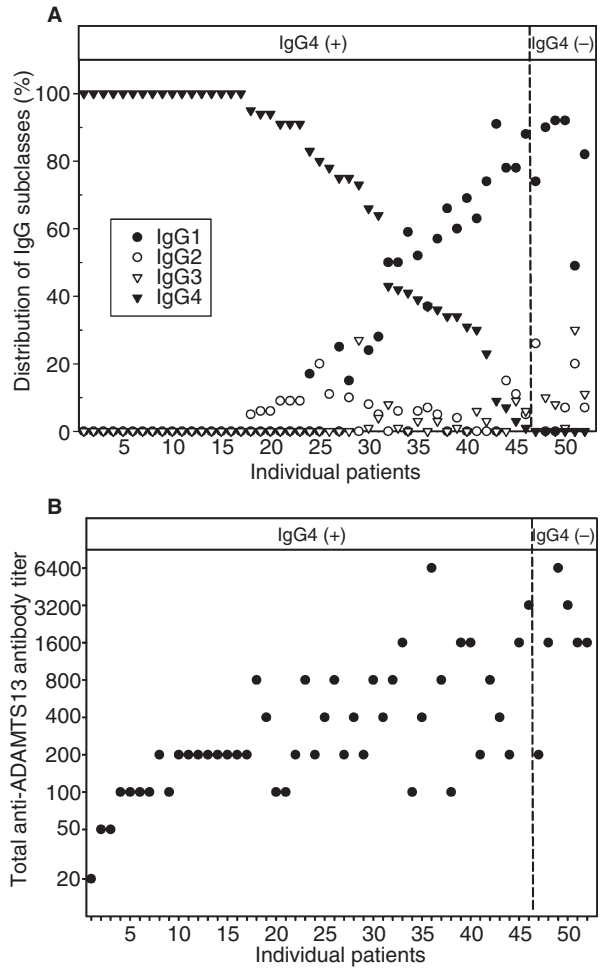

Fig. 2. Relative concentrations of anti-ADAMTS13 IgG subclasses and total IgG anti-ADAMTS13 antibody titers within a patient. (A) The relative concentrations of the different IgG subclasses were calculated for plasma of 52 TTP patients. The individual IgG subclass distribution of each patient is shown. Patients are numbered 1 to 52 according to decreasing levels of IgG_4. IgG_4 and IgG_1 are the predominant IgG subclasses. (B) Total IgG anti-ADAMTS13 antibody titers corresponding to each patient [numbered from 1 to 52 as in (A)]. In both (A) and (B), individual patient numbers are shown in steps of five, and patients 21, 29, 31, 41, 48 and 52 have no detectable functional inhibitors.

IgG subclasses (Fig. 2B). The combination of IgG_4 with one, two or more IgG subclasses was associated with the highest IgG_{tot} titers (range 100–6400). This was even more pronounced in patients with undetectable IgG_4 (range 1600–6400). Patients without detectable functional inhibitors generally had low IgG_{tot} antibody titers (100–400), except for two patients who had undetectable IgG_4 (1600 for both) (Fig. 2B).

The relationship between ADAMTS13:Ag levels and the IgG subclass profile was variable, with a trend towards lower ADAMTS13:Ag levels with increasing numbers of IgG subclasses. Patients with the lowest IgG_4 levels (< 10%) or highest IgG_1 levels (> 85%) had significantly lower ADAMTS13:Ag levels than patients with the highest IgG_4 levels or IgG_4 as the only subclass present ($P < 0.01$ and $P < 0.05$, respectively; the six patients with IgG_4 OD values outside the linear range were excluded from this analysis).

Subclass distribution of anti-ADAMTS13 IgG antibodies and clinical outcome

Four patients ($n = 4$) who had very low (< 5%) or undetectable IgG_4 with high levels of IgG_1 died during the first acute TTP event (Table 2). These patients also showed high anti-ADAMTS13 IgA antibody titers, three of them in combination with anti-ADAMTS13 IgM antibodies.

The association between levels of IgG_4 and IgG_1 and the recurrence of TTP was analyzed in 40 patients, who were divided into three groups: those who had experienced only a single TTP episode [group 1; IgG_4 and IgG_1 median values were 44% (IQR 30–85) and 50% (IQR 15–63), respectively]; those who relapsed during the 36 months of follow-up [group 2; IgG_4 and IgG_1 median values were 91% (IQR 72–95.5) and 0% (IQR 0–15), respectively]; and those who had had relapse(s) before [group 3; IgG_4 and IgG_1 median values were 100% (IQR 91.2–100) and 0% (IQR 0–0), respectively] (Fig. 3) (for inclusion criteria, see Materials and methods, Statistical analysis). A second analysis was performed in parallel, in which the six patients with IgG_4 OD values outside the linear range (most likely with underestimated IgG_4 levels) were included. Three of these patients were included in

Table 2 ADAMTS13 laboratory findings and clinical outcome in patients with very low (< 5%) or undetectable levels of IgG_4 and high levels of IgG_1

Patient no.*	ADAMTS13:Ac (U mL^{-1})	ADAMTS 13 inhibitor	ADAMTS 13:Ag (ng mL^{-1})	IgG anti-ADAMTS 13 (titer)	IgM anti-ADAMTS 13 (titer)	IgA anti-ADAMTS 13 (titer)	IgG_1 (%)	IgG_4 (%)	Outcome
49	< 0.05	+ + +	< 65	6400	3200	> 10 000	92	0	Death
50	< 0.05	+	< 65	3200	0	0	92	0	Remission
48	< 0.05	–	< 65	1600	0	> 10 000	91	0	Death
52	< 0.05	–	< 65	1600	50	800	85	0	Death
46	< 0.05	+	< 65	3200	0	3200	88	1	Remission
45	< 0.05	+	83	1600	20	3200	78	3	Death
47	< 0.05	+	560	200	0	0	71	0	Remission

ADAMTS13:Ac, ADAMTS13 activity; ADAMTS13:Ag, ADAMTS13 antigen. *Patients' numbers correspond to the individual numbers in Fig. 2. All patients had idiopathic thrombotic thrombocytopenic purpura.

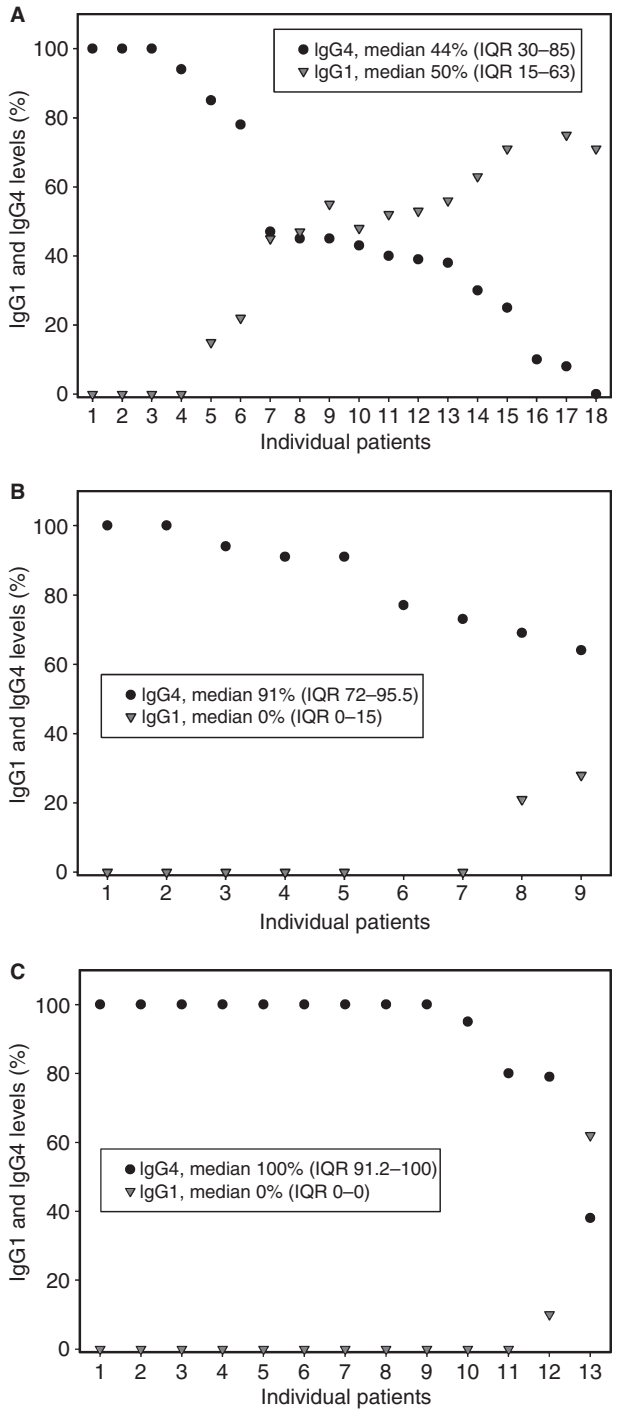

Fig. 3. Levels of IgG_1 and IgG_4 anti-ADAMTS13 antibodies in patients with acute acquired thrombotic thrombocytopenic purpura (TTP) patients. (A) Patients who had experienced only a single TTP event. (B) Patients who relapsed during the 36 months of follow-up. (C) Patients who had had relapse(s), including patients without further relapses during follow-up and patients lost to follow-up after the acute relapsing event. IQR, interquartile range.

group 1, two in group 2, and one in group 3. High levels of IgG_4 were associated with relapse ($P = 0.03$ and $P = 0.001$ for comparison between groups 1 and 2, and groups 1 and 3, respectively). Similar *P*-values were obtained when patients with IgG_4 OD values outside the linear range were included ($P = 0.017$ and $P < 0.001$ for comparison between groups 1 and 2, and groups 1 and 3, respectively).

Likewise, patients with undetectable or very low IgG_1 levels experienced more TTP events than patients with detectable (moderate or low) levels ($P = 0.005$ and $P < 0.001$ for comparison between groups 1 and 2, and groups 1 and 3, respectively). *P*-values of 0.004 and < 0.001 for comparison between groups 1 and 2, and groups 1 and 3, respectively were obtained when patients with IgG_4 OD values outside the linear range were included.

Taken together, our findings suggest that patients with high levels of IgG_4 and undetectable IgG_1 are more prone to relapse than patients with low levels of IgG_4 and detectable IgG_1.

Discussion

We investigated the IgG subclass distribution of anti-ADAMTS13 IgG antibodies in patients with acquired TTP, using an ELISA as described [7], but slightly modified, with anti-human IgG_1–IgG_4 antibodies as detecting antibodies. We reasoned that, because the various IgG subclasses differ in their biological functions, the characterization of the subclass profile of anti-ADAMTS13 antibodies could provide insights into the pathophysiology of acquired TTP.

IgG_4 was the only subtype detected in a remarkable number of patients (17/58), implying a dominant role for IgG_4 in this autoimmune disease. The second most frequent subtype was IgG_1, and IgG_2 and IgG_3 were less represented. Our findings indicate that the autoimmune response against ADAMTS13 is polyclonal and heterogeneous.

IgG_1 and IgG_4 also predominated in patients with inhibitors in acquired and in congenital hemophilia A [15], in blistering skin diseases [16], and in other autoimmune diseases [17]. Interestingly, and inexplicably, absolute levels of IgG_1 and IgG_4 were always inversely correlated.

IgG_4 is predominantly produced after prolonged antigenic stimulation [18]. The strong bias towards production of IgG_4 autoantibodies against ADAMTS13 therefore suggests chronic antigenic stimulation of the immune system when systemic tolerance mechanisms fail.

Although our study is limited by the number of patients, and further studies are needed, we observed that patients are less likely to survive their first TTP event (four of seven patients died) if they have IgG_1 and very low or undetectable IgG_4 levels plus higher titers of other classes of anti-ADAMTS13 antibodies (particularly IgA). Our findings are supported by a recent report of a TTP patient with high IgG_1 levels who died during a relapsing event, although IgG_4 antibodies were not investigated [19]. IgG_1 is known to activate the classical complement pathway, and binds to activating FcγRs expressed on innate immune effector cells, whereas IgG_4 fails to activate complement, and binds to cellular FcγRs with less efficiency than IgG_1 [9]. Furthermore, IgA antibodies may also contribute to the severity of the pathogenesis, because they have been shown to activate complement via the mannan-binding lectin

pathway [20], favoring an enhancement of complement-mediated inflammation.

IgG_4 antibodies are thought to be functionally monovalent [18,21] through a process described as '*in vivo* Fab arm exchange of IgG_4 half-molecules', in which a heavy–light chain pair of one IgG_4 molecule joins with another, unrelated, half IgG_4 molecule, leading to a newly combined IgG_4 molecule with two different antigen-binding sites [22]. Such hybrid molecules are unable to crosslink antigen and, consequently, to form large immune complexes. However, only large immune complexes can crosslink FcγRs, thereby activating innate immune effector cells [23]. Functional monovalency might decrease the pathologic potential of IgG_4 antibodies and might, during contact with antigen, result in only small and relatively harmless immune complexes with a low potential for induction of immune activation [24]. Although anti-ADAMTS13 IgG_4 antibodies have yet to be shown to be functionally monovalent and to interfere with complement activation *in vivo*, it is tempting to speculate that IgG_4 autoantibodies, to some extent, act as 'protective' antibodies in TTP patients, inducing a milder and treatable form of TTP, whereas IgG_1 (and IgA) anti-ADAMTS13 antibodies might have higher pathogenic potential, particularly in the absence of IgG_4.

Relapse occurs in 30–50% of the patients who survive an initial episode of TTP, being more frequent during the first year after the onset [25,26]. *S*everal studies have investigated the significance of ADAMTS13 activity and inhibitors in predicting outcomes. Most concluded that severe ADAMTS13 deficiency and the presence of antibodies against ADAMTS13 at presentation and/or remission are associated with a higher risk of disease recurrence [8,25,27]. We investigated the possibility of an association between IgG subclasses and relapse, and found that high levels of IgG_4 with undetectable IgG_1 were statistically significantly associated with a trend towards recurrence of TTP. This suggests that high levels of IgG_4 could help to identify patients who are at risk of recurrence, and could be used, in association with ADAMTS13 activity and inhibitors, as a prognostic marker to predict possible relapse.

In conclusion, we found that the subclass profile of anti-ADAMTS13 IgG antibodies in patients with acquired TTP is dominated by IgG_4 and IgG_1 antibodies with inversely correlated titers. An immune response characterized by high levels of IgG_4 predicts, at least partially, a more treatable form of TTP than if IgG_1 antibodies are present. We conclude that IgG_4 levels could be a recurrent predicting biomarker, although prospective trials with well-characterized patients are needed to substantiate our preliminary observations. The contribution made by IgG_4 and IgG_1 autoantibodies to the pathologic mechanism in acquired TTP needs further investigation.

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Disclosure of Conflict of Interests

S. Ferrari, M. Rieger and F. Scheiflinger are employees and G. C. Mudde is a former employee of Baxter BioScience. J. A. Kremer Hovinga receives an honorarium as a consultant for Baxter BioScience.

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Inverse correlation of free and immune complex-sequestered anti-ADAMTS13 antibodies in a patient with acquired TTP

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Acquired thrombotic thrombocytopenic purpura (TTP) is characterized by the presence of neutralizing and/or non-neutralizing anti-ADAMTS13 autoantibodies which are predominantly of the IgG class and less frequently of the IgM and/or IgA classes [1-4]. However, the extent to which these antibodies circulate in plasma in their free form or as a complex with ADAMTS13 and how this proportion changes over time is unclear. We and others have recently reported indirect detection by an ADAMTS13 antigen (ADAMTS13:Ag) ELISA of IgG ADAMTS13-specific immune complexes (ICs) in plasma from patients with acquired TTP [5,6]. In the present study we used another more direct method for detecting ICs and compared the levels of free anti-ADAMTS13 antibodies, ADAMTS13:Ag, ADAMTS13 activity (ADAMTS13:Ac) and inhibitor with those of ICs.

Samples from a patient with acquired idiopathic TTP, whose case history has in part been described [1], were analyzed. Treatment modalities of this patient are summarized in Fig. 1A. The patient presented with undetectable ADAMTS13:Ac and ADAMTS13:Ag, but also without ADAMTS13:Ac inhibition as determined by the CBA assay [1,5,7]. Re-assessment of the inhibitor with the more sensitive fluorometric FRETS-VWF73 assay [7] revealed low level inhibition of ADAMTS13:Ac, corresponding to ~0.8 BU/mL.

Fig. 1B summarizes the dynamic course of ADAMTS13:Ac, ADAMTS13:Ag, and ADAMTS13 inhibitor during the 64 days' follow-up until the patient's death. Analyses of specific IgG, IgM and IgA anti-ADAMTS13 antibodies by ELISA [8] detected IgG and IgA antibodies at most time points, whereas IgM antibodies were only measurable until day 12 (Fig. 1C-E, Supplementary Fig. 1). The anti-ADAMTS13 IgG subclass profile on admission (82% IgG1, 7% IgG2, 11% IgG3, no IgG4) did not change significantly during the course of the disease (not shown). The IgG and IgA

antibody titers decreased during periods of transient response to plasma exchange (PEX) treatment, but this drop was not always accompanied by a recovery in ADAMTS13:Ac and ADAMTS13:Ag levels. This lack of ADAMTS13 activity recovery and the presence of high levels of IgG1 combined with undetectable IgG4 have been described as indicators of poor prognosis and increased risk of mortality in patients with acquired TTP [6,8] and may, at least in part, explain the poor outcome of the patient.

The presence of ICs was demonstrated by co-immunoprecipitation of ADAMTS13 with immunoglobulins, using protein G for the isolation of total IgG, and anti-IgA- and anti-IgM-specific affinity matrixes for isolation of total IgA and IgM, respectively (Fig. 1C-E). Interestingly, ADAMTS13-specific IgG-ICs were detectable in significant amounts at most times during the 2-month follow up, particularly when the free IgG antibody titers and inhibitor were low. By contrast, IgG-ICs became almost undetectable when the free IgG antibody titers and ADAMTS13 inhibitor were high (Fig. 1B, C). Thus, IgG-ICs followed an inverse kinetics to both the free IgG antibody in plasma and the inhibitory titer. Similar trends were seen for the IgA- and IgM-specific ICs, but with an apparently lower total concentration than IgG-ICs (Fig. 1D, E). Notably, low amounts of IgM-ICs were detected at most time points although no free IgM antibodies were detected after day 12 (Fig. 1E), suggesting that all free IgM antibodies had been complexed by ADAMTS13. Overall it appears that anti-ADAMTS13 antibodies are not detected by ELISA when they are part of an immune complex and the inhibitor titer measured correlates with the amount of free anti-ADAMTS13 antibodies.

In the patient studied, samples from most time points were devoid of free ADAMTS13:Ag but contained circulating ICs, indicating that ADAMTS13, either newly synthesized or exogenously administered by plasma infusion during PEX, had been quantitatively sequestered through formation of ICs. ADAMTS13:Ag was also undetectable in samples that had high levels of free anti-ADAMTS13 antibodies but lacked circulating ICs (Fig. 1; days 8-11 and 16-21), suggesting that ICs that had formed were already cleared at these time points.

Considering that the patient was receiving daily PEX, the plasma concentration of antibodies and antigen changed continuously throughout therapy, thereby influencing the dynamic processes of formation, biological activity, deposition and clearance of ICs. Under normal conditions, ICs are thought to be quickly cleared by the reticulo-

endothelial system, and only a continuous formation eventually exceeds the clearance capacity of the system, causing a build-up of ICs in the circulation. There, IgM- or IgG-containing ICs can either activate the complement classical pathway or bind to cellular Fc receptors [9,10]. Circulating IgA-ICs have the potential to activate complement via the mannan-binding lectin pathway and immune cells when binding to Fcα receptors. These features can lead to endothelial cell activation, promoting inflammation and disturbing the hemodynamic flow, which in turn may contribute to enhanced VWF-dependent platelet adhesion and aggregation. The continuous presence of excessive amounts of circulating ICs might therefore perpetuate a pro-inflammatory state promoting thrombosis and predisposing to relapse.

In conclusion, our study on the dynamic course of the ADAMTS13-specific autoantibody response in a patient with refractory TTP suggests that in addition to a characterization of the whole panel of autoantibodies against ADAMTS13, circulating ADAMTS13-specific ICs also need to be determined to obtain a full picture of the status of the disease. Formation of ADAMTS13-specific ICs capturing all freely available ADAMTS13 should be particularly considered in patients who are refractory to treatment. Future studies on a representative cohort of patients with acquired TTP will show whether our observation of an inverse correlation of free and IC-sequestered anti-ADAMTS13 antibodies in a single patient is a general phenomenon.

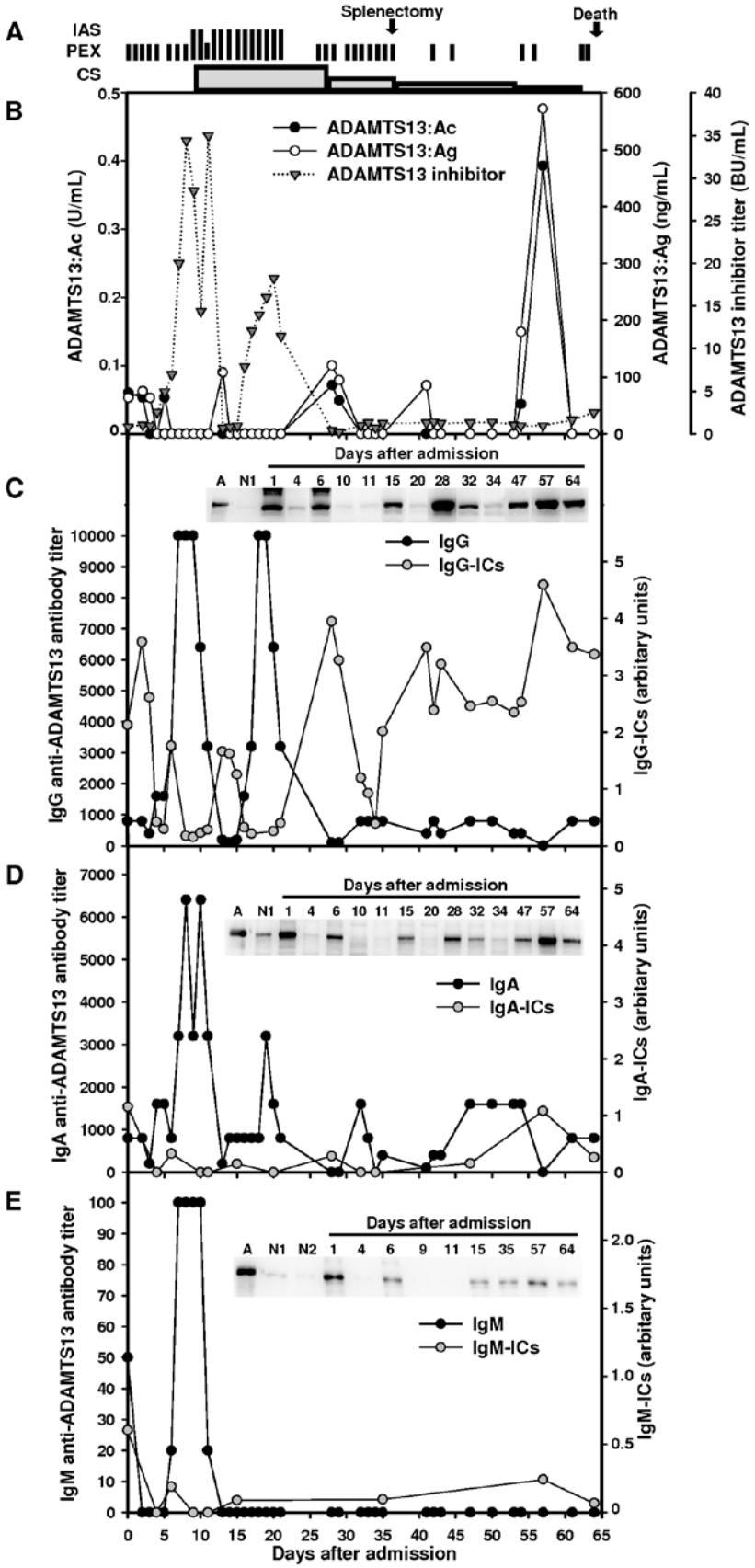

Figure 1. Course of ADAMTS13-related variables in a patient with acquired idiopathic TTP. (A) Treatment regimen over 64 days until the patient's death. IAS,

immunoadsorption (on staphylococcal protein A Sepharose columns); PEX, plasma exchange (40 mL fresh-frozen plasma/kg body weight); CS, corticosteroids (prednisone 100,50,25,12.5 mg/day). The date of splenectomy is also indicated. The following ADAMTS13-related variables were determined at the time points indicated: (B) ADAMTS13:Ac (FRETS-VWF73, U/mL), ADAMTS13:Ag (ng/mL, reference range 740–1420 ng/mL) and ADAMTS13 inhibitor (BU/mL); (C) IgG anti-ADAMTS13 antibodies (titer) and IgG-ICs (arbitrary units), (D) IgA anti-ADAMTS13 antibodies (titer) and IgA-ICs (arbitrary units); (E) IgM anti-ADAMTS13 antibodies (titer) and IgM-ICs (arbitrary units). ADAMTS13-specific ICs were analyzed by co-immunoprecipitation of ADAMTS13 with either Protein G Sepharose or anti-IgA- or anti-IgM-specific affinity matrixes. Plasma-bound antibodies were eluted and co-immunoprecipitated ADAMTS13 was analyzed by Western blot using an affinity-purified polyclonal rabbit IgG anti-human ADAMTS13 antibody. The relative amount of ICs (expressed in arbitrary units) was quantified by densitometry analysis and rADAMTS13 (A; 1 ng) as standard. Two pooled normal human plasmas (N1, N2) were used as a negative control. The insets in Fig. 1C-E show a section of the respective blots. The entire blots and a detailed description of the method are depicted in Supplementary Fig. 1.

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Supporting Information

Figure S1

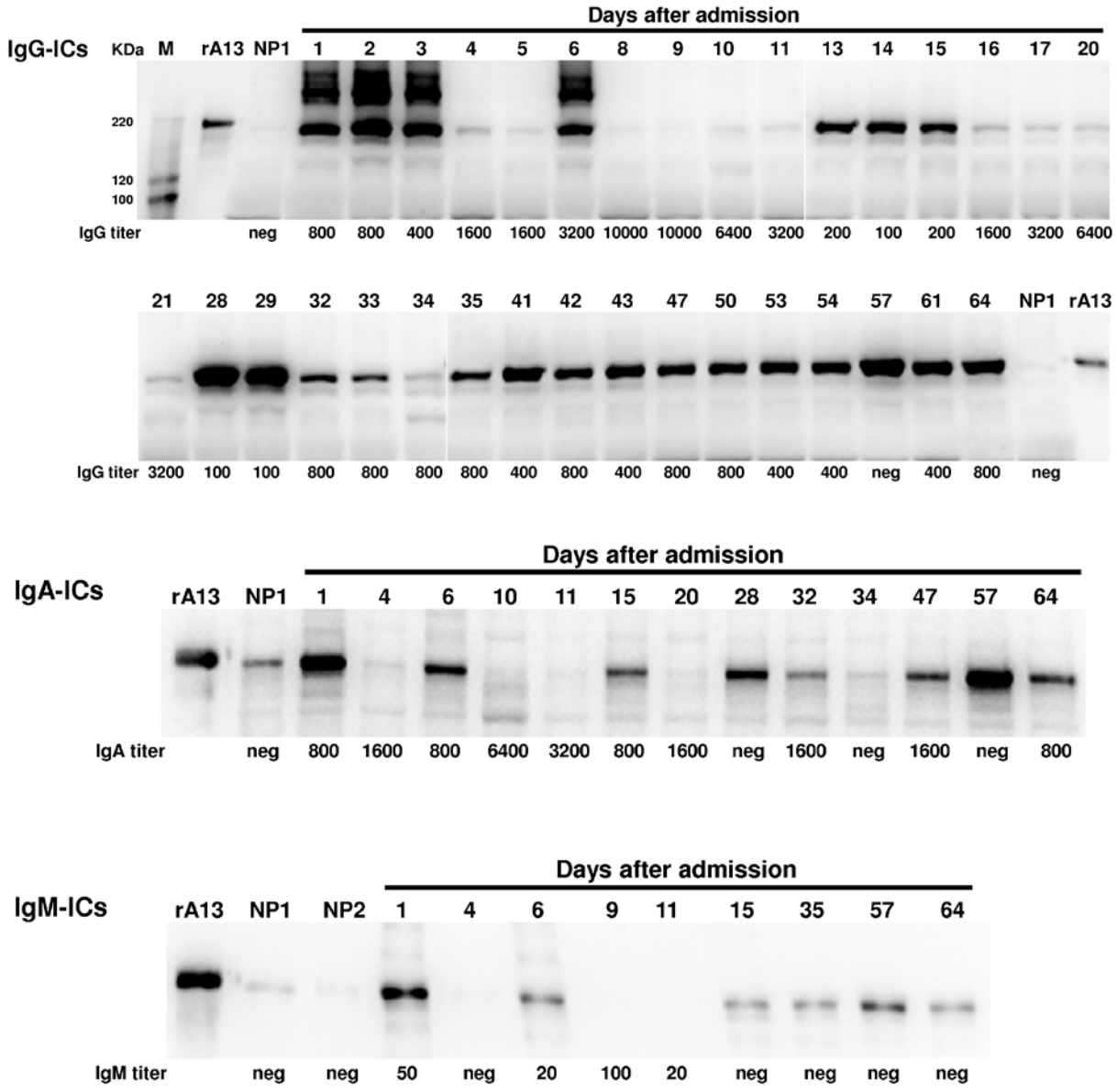

Figure S1. Detection of circulating ADAMTS13-specific ICs at different time points by co-immunoprecipitation and Western blotting. Circulating ADAMTS13-specific ICs were analyzed by co-immunoprecipitation of ADAMTS13 with either Protein G Sepharose (GE Healthcare, Buckinghamshire, England) or anti-IgA- or anti-IgM-specific affinity matrixes (BAC CaptureSelect, Naarden, The Netherlands). Patient plasma (200 µl) was incubated with 100 µl of beads at room temperature for 60 min. Bound antibodies were eluted by adding sample loading buffer (Thermo, Rockford, IL, USA) and heating at 95°C for 10 min. The co-immunoprecipitated ADAMTS13 was analyzed by Western blot using an affinity purified polyclonal rabbit IgG anti-human ADAMTS13 antibody (Baxter Innovations, Vienna, Austria) in

combination with a peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) and a chemiluminescent substrate (Thermo). Immunoreactive bands were visualized with the Fusion FX7 image system (Vilber Lourmat, Germany) and the relative amount of ICs (expressed in arbitrary units) was quantified by densitometry analysis and rADAMTS13 (rA13; 1 ng) as standard. Pooled normal human plasma (NP) was used as a negative control. The average background signal intensity from the NP was subtracted from the signal intensities of the samples. M denote molecular marker. Each lane contains equal amounts of total IgG. The anti-ADAMTS13 antibody (IgG, IgA and IgM) titers of the samples are shown below the diagram.