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Original Article

Pathogenicity of Anti-ADAMTS13 Autoantibodies in Acquired Thrombotic Thrombocytopenic Purpura☆

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ABSTRACT

Background: Acquired thrombotic thrombocytopenic purpura (TTP) is an autoimmune disease in which anti-ADAMTS13 autoantibodies cause severe enzyme deficiency. ADAMTS13 deficiency causes the loss of regulation of von Willebrand factor multimeric size and platelet-tethering function, which results in the formation of disseminated microvascular platelet microthrombi. Precisely how anti-ADAMTS13 autoantibodies, or antibody subsets, cause ADAMTS13 deficiency (ADAMTS13 activity generally <10%) has not been formally investigated.
Methods: We analysed 92 acquired TTP episodes at presentation, through treatment and remission/relapse using epitope mapping and functional analyses to understand the pathogenic mechanisms of anti-ADAMTS13 IgG.
Results: 89/92 of TTP episodes had IgG recognising the ADAMTS13 N-terminal domains. The central spacer domain was the only N-terminal antigenic target detected. 38/92 TTP episodes had autoantibodies recognising the N-terminal domains alone; 54/92 TTP episodes also had antibodies against the ADAMTS13 C-terminal domains (TSP2–8 and/or CUB domains). Changes in autoantibody specificity were detected in 9/16 patients at relapse, suggesting a continued development of the disease. Functional analyses on IgG from 43 patients revealed inhibitory IgG were limited to anti-spacer domain antibodies. However, 15/43 patients had autoantibodies with no detectable inhibitory action and as many as 32/43 patients had autoantibodies with inhibitory function that was insufficient to account for the severe deficiency state, suggesting that in many patients there is an alternative pathogenic mechanism. We therefore analysed plasma ADAMTS13 antigen levels in 91 acquired TTP presentation samples. We demonstrated markedly reduced ADAMTS13 antigen levels in all presentation samples, median 6% normal (range 0–47%), with 84/91 patients having <25% ADAMTS13 antigen. ADAMTS13 antigen in the lowest quartile at first presentation was associated with increased mortality (odds ratio 5.7).
Conclusions: Anti-spacer domain autoantibodies are the major inhibitory antibodies in acquired TTP. However, depletion of ADAMTS13 antigen (rather than enzyme inhibition) is a dominant pathogenic mechanism. ADAMTS13 antigen levels at presentation have prognostic significance. Taken together, our results provide new insights into the pathophysiology of acquired TTP.

1. Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare, life-threatening disorder associated with inherited or, more commonly, acquired deficiency in the plasma metalloprotease, ADAMTS13 (Levy et al., 2001; Fujikawa et al., 2001). Severe ADAMTS13 deficiency (activity generally <10%) results in insufficient processing of von Willebrand factor (VWF) — a critical mediator of normal platelet tethering. ADAMTS13 deficiency results in the accumulation of the most haemostatically active "ultra-large" forms of VWF in plasma. These UL-VWF multimers can unravel during passage through the microcirculation, which precipitates unwanted platelet aggregation and multi-organ microvascular thrombosis. This accounts for the clinical sequelae of TTP, namely thrombocytopenia and microangiopathic haemolytic anaemia, variably with neurological, cardiac, gastro-intestinal and/or renal involvement.

Autoantibodies against ADAMTS13, predominantly immunoglobulin class G (IgG), are present in the majority of acquired TTP patients and cause profound loss of VWF-cleaving function (Hovinga et al., 2010; Ferrari et al., 2007, 2009; Peyvandi et al., 2008). Antibodies that bind the N-terminal domains of ADAMTS13 (herein termed MDTCS) are detected in most patients, although antibodies recognising the C-terminal domains of ADAMTS13 have also been reported (Klaus et al., 2004; Luken et al.,

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2005, 2006; Soejima et al., 2003; Zheng et al., 2010; Pos et al., 2011). However, epitope mapping studies alone do not identify the antibodies that are inhibitory and/or pathogenic. For example, non-inhibitory IgG antibodies that do not impair ADAMTS13 function in vitro may still be pathogenic and compromise VWF processing in vivo (Scheiflinger et al., 2003). Autoantibodies against different ADAMTS13 domains likely inhibit enzyme function to different extents, and may cause deficiency in vivo via distinct mechanisms.

Treatment of acquired TTP involves plasma exchange (PEX) to provide a new source of ADAMTS13. Steroids are used to target the autoimmune component of the disease. Therapy with rituximab reduces rates of recurrence (Scully et al., 2011; Westwood et al., 2013). Recombinant ADAMTS13 is currently undergoing trials for the treatment of inherited TTP. However, its effectiveness in the more prevalent acquired form of the disease (~95% cases), with inhibitory anti-ADAMTS 13 IgG antibodies, is unknown.

In this study, we characterised the repertoire of antibodies in patients with acute idiopathic TTP at presentation and, for the first time, through therapy, remission and relapse, and explored the inhibitory potential and other pathogenic mechanisms of these antibodies. Identification of the pathogenic mechanisms that cause loss of ADAMTS13 activity is critical to our understanding of acquired TTP, as well as potentially for monitoring and treating acquired TTP patients in the future.

2. Methods

2.1. Patients

Citrated plasma samples from a non-sequential cohort of 78 patients with acquired idiopathic TTP referred to our reference centre between 2000 and 2012 were analysed. Presenting samples from 92 acute episodes of TTP were included in the domain-specificity study, of which 43 subsequently underwent IgG extraction for functional analysis. A flowchart of sample distribution of the 92 acute episodes is shown in Fig. 1.

TTP patients were diagnosed clinically based on the combination of microangiopathic haemolytic anaemia with thrombocytopenia and end-organ damage with no other known cause (Scully et al., 2012). Patients with other thrombotic microangiopathies, or TTP secondary to HIV or pregnancy were excluded. All presentation samples were taken before plasma exchange or rituximab were commenced. All patients had severe deficiency in plasma ADAMTS13 activity (<10%), with the exception of one patient with 12% activity, and one with 27% activity (although this sample was taken after plasma infusion had been given) and were positive for anti-ADAMTS13 IgG (Gerritsen et al., 1999; Yarranton et al., 2004; Kokame et al., 2005; Scully et al., 2007).

Patients were selected based on their medium/high anti-ADAMTS13 titre (i.e. >15% using a well-established in-house anti-ADAMTS13 IgG ELISA (Scully et al., 2007)). Through exclusion of patients with low-titre anti-ADAMTS13 antibodies, this cohort of patients was consequently enriched for those that relapsed patients and/or died during a TTP episode, facilitating analysis of the longitudinal humoral response and disease severity. For the purpose of the study, relapse was defined as either clinical relapse, or acute drop in plasma ADAMTS13 activity (to <10%) during follow-up, despite normal routine laboratory parameters, necessitating treatment with elective rituximab. Follow-up ended on May 14th 2014.

Citrated plasma samples were also collected from 67 normal healthy adult volunteers for use as controls. The research was approved by the Research Ethics Committee (08/H0810/54, 08/H0810/54, 08/H0716/72). Informed consent was obtained from all patients and healthy volunteers. Assent was obtained from the patient representatives for those TTP patients that lacked capacity to give informed consent.

2.2. Expression and Purification of ADAMTS13 and VWF Fragments

Full-length ADAMTS13 and fragments; metalloprotease to disintegrin-like domain (MD), metalloprotease to cysteine-rich domain (MDTC), metalloprotease to spacer domain (MDTCS), TSP2–8 domains and CUB1/2 domains were expressed in HEK293 cells and

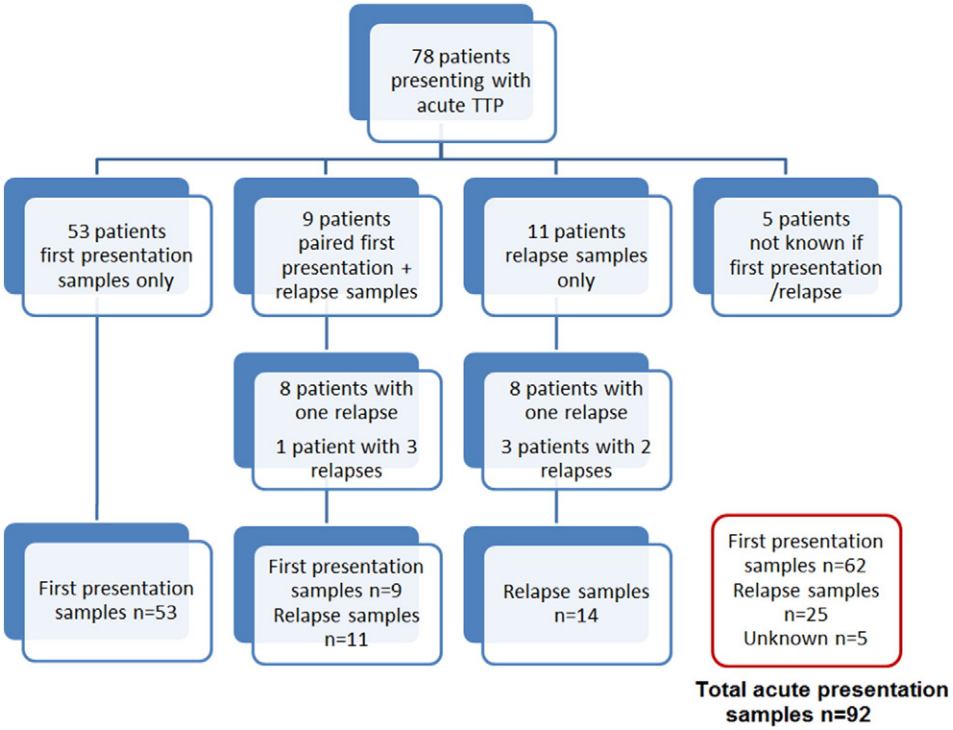

Fig. 1. Flowchart of the 92 acute TTP episodes.

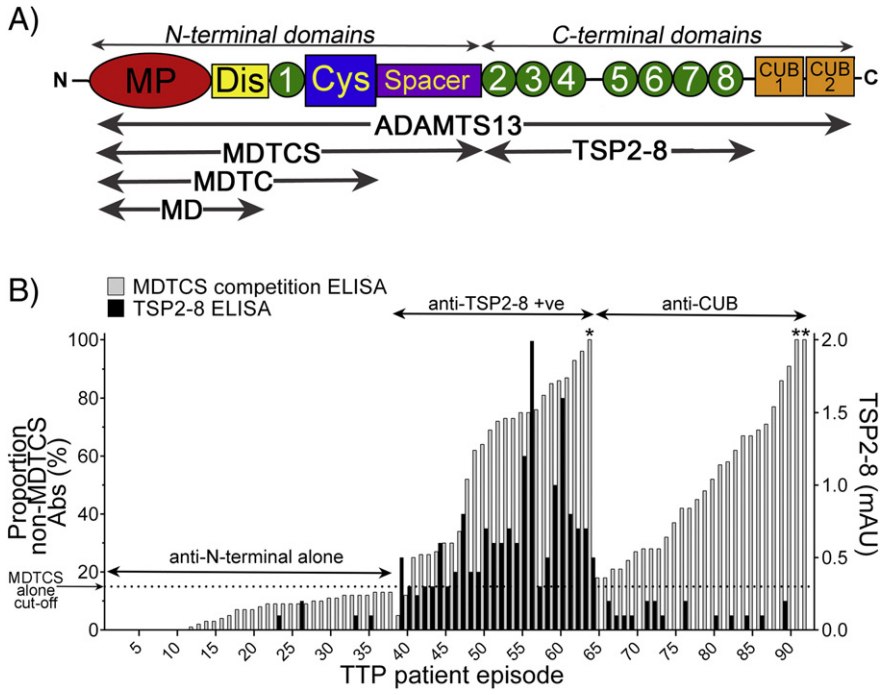

Fig. 2. Domain organisation of ADAMTS13 and domain specificity of anti-ADAMTS13 antibodies at TTP presentation. A) Domain organisation of ADAMTS13 consisting of the metalloprotease domain (MP), disintegrin-like domain (Dis), eight thrombospondin repeats (1–8, green), Cysteine-rich domain (Cys), spacer domain and two C-terminal CUB domains. Underneath are denoted the domain fragments used in this study — the N-terminal domain fragments, MDTCS, MDTC and MD, and the C-terminal TSP2–8. B) Graph depicting summary of anti-ADAMTS13 domain specificity ELISAs for 92 TTP patient episodes. TTP patient plasmas (diluted 1/50) were analysed for IgG that recognised immobilised full length ADAMTS13 both with and without preincubation of plasmas with 10 nM MDTCS. For each patient, the proportion of full length ADAMTS13 binding that MDTCS could not compete for is plotted in grey (left axis; proportion non-MDTCS Abs, %). Plasma samples were also analysed for the presence of anti-TSP2–8 IgG in a separate ELISA plotted in black (right axis: TSP2–8, mAU). All patients were positive for anti-N-terminal antibodies, except samples labelled with *, denoting the three patients in which MDTCS could not compete at all for full-length ADAMTS13 binding. Patients 1–38 are termed anti-N-terminal alone, as MDTCS competed for >85% (dotted line) full length ADAMTS13 binding and these samples exhibited no or very low recognition of TSP2–8. Patients 39–64 are positive for anti-TSP2–8 antibodies. Patients 65–92 are termed anti-CUB, as MDTCS competed for <85% (dotted line) of full length ADAMTS13 binding, consistent with the presence of anti-C-terminal antibodies, but these samples exhibited no or very low recognition of TSP2–8.

purified from conditioned media (Fig. 2A). VWF115 and VWF106 were expressed and purified as previously described (Supplementary Methods) (Pos et al., 2010).

2.3. Domain Specificity of Anti-ADAMTS13 Antibodies in TTP Patient Plasmas

Maxisorb plates (Nunc) were coated with 2.5 μg/ml purified ADAMTS13, MDTCS or TSP2–8 in 50 mM sodium carbonate buffer pH 9.6 and blocked with PBS/2.5% BSA/1% fetal calf serum (FCS) for 2 h. TTP patient, or pooled control plasma were diluted 1/20 to 1/50 in PBS/1% BSA/1% FCS and incubated in duplicate with wells for 1 h. Wells were washed and bound anti-ADAMTS13 antibodies detected with anti-human IgG-HRP (Dako). OPD (Sigma) was used for colour development. In parallel, identical experiments were set up, except that the diluted plasma samples were preincubated with either 20 nM MD, 20 nM MDTC or 10 nM MDTCS for 30 min, before applying to wells coated with full-length ADAMTS13. The ability of these fragments to compete for autoantibody binding to immobilised ADAMTS13 was assessed by comparison to samples that were preincubated without an ADAMTS13 fragment.

Based on the results of these assays, patients were divided into categories:

Anti-N-terminal autoantibodies alone	MDTCS competed for >85% of full-length ADAMTS13 binding, and patients exhibited no specific immunoreactivity with TSP2–8 domains in a separate ELISA.
Anti-TSP2–8 autoAb	immunoreactivity with TSP2–8 domains.
Anti-CUB1/2 autoAb	MDTCS competed for <85% of full-length ADAMTS13 binding and patient plasma exhibited no specific immunoreactivity with TSP2–8 domains by ELISA.

2.4. IgG Extraction

Total IgG was isolated from TTP patient and healthy control plasmas using Melon™ gel spin columns according to manufacturer's instructions (Thermo Scientific). IgG was dialysed into 20 mM Tris, pH 7.6 150 mM NaCl and quantified using an IgG ELISA (Bethyl Laboratories).

2.5. Antibody-Mediated Inhibition of ADAMTS13 Proteolysis of VWF115 and VWF106

The ability of isolated total IgG from TTP patients to inhibit ADAMTS13 function was assessed using VWF115 and VWF106 (Pos et al., 2010). Briefly, 2 nM ADAMTS13 (final concentration) in 20 mM Tris pH 7.6, 150 mM NaCl was preincubated for 1 h with 5 mM $CaCl_2$ both with and without 17 μM total IgG isolated from TTP or control plasmas. 5 μM VWF115 was added to start the reaction and incubated at 37 °C. At 0 to 60 min, subsamples were stopped and proteolysis monitored by SDS-PAGE and Coomassie staining. For analysis of VWF106 proteolysis, similar reactions were set up, using 3.5 nM ADAMTS13, 29 μM total IgG, and reaction times extended to 2 h.

2.6. Antibody-Mediated Inhibition of MDTCS Proteolysis of FRETS-VWF73 and Competition With MDTC

MDTCS (0.125 nM; final concentration) in 5 mM Bis Tris, 25 mM $CaCl_2$, 0.005% Tween, pH 6.0 was preincubated at 37 °C for 30 min with 0–5.6 μM total IgG isolated from TTP or healthy plasmas. 1 μM FRETS-VWF73 substrate (Peptide International) was added and fluorescence measured for 1 h to monitor substrate proteolysis. After titration of each IgG preparation, assays were repeated at three different IgG concentrations spanning the IC_{50} for each sample. This was performed with and without preincubation with 10 nM purified MDTC, to ascertain the proportion of inhibition attributable to anti-spacer domain antibodies.

2.7. ADAMTS13 ELISA

ADAMTS13 concentrations in healthy control and TTP patient plasma samples were quantified by ELISA using a rabbit anti-ADAMTS13 antibody (anti-TSP2–4 depleted) for capture, and biotinylated anti-TSP2–4 polyclonal antibody and streptavidin-conjugated peroxidase for detection, as previously described (Chion et al., 2007; Andersson et al., 2012). Plasmas were diluted from 1/25 to 1/200 in PBS 1% BSA, each analysed in duplicate. To test whether anti-ADAMTS13 autoantibodies interfere with the ELISA, 2 μl pooled normal plasma was preincubated with and without 4 μl of isolated IgG from 14 different TTP patients prior to incubation with wells.

2.8. Statistical Analyses

Statistical analyses were performed using SPSS and GraphPad Prism software. For continuous variables, differences between patients with varying patterns of domain specificity were evaluated by the Mann Whitney U test or the Kruskal Wallis test. For discrete variables, differences were evaluated using the χ^2 test or Fisher's exact test. Cumulative incidence and Gray's test were used to compare the incidence of relapse between groups. Logistic regression analysis was applied to compute odds ratios (ORs) and 95% confidence intervals (CIs), which were used as an estimate of the likelihood of mortality. A probability (p) value of <0.05 was deemed statistically significant.

3. Results

3.1. Patient Characteristics

The clinical and laboratory characteristics of the 92 TTP episodes in 78 non-consecutive patients are summarised in Table 1. Further clinical and biochemical characteristics of this group are available in the Supplementary Results.

3.2. Domain Specificity of TTP Autoantibodies at Presentation

To examine the domain specificity of anti-ADAMTS13 autoantibodies in TTP patients at presentation, we developed ELISA-based and competition ELISA assays. All TTP patient plasmas (diluted 1/50) contained IgG that strongly recognised immobilised full length ADAMTS13 by ELISA (Supplementary Fig. 1). The ADAMTS13 N-terminal domains (MDTCS — see Fig. 2A) were poorly recognised when immobilised onto wells, suggesting that direct coupling of MDTCS appreciably compromises its immunoreactivity with TTP autoantibodies (data not shown). Therefore, we used a competition assay in which TTP plasmas were preincubated with and without 10 nM MDTCS in solution (to preabsorb IgG recognising these domains) prior to incubation with wells coated with full-length ADAMTS13. This assay thus enabled estimation of the proportion of anti-ADAMTS13 antibodies that recognised MDTCS, with the residual binding detected representing IgG recognising the ADAMTS13 C-terminal domains (i.e. TSR2–8 and/or CUB1/2) (Supplementary Fig. 1). Using this approach, 89/92 (97%) of TTP presentation samples were designated to have immunoreactivity against the ADAMTS13 N-terminal domains (i.e. MDTCS competed for >5% of binding to full-length ADAMTS13). Only in 3/92 patient samples (patients #64, #91, #92) did MDTCS fail to compete for any full-length ADAMTS13 binding, suggesting that these patients' IgG recognise the ADAMTS13 C-terminal domains alone. These three patients were amongst the patients with the lowest anti-ADAMTS13 titre in this cohort.

We identified 38/92 (41%) patients (patients #1–38 in Fig. 2B) with autoantibodies that recognised the ADAMTS13 N-terminal domains alone, as MDTCS competed for >85% of full-length ADAMTS13 binding (Fig. 2B) and they exhibited no specific immunoreactivity with TSP2–8 domains in a separate ELISA (Supplementary Fig. 1). 26/92 (28%) patients had IgG recognising the ADAMTS13 TSR2–8 domains (patients #39–64), of which 25/26 of these patients also had antibodies recognising MDTCS. The remaining 28/92 (31%) patients (patients #65–92) exhibited little/no immunoreactivity against TSP2–8 domains, were, by elimination, determined to have an appreciable proportion of their autoantibodies recognising the CUB1/2 domains. 26 of these 28 patients also had antibodies recognising MDTCS. Patients could be assigned to different groups based on the domain specificity (i.e. 38/92 “anti-N-terminal”, 51/92 “anti-N- and anti-C-terminal” and 3/92 “anti-C-terminal”).

In 25 patients with a high proportion of antibodies recognising MDTCS (total anti-ADAMTS13 IgG >40%, >80% anti-MDTCS), no evidence of immunoreactivity against either MD or MDTC was detected in separate competition ELISAs (Supplementary Fig. 2), strongly suggesting that the spacer domain is the primary antigenic target amongst those antibodies that recognise the N-terminal domains.

3.3. Domain Specificity, Patient Characteristics, Disease Severity and Relapse

There was no difference in sex, ethnicity or anti-ADAMTS13 IgG titre between the 38 patient samples assigned to the “anti-N-terminal” group, and the 54 patient samples with anti-C-terminal antibodies (of which 51 also had anti-N-terminal IgG). Analysis of the prognostic implications of domain specificity of anti-ADAMTS13 was performed on the 62 first presentation samples. Domain specificity did not differ in patients who died ($n = 16$) from those who survived ($n = 46$). There was also no difference in median platelet count, Hb or LDH, number of PEX to remission, frequency of cardiac involvement or relapse rate between patients with antibodies directed against C-terminal domains and those with anti-N-terminal antibodies alone. This suggested to us that the domain specificity, per se, of anti-ADAMTS13 autoantibodies was not necessarily a major determinant of disease severity.

3.4. Identification of Inhibitory Antibodies Against ADAMTS13

To explore the inhibitory potential of TTP patient autoantibodies, we performed functional analyses using isolated total IgG (Fig. 3). Purified total IgG (17 μM) from a TTP patient with high titre (105% by comparison to reference plasma (18, 20)) anti-ADAMTS13 IgG directed against the N-terminal domains alone (#16 in Fig. 2B) appreciably inhibited VWF115 proteolysis, although specific cleavage products were still detected at this IgG concentration, suggesting that inhibition was not complete under these conditions. Total IgG (17 μM) from another patient (#81, remission sample) with similar anti-ADAMTS13 titre but only anti-C-terminal antibodies, had no inhibitory effect upon VWF115 proteolysis. Patient #61 at presentation (anti-ADAMTS13 IgG titre 99%) with antibodies against both anti-N- and C-terminal domains partially inhibited VWF115 cleavage at this IgG concentration.

VWF106, which is identical to VWF115 but lacks 9 residues from its C-terminus critical to ADAMTS13 spacer domain binding, is proteolysed

Table 1
Summary table of acquired TTP patient characteristics, clinical features and test results.
Tabulation of the parameters for all cases, subsequently divided into first episode and relapses, in a selected cohort based on high anti-ADAMTS 13 IgG antibody levels. Differences in clinical and laboratory parameters are statistically presented. First presentation episodes were more severe than relapse episodes as determined by increased frequency of neurological symptoms, lower Hb, higher LDH and increased number of PEX to achieve clinical remission.

	TTP episodes			
	All (n = 92)	First (n = 62)	Relapse (n = 25)	p[a]
Age, years (range)	43 (13–78)	44 (13–78)	40 (14–75)	0.38
Sex, n (%)				
Female	64 (70%)	43 (69%)	18 (72%)	1.0
Ethnicity, n (%)				
Caucasian	49 (53%)	37 (60%)	11 (44%)	–
Afro-Caribbean	23 (25%)	14 (23%)	9 (36%)	–
Asian	9 (10%)	5 (8%)	4 (16%)	–
SE Asian	2 (2%)	2 (3%)	0 (0%)	–
Mixed race	3 (3%)	2 (3%)	1 (4%)	–
Unknown	6 (7%)	2 (3%)	0 (0%)	–
Clinical features, n (%)				
Neurology	65 (71%)	50 (81%)	15 (60%)	<0.05
Cardiac	38 (41%)	28 (45%)	10 (40%)	0.63
Renal	34 (37%)	25 (40%)	9 (36%)	0.81
GI	29 (32%)	22 (35%)	7 (28%)	0.61
Fever	30 (33%)	25 (40%)	5 (20%)	0.08
Blood results (range)				
Hb, g/dl	8.4 (3.6–13.8)	8.0 (3.6–11.8)	10.8 (4.8–13.8)	<0.0005
Platelets, $\times 10^9$/l	13 (3–89)	13 (3–60)	18 (3–89)	0.17
LDH, IU/l (NR 470–900 IU/l)	1569 (165–5000)	2046 (165–5000)	921 (276–3174)	<0.005
ADAMTS13 assays (range)				
ADAMTS13, % act (NR 55–126%)	<5 (<5–27)	<5 (<5–12)	<5 (<5–27)	–
Anti-ADAMTS13, % titre (NR < 6.1%)	58 (9–164)	65 (18–164)	34 (9–101)	<0.0001
PEX to remission, pv (range)	16 (4–92)	21.5 (4–92)	12.25 (4–33)	<0.005
Subsequent relapses				
Number (%)	27 (29%)	12 (19%)	15 (60%)	<0.005
Time to relapse, months (range)	19 (2–52)	28.5 (4–50)	16 (2–52)	0.24
Deaths (%)	21 (23%)	16 (26%)	3 (12%)	0.25
Follow-up, years (range)	5.6 (1.7–13.6)			

Hb = haemoglobin, LDH = lactate dehydrogenase, NR = normal range, PV = plasma volumes.
[a] Comparison of first presentation and relapses.

by ADAMTS13 more slowly than VWF115 (Pos et al., 2010). Using 3.5 nM ADAMTS13 and 2 hour reaction times, VWF106 was partially proteolysed by ADAMTS13 after 120 min (Fig. 3). This cleavage was minimally affected by isolated IgG from patient samples #61 and #16, strongly suggesting that in both cases the inhibition observed in the VWF115 assay is mediated by antibodies that recognise the spacer domain, and that any autoantibodies that recognise the MDTC domains either do not impair ADAMTS13 function or are only present at very low concentrations.

To examine further the inhibitory potential of the autoantibodies in a larger number of samples, we assayed the ability of isolated total IgG to inhibit proteolysis of FRETS-VWF73 by the ADAMTS13 N-terminal domains, MDTCS. Total IgG from 43 patients (29 first presentation and 14 relapse samples) was isolated and titrated into FRETS-VWF73 activity assays to estimate the IgG concentration at which 50% enzyme inhibition was achieved (IC_{50}) (Fig. 4A–G).

IgG from 10 patients assigned to the anti-N-terminal alone group (termed *Group I*) dose-dependently inhibited 125 pM MDTCS (median

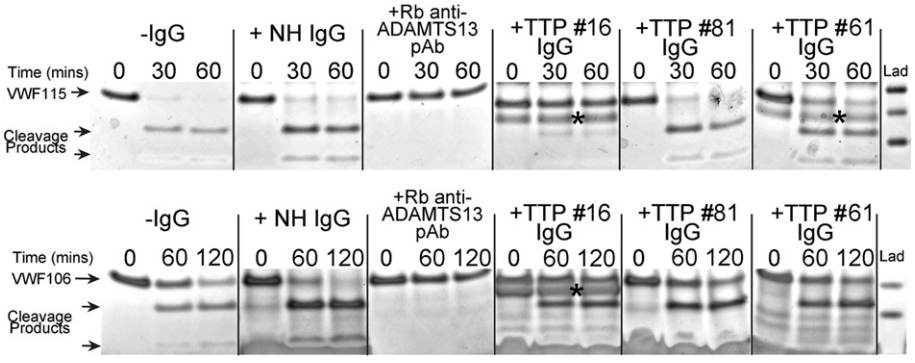

Fig. 3. ADAMTS13 inhibition is mediated by anti-spacer antibodies. ADAMTS13 activity assays using VWF115 (top) and VWF106 (bottom) in the presence and absence of isolated IgG samples. 2 nM ADAMTS13 proteolysed VWF115 into 10 kDa and 6.9 kDa fragments (−IgG), within 60 min. 17 μM normal human IgG (NH IgG) did not inhibit this reaction, whereas 7 μM rabbit polyclonal anti-ADAMTS13 led to complete inhibition. Identical reactions containing isolated total IgG (17 μM) from TTP patient samples #16, #81 (remission sample) and #61 are shown (* denotes contaminating band from IgG extraction). In parallel, proteolysis of VWF106, which lacks 9 residues that are critical to ADAMTS13 spacer domain binding, was investigated using 3.5 nM ADAMTS13 and 2 hour reaction times. Under these conditions, VWF106 was only partially proteolysed by ADAMTS13 after 120 min (−IgG). Cleavage was unaffected by normal IgG (NH IgG), but completely inhibited by rabbit polyclonal anti-ADAMTS13. Reactions containing isolated total IgG (29 μM) from TTP patient samples #16, #81 and #61 are shown. TTP patient IgG does not inhibit proteolysis of VWF106.

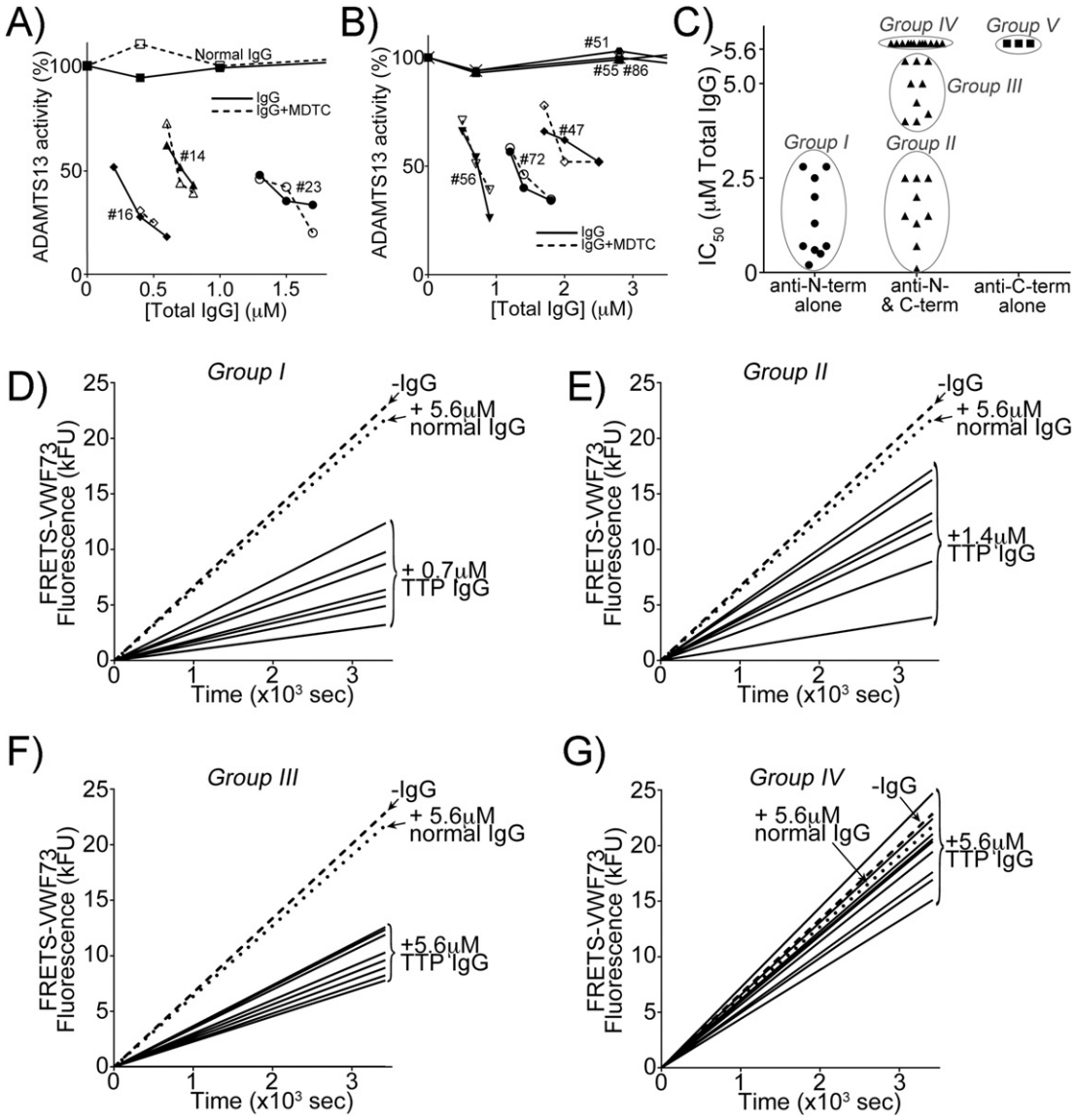

Fig. 4. Analysis of the inhibitory potential of total IgG isolated from acquired TTP patients. A) and B) 125 pM MDTCS was incubated with increasing concentrations of IgG isolated from either normal or TTP patient plasmas in the absence (solid lines) and presence of preincubation with 10 nM MDTC (dotted lines). A) Normal IgG had no effect on MDTCS activity, and addition of MDTC did not affect the activity detected. Three examples of TTP patients with anti-N-terminal alone antibodies are shown ± MDTC. All samples were inhibitory and this inhibition was not influenced by MDTC. B) as in A, except examples of IgG isolated from patients with both anti-N- and C-terminal antibodies are shown. Note the different x-axis scale highlighting that these IgG preparations are not as inhibitory. IgG from patients #51, #55 and #86 had no inhibitory effect upon MDTCS activity. C) Graph depicting the IgG concentration at which 50% enzyme inhibition was achieved (IC_{50}) for each patient tested with antibodies with different domain specificities. Patients are separated in five groups (*Groups I–V*) based on their domain specificity and the inhibitory potential of their IgG. D) Inhibition of 125 pM MDTCS by 0.7 μM total IgG isolated from *Group I* samples, all samples shown inhibit MDTCS appreciably at this concentration. E) Inhibition of 125 pM MDTCS by 1.4 μM total IgG isolated from *Group II* samples. Samples shown inhibit MDTCS variably at this concentration. F) Inhibition of 125 pM MDTCS by 5.6 μM total IgG isolated from *Group III* samples. Samples shown inhibit MDTCS by ~50% at 5.6 μM total IgG. G) Inhibition of 125 pM MDTCS by 5.6 μM total IgG isolated from *Group IV* samples. At 5.6 μM total IgG, little or no inhibition of MDTCS was detected for these samples.

IC_{50} 1.0 μM; range 0.2–2.8 μM) (Fig. 4A and D). To determine the contribution of anti-spacer domain antibodies to this inhibition, in parallel, varying concentrations of total IgG were preincubated with an 80-fold molar excess (10 nM) of purified MDTC (Fig. 4A). At this concentration, this purified preparation of MDTC had no effect upon activity detected using normal IgG. For all 10 patient IgG samples, preincubation with MDTC failed to detectably alter the IC_{50} (Fig. 4A), further suggesting that anti-spacer domain antibodies are the primary inhibitory antibodies.

As expected, IgG isolated from the 3 patients with only anti-C-terminal domain antibodies (termed *Group V*, Fig. 4C) failed to inhibit the activity of MDTCS even at the highest concentration tested (5.6 μM).

IgG was isolated from 30 TTP patient episode samples containing both anti-N- and anti-C-terminal antibodies. 12/30 (40%) patient samples (termed *Group IV*), exhibited no, or minimal inhibition of MDTCS using 5.6 μM total IgG (Fig. 4C and G). The remaining 18/30 (60%) patient samples with both anti-N- and anti-C-terminal antibodies were inhibitory. However, the median IC_{50} (3.3 μM; range 0.2–5.6 μM) was appreciably higher than for the samples containing only anti-N-terminal antibodies ($p < 0.05$). These patients could be separated into two groups: those with an inhibitory profile similar to *Group I* (*Group II*, $n = 9$) (Fig. 4C and E), and those with modest inhibitory potential (*Group III*, $n = 9$) (Fig. 4C and F). In 9/9 *Group II* samples, MDTC failed to alter the

estimated IC_{50}, again, strongly suggesting that the inhibitory antibodies in these samples are those that recognise the spacer domain.

3.5. ADAMTS13 Antigen Levels

The inhibition assays suggested that the anti-ADAMTS13 autoantibodies from patients in *Groups IV* (n = 12) and *V* (n = 3) (and potentially also those from *Group III*, n = 9) may be unlikely to cause severe functional deficiency of ADAMTS13 through inhibition alone, due to their limited inhibitory capacity. We therefore hypothesised that the loss in ADAMTS13 activity at TTP presentation in these patients may be due to antibody-mediated depletion of ADAMTS13 antigen.

In 91 TTP presentation samples (one sample was lost to analysis due to insufficient sample size), ADAMTS13 concentrations were significantly lower [median 58 ng/ml (6% normal); range 0–450 ng/ml (0%–47%)] than in 67 normal volunteers [median 951 ng/ml (range 515–1829 ng/ml) $p < 0.0001$] (Fig. 5A). Indeed, 84/91 (92%) patients had antigen levels <25%.

At first presentation, patients with anti-N-terminal antibodies alone (n = 23) had a median ADAMTS13 concentration of 81 ng/ml (8.5%); range 13–331 ng/ml (Fig. 5B). Patients' samples with no detectable inhibitory antibodies (n = 9) had a significantly lower ADAMTS13 concentration [median 2 ng/ml (0.2%); 0–141 ng/ml; $p = 0.005$]. Patients with both anti-N- and C-terminal antibodies with inhibitory function showed a trend to lower ADAMTS13 antigen levels, compared to those with anti-N-terminal alone [median 30 ng/ml (3.2%); range 0–356 ng/ml; $p = 0.08$] (Fig. 5B). However, the median ADAMTS13 antigen levels in both were significantly higher than in episodes in which no inhibitory antibodies were detected ($p < 0.05$). The same pattern was observed examining the ADAMTS13 antigen levels in patients assigned to *Groups I–IV* (Supplementary Fig. 3) — *Group V* patients were not included as these were not first presentation samples.

Interestingly, median ADAMTS13 antigen levels were significantly lower at presentation in patients that died during their first episode [12 ng/ml (1.3% normal); range 0–165)] than in survivors [57 ng/ml (6% normal); (0–356); $p < 0.05$] (Fig. 5C). Moreover, ADAMTS13 antigen levels in the lowest quartile (<13.5 ng/ml, <1.4% normal) were associated with increased mortality (OR 5.4; 95% CI 1.5–19.3; $p = 0.008$), and this remained statistically significant when multivariate analysis was performed taking age and sex as co-variates (OR 5.7; 95% CI 1.5–21.8; $p = 0.01$). These results suggest that autoantibody-mediated ADAMTS13 clearance is a major pathogenic mechanism and determinant of disease severity.

3.6. Longitudinal Analysis of TTP Patients

Four different patterns of clinical response to therapy were identified in 26 patients surviving their index episode of TTP and for whom longitudinal/follow-up samples were available.

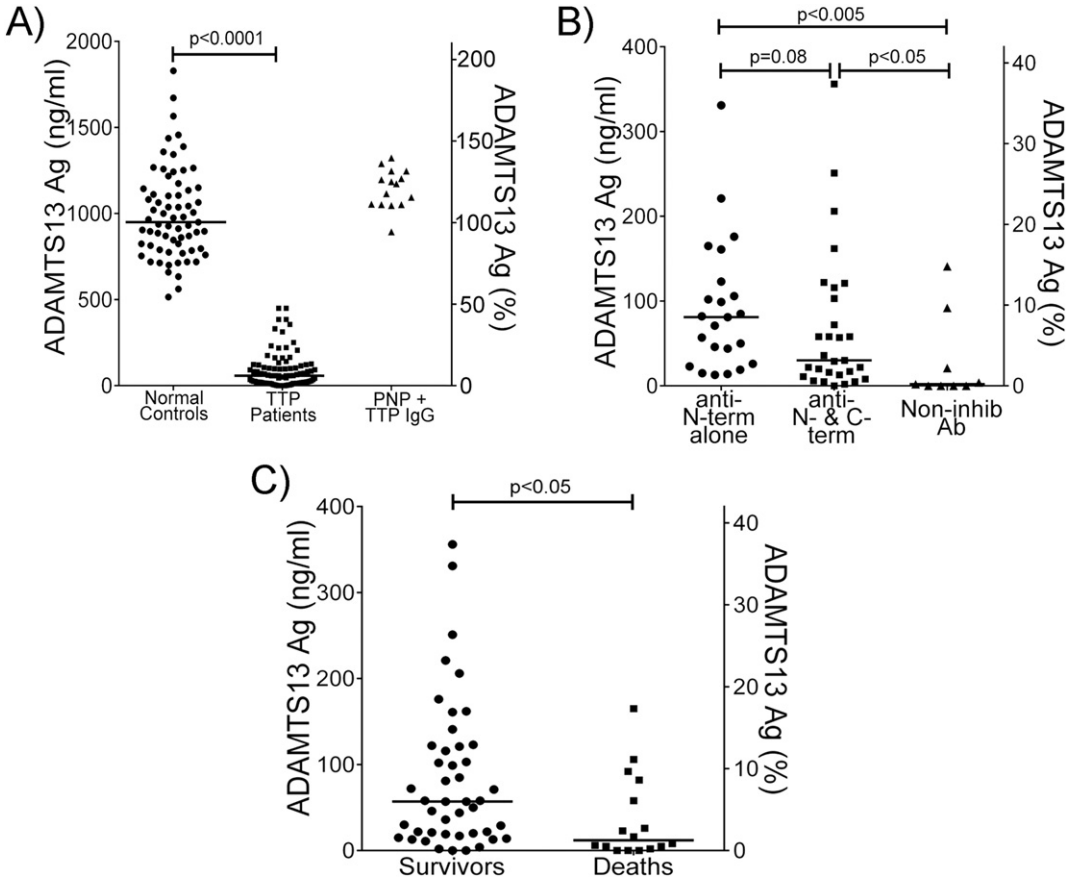

Fig. 5. ADAMTS13 plasma antigen levels in acquired TTP patients. A) Plasma ADAMTS13 antigen levels (left axis, ng/ml; right axis % normal levels) were measured by ELISA in 67 normal healthy controls and 91 acquired TTP patient samples at presentation. To ensure that reduced ADAMTS13 levels were not attributable to epitope masking by the autoantibodies, ADAMTS13 levels were measured in pooled normal plasma (PNP) that was preincubated in the presence of IgG isolated from 14 different TTP patient episodes. Patient antibodies did not appreciably influence the ELISA. B) Plasma ADAMTS13 antigen levels at first presentation in acquired TTP patients separated into anti-N-terminal alone, anti-N- and C-terminal antibodies, and patients with apparently non-inhibitory antibodies (*Group IV* patients from Fig. 4). C) Plasma ADAMTS13 antigen levels in acquired TTP patients at first presentation that survived their first episode or died. In A–C, individual concentrations are shown, the median is represented by a horizontal line. Statistical comparisons made are marked.

1) No relapse (n = 6) All patients received standard PEX and steroid therapy in conjunction with rituximab. Median follow-up was 3.3 years (range 1–6.3 years) — Supplementary Table 1. Total anti-ADAMTS13 IgG titre fell following therapy with PEX, steroids and rituximab (Scully et al., 2007). There were two cases with solely anti-N-terminal antibodies at presentation that disappeared with time (one example is given in Fig. 6A). Four patients with both anti-N and C-terminal antibodies at presentation all cleared the anti-C-terminal antibodies prior to anti-N-terminal antibodies.

At one month, two patients had normal ADAMTS13 activity, but low titre autoantibodies (19% and 22%) suggesting that these autoantibodies were not pathogenic. Anti-ADAMTS13 antibodies took 3 to 12 months to clear, which coincided with the recovery of ADAMTS13 antigen to normal levels.

2) Relapse (n = 16) The median time to either clinical relapse or to elective rituximab given in response to a severe drop in ADAMTS13 activity during follow-up was 31 months (4–52 months) (Table 2). 13/16 patients had received rituximab during their initial treatment. 9/16 patients exhibited altered domain specificity profile at relapse. The most frequent pattern (5/9 patients) was the loss of anti-C-terminal reactivity, but reappearance of anti-N-terminal antibodies at relapse (Fig. 6B). 3/9 patients had anti-N-terminal and high titre anti-TSR2–8 antibodies at initial presentation, but at relapse no longer had detectable anti-TSR2–8 antibodies (although still had anti-C terminal antibodies, suggesting development of anti-CUB antibodies). 1/9 patients developed novel anti-C-terminal antibodies at relapse, in addition to the reappearance of anti-N-terminal antibodies (episode #14 Fig. 6C).

3) Low ADAMTS13 activity during clinical remission There is a rare group of patients that respond clinically to rituximab, but have persistent low plasma ADAMTS13 activity and anti-ADAMTS13 antibodies. These patients are susceptible to multiple relapses. The domain specificity of anti-ADAMTS13 antibodies in two such patients revealed the persisting inhibitory IgG was directed against the N-terminal domains of ADAMTS13 (Fig. 6D). ADAMTS13 antigen levels varied between 20 and 60% in the remission samples, but plasma ADAMTS13 activity was persistently <10%, indicating the inhibitory nature of the persisting anti-ADAMTS13 antibodies.

4) Non-pathogenic IgG in remission Two patients achieved sustained remission (follow-up 5.1 years and 6.3 years) after standard therapy and up-front rituximab.

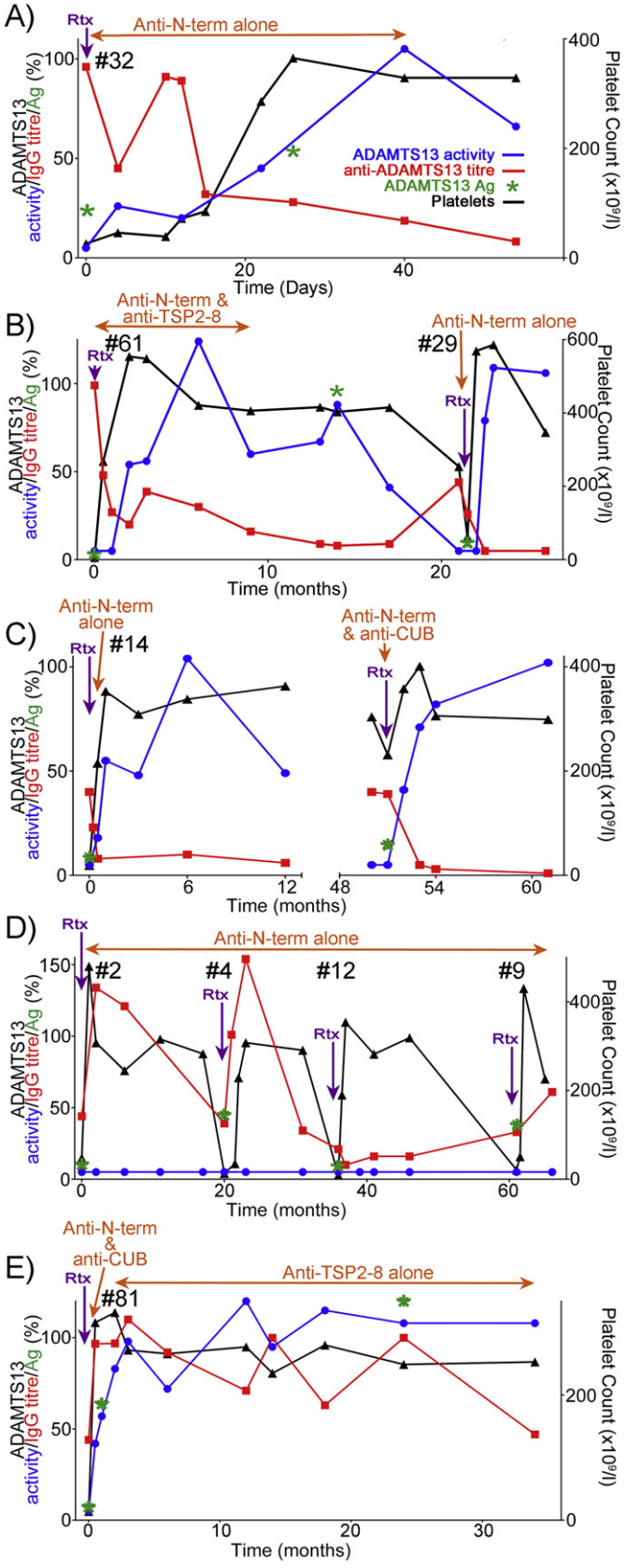

Fig. 6. Longitudinal analysis of acquired TTP patients. A–E) Longitudinal analysis of five acquired TTP patients following a first initial presentation. ADAMTS13 activity (blue), antigen (green), anti-ADAMTS13 IgG titre (red) are shown as % normal (left axis). Platelet counts are also shown (black, right axis). TTP episode number (e.g. # 32) refers to episodes denoted in Fig. 1. Points at which rituximab (Rtx) was given are highlighted by purple arrows. ADAMTS13 domain specificity results at different time points are marked in orange. A) represents a patient that did not relapse after their first episode. B) and C) are patients that relapsed and whose anti-ADAMTS13 domain specificity had changed at relapse. D) represents a patient that entered clinical remission, but had persistent low ADAMTS13 activity and inhibitory IgG and relapsed repeatedly. E) represents a patient that responded well to rituximab and entered remission. Despite persistent anti-ADAMTS13 antibodies, ADAMTS13 antigen and activity normalised.

Table 2
Longitudinal analysis of domain specificity of anti-ADAMTS13 IgG titre, ADAMTS13 antigen and activity in relapsing acquired TTP patients (n = 16).
TTP episode number refers to episodes denoted in Fig. 1.

First Presentation					Relapse						
TTP episode	AutoAb specificity	Act (%)	Total IgG	Ag (%)	TTP episode	AutoAb specificity	Act (%)	Total IgG	Ag (%)	Relapse/Elect Rtx	TTR (mo)
#72[a]	N & CUB	<5	40	26	#19	N	5	12	47	Relapse	6
#65	N & CUB	<5	84	6	–	N	<5	20	14	ER	34
#61	N & 2–8	<5	99	2	#29	N	<5	26	10	Relapse	21
#46	N & 2–8	<5	25	3	#27	N	<5	34	6	Relapse	27
#42	N & 2–8	<5	30	4	–	N	15	18	0	ER	52
#55[a]	N & 2–8	6	68	0	#92	N & CUB	<5	68	1	Relapse	34
#56[a]	N & 2–8	<5	126	n.d.	#87	N & CUB	<5	13	2	Relapse	36
#48	N & 2–8	<5	92	6	#64	N & CUB	27[b]	9	40	Relapse	4
#14	N	<5	40	9	–	N & CUB	<5	39	15	ER	50
#23	N	<5	23	7	#13	N	<5	53	13	Relapse	17
#22	N	<5	62	24	–	N	11	13	44	ER	42
#34	N	<5	23	23	–	N	<5	9	88	ER	31
#49	N & 2–8	<5	18	17	#44	N & 2–8	<5	23	3	Relapse	30
#62	N & 2–8	<5	34	22	#58	N & 2–8	<5	n.d.	7	Relapse	46
#86	N & CUB	<5	51	10	#90	N & CUB	<5	84	10	Relapse	5
#84	N & CUB	<5	44	2	#80	N & CUB	<5	48	2	Relapse	6

N = anti-N-terminal Abs, CUB = anti-CUB, 2–8 = anti-TSP2–8 Abs. Relapse = clinical relapse, Elect Rtx = acute drop in ADAMTS13 activity, prompting elective rituximab therapy. – = elective rituximab, patient episodes are not presented in Figs. 1 and Supplementary Fig. 1. TTR = time to relapse (months).
[a] No rituximab at initial presentation. n.d. = not determined (insufficient sample).
[b] Post PEX sample.

ADAMTS13 activity returned to, and remained at, normal levels within 1–2 months, but both cases had persistent detectable medium-high titre anti-ADAMTS13 IgG (25–100%) in clinical remission. ADAMTS13 antigen levels in remission were normal, suggesting that their anti-ADAMTS13 IgG did not promote clearance. The persisting non-inhibitory IgG was directed predominantly against the TSP2–8 domains (Fig. 6E).

4. Discussion

To date, this is the largest cohort in which the domain specificity of anti-ADAMTS13 IgG at presentation has been analysed (Zheng et al., 2010; Pos et al., 2011), and the first to report longitudinally through therapy, remission and relapse. The accompanying clinical and outcome data enabled the clinical significance of different antibody patterns to be explored. Unlike previous studies, our competition ELISA enables estimation of the proportion of anti-ADAMTS13 antibodies recognising the N-terminal domains (Fig. 2B), rather than merely assessing whether a domain fragment-specific antibody is present/absent. Our results at presentation broadly support findings from other studies, with 97% of patient samples having antibodies against the MDTCS domains (Klaus et al., 2004; Zheng et al., 2010; Pos et al., 2011). 41% of presentation samples had antibodies that only recognise these N-terminal domains. The remaining 59% of patient samples had antibodies against C-terminal domains (28% TSP2–8 and 31% CUB1/2). In contrast to previous studies, we found no evidence of antibodies recognising either MD or MDTC (Klaus et al., 2004; Zheng et al., 2010).

Domain specificity of anti-ADAMTS13 antibodies at presentation had no prognostic implication for disease severity in terms of mortality, likelihood of a neurological/cardiac presentation, or number of PEX to remission, suggesting that domain specificity of anti-ADAMTS13 IgG is unlikely a major determinant of their pathogenicity. A previous study found the presence of IgG antibodies against TSP2–8 and/or CUB was inversely correlated with patient platelet counts on admission (Zheng et al., 2010). However in our cohort, there was no difference in median platelet count between patients with antibodies directed against the C-terminal domains and those with solely anti-N-terminal antibodies, once the potential confounding factor of first versus relapsed presentation was removed. In keeping with two previous studies, we found no association between autoantibody/inhibitor titre and domain specificity (Klaus et al., 2004; Zheng et al., 2010).

Analysis of longitudinal samples revealed that patients that relapsed may have an altered domain specificity profile from those at first presentation (Fig. 6B–C). Whilst this may, in part, be explained by the failure of rituximab to eradicate all the clones of autoimmune B cells responsible for the anti-ADAMTS13 immune response, the epitope spreading seen in one patient (Fig. 6C) suggests a further development of the autoimmune response in some patients, rather than simple re-emergence of the clones responsible for the initial anti-ADAMTS13 immune response. It may be that the autoimmune response to ADAMTS13 can be reconstituted from escaped CD20 positive B cells or long lived memory cells (CD20 positive or negative) hiding in secondary lymphoid organs: indeed the spleen has been shown to harbour ADAMTS-13 specific memory B cells following acute acquired TTP (Schaller et al., 2014).

Our results are the first to formally demonstrate the critical role of anti-spacer antibodies in mediating ADAMTS13 inhibition (Figs. 3, 4A–B). We detected no antibodies other than those directed against the spacer domain that were capable of inhibiting MDTCS function. This suggests that, even if antibodies that recognise MDTC are present, their inhibitory contribution relative to those targeting the spacer domain is not significant. The spacer domain has long been suspected as the primary antigenic target for inhibitory antibodies, corroborated by mutagenesis studies (Jian et al., 2012), and also a recent analysis of monoclonal antibodies derived from two acquired TTP patients (Schaller et al., 2014). However, no other study has demonstrated in this many patients that inhibitory antibodies are limited to those that recognise the spacer domain. Despite their high frequency, anti-spacer domain antibodies are not a prerequisite for the development of TTP, as 3/92 patients at presentation had no evidence of anti-MDTCS antibodies and yet presented with severe ADAMTS13 deficiency.

Although anti-spacer domain IgG appears to be the major inhibitory antibody species in TTP, ADAMTS13 inhibition is not the exclusive pathogenic mechanism responsible for severe ADAMTS13 deficiency in TTP. We identified 15/43 TTP patients (*Groups IV* and *V*) with anti-ADAMTS13 IgG with little or no inhibitory function, suggesting that the inhibitory potency of autoantibodies in many TTP patients may, by itself, be insufficient to cause the severe ADAMTS13 deficiency (i.e. < 10% plasma activity). In these assays, we used 0.125 nM ADAMTS13, which is 1/40th of the plasma ADAMTS13 concentration. Based on the normal plasma IgG concentration of 80 μM, this might suggest that in those patients with and IC_{50} values >2 μM (i.e. 1/40th

plasma IgG concentration) the inhibitory potential of the autoantibodies would be insufficient to cause severe deficiency in the absence of any other pathogenic mechanism. This, in turn, implies that in potentially 32/43 of the patient samples from this cohort, inhibition cannot account for the deficiency state, and that only in 11/43 patient samples might the inhibitory actions of the autoantibodies be of sufficient potency to appreciably contribute to ADAMTS13 deficiency.

ADAMTS13 antigen was significantly reduced (median 6% normal) in TTP patient plasma samples, with 92% of samples having <25% normal ADAMTS13 antigen levels (Fig. 5). Antibody-mediated clearance of ADAMTS13 antigen is likely the major cause of loss of protease activity in acquired TTP. Low ADAMTS13 antigen levels in smaller groups of acquired TTP patients have been previously reported (Feys et al., 2006; Rieger et al., 2006), but despite this far greater attention has been placed on the contribution of inhibitory antibodies. One group investigated the relationship of ADAMTS13 antigen levels to anti-ADAMTS13 IgG and inhibitor titre, and concluded that antigen levels were lower in idiopathic TTP patients with inhibitory autoantibodies than those with non-inhibitory IgG or no IgG/inhibitor (Shelat et al., 2006). However, this finding was significantly limited by the inclusion of patients with only moderately reduced/normal ADAMTS13 activity or no autoantibody in their cohort.

Crucially, ADAMTS13 antigen levels at presentation have prognostic significance. ADAMTS13 antigen levels were lower in presentation samples of patients who died (median 1.0% vs. 5.5%). This finding is in keeping with a smaller study of 4 patients by Yang et al. (2011). Indeed ADAMTS13 antigen levels in the lowest quartile at first presentation increased the likelihood of mortality by 5.7-fold.

Patient samples without detectable inhibitory antibodies had the lowest ADAMTS13 antigen levels (Fig. 5B), suggesting that the primary pathogenic mechanism of anti-C-terminal antibodies is through increased ADAMTS13 clearance. It remains possible that some anti-C-terminal antibodies may also compromise ADAMTS13 function. For example, we identified two patients at first presentation without evidence of inhibitory antibodies against MDTCS, but with antigen levels >10% (Fig. 5B). These two individuals may thus harbour autoantibodies that recognise the C-terminal tail, which may be capable of compromising the function of full-length ADAMTS13.

Importantly, whilst anti-spacer domain autoantibodies have the potential to be inhibitory, they also promote ADAMTS13 clearance, as ADAMTS13 antigen levels were also severely reduced in patients with only anti-N-terminal antibodies. However, not all anti-ADAMTS13 antibodies promote clearance, as anti-ADAMTS IgG titre did not correlate with ADAMTS13 antigen. This was further exemplified by two patients that entered clinical remission with normalisation of ADAMTS13 activity and antigen levels (Fig. 6E) but persistent high-titre antibodies that recognised ADAMTS13.

Given the apparent importance of antibody-mediated clearance of ADAMTS13 as a significant pathogenic mechanism underlying ADAMTS13 deficiency in acquired TTP, characterisation of the underlying mechanism(s) is now necessary. ADAMTS13 antigen/antibody immune complexes (IC) have been described in acute TTP and during remission (Ferrari et al., 2012, 2014; Lotta et al., 2014), and are likely to play an important role. Naturally, detection and assessment of the importance of ICs at TTP presentation is potentially challenging if the ADAMTS13 antigen levels are already very low. The clearance of IgG-containing IC is known to occur primarily in the liver, both through both Fc receptor-dependent and independent mechanisms (Vugmeyster et al., 2012; Schifferli and Taylor, 1989; Emlen et al., 1992; Johansson et al., 2002; Kosugi et al., 1992). Complement also plays an important role in the elimination of immune complexes, with C3b binding keeping IC soluble (Schifferli and Taylor, 1989). Erythrocytes bind these opsonised immune complexes in the circulation via C3b receptors, and deliver them to tissue macrophages for elimination (Emlen et al., 1992). The spleen has also been implicated in the clearance of ICs in some studies (Johansson et al., 2002), and the size and type of immune complexes may influence the relative contribution of different clearance mechanisms (Vugmeyster et al., 2012).

Insight into how antibodies alter the kinetics of clearance of ADAMTS13 perhaps has more profound and immediate implications. ADAMTS13 has a relatively long active plasma half-life of 2–3 days (Furlan et al., 1999), suggesting its baseline rate of clearance is normally relatively slow. As over 70% of the TTP patients' samples that we analysed had no or low inhibitory potential, this could suggest that provision of recombinant ADAMTS13 to acquired TTP patients may not result in rapid inhibition of the enzyme in an appreciable proportion of patients. If the autoantibodies in these patients are non-inhibitory and their enhancement of clearance is not very rapid (which seems unlikely), this may allow recombinant ADAMTS13 a window of therapeutic benefit in these patients, and potentially reduce the number of PEX required to achieve remission.

This study has improved our understanding of the immunological basis of acquired TTP, which accounts for the vast majority of TTP cases. It has for the first time investigated the contribution of antibodies against different ADAMTS13 domains to the inhibitory potential in plasma, and revealed that antibodies against the spacer domain are the primary inhibitory species. However, our results implicate antibody-mediated ADAMTS13 depletion as a significant pathogenic mechanism underlying severe loss of enzyme activity in acquired TTP. This has considerable relevance, not only to how the diagnosis of acquired TTP is confirmed with ADAMTS13 assays, but also to prognosis and the possible benefit of ADAMTS13 replacement in acute therapy for TTP. The appreciable proportion of acquired TTP patients with non-inhibitory/weakly inhibitory anti-ADAMTS13 IgG, may imply that recombinant ADAMTS13 could have therapeutic potential as an adjunct to standard therapy, without the need for provision of very high ADAMTS13 concentrations to overcome antibody-mediated inhibition. It is now imperative to understand the mechanisms and kinetics of clearance to ascertain whether there is a therapeutic window for such treatment.

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The funding source had no involvement in the study.

Authorship Contributions

MRT designed the research, performed experiments, interpreted data and wrote the paper.

RdeG performed experiments and interpreted data.

MAS designed the research, interpreted data and wrote the paper.

JTBC designed the research, performed experiments, interpreted data and wrote the paper.

Conflict of Interest Disclosures

MRT, RdeG, MAS and JTBC declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.06.007.

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