The Role of Antibodies in Acquired Thrombotic Thrombocytopenic Purpura

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DECLARATION OF ORIGINALITY

I, Mari Rebeca Thomas, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

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.

The aims of my thesis were to develop novel assays to determine the domain specificity of anti-ADAMTS13 antibodies and use these to characterise the repertoire of antibodies in patients with acquired TTP. Functional analyses were also performed to explore the pathogenic mechanisms of these antibodies.

92 acquired TTP episodes were analysed at presentation, and through treatment and remission/relapse. Epitope mapping revealed 97% of episodes had autoantibodies that recognised the ADAMTS13 N-terminal domains. 41% episodes had antibodies recognising the N-terminal domains alone; 59% had antibodies against the C-terminal 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 antibodies with inhibitory function insufficient to account for the severe deficiency state, suggesting that in many patients there is an alternative pathogenic mechanism. There were markedly reduced ADAMTS13 antigen levels in all presentation samples, median 6% normal (range 0-47%). ADAMTS13 antigen in the lowest quartile at first presentation was associated with increased mortality (odds ratio 5·7).

This work has shown that 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, these results provide new insights into the pathophysiology of acquired TTP.

LIST OF ABBREVIATIONS USED IN THIS THESIS

Ab	antibody
A13	ADAMTS13
ADAMTS13	A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats
Ag	antigen
APC	antigen presenting cells
AutoAb	autoantibody
β-ME	β-mercaptoethanol
BCA	bicinchoninic acid
BSA	bovine serum albumin
CBA	collagen binding assay
CUB	Complement components C1r/C1s, sea urchin Uegf protein, Bone morphogenetic protein-1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoide triphosphates
ELISA	enzyme-linked immunoassay
ER	endoplasmic reticulum
FCS	fetal calf serum
FLA13	full length ADAMTS13
FPLC	fast protein liquid chromatography
GoF	gain of function
FRET	fluorescence resonance energy transfer
Hb	haemoglobin
HEK	human embryonic kidney fibroblast
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HRP	horseradish peroxidase
HUS	haemolytic uraemic syndrome
IC	immune complex

IgG	immunoglobulin G
IP	immunoprecipitation
LDH	lactate dehydrogenase
MAHA	microangiopathic haemolytic anaemia
mAb	monoclonal antibody
mAU	milli absorbance unit
MD	metalloprotease, disintegrin-like domain fragment of ADAMTS13
MDTC	metalloprotease, disintegrin-like, thrombospondin type 1 motif, cysteine-rich domain fragment
MDTCS	metalloprotease, disintegrin-like, thrombospondin type 1 motif, cysteine-rich, spacer domain fragment
MEM	minimal essential media
MHC	major histocompatibility complex
MW	molecular weight
NA	not available
NaCl	sodium chloride
OD	optical density
OPD	o-phenylenediamine dihydrochloride
PEX	plasma exchange
Plt	platelets
PBS	phosphate buffered saline
PBST	phosphate buffered saline Tween
PCR	polymerase chain reaction
PEI	linear polyethylenimine
PNGase	peptide-N-glycosidase
PNP	pooled normal plasma
rADAMTS13	recombinant ADAMTS13
SDS-PAGE	sodium docecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TFF	tangential flow filtration

TMA	thrombotic microangiopathy
TSP	thrombospondin
TTP	thrombotic thrombocytopenic purpura
ULVWF	ultra large von Willebrand factor
VWF	von Willebrand factor
WB	Western blot
WT	wild type

PUBLICATIONS AND PRESENTATIONS ARISING OUT OF WORK PERFORMED FOR THIS THESIS

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Presentations

Domain specificity of anti-ADAMTS13 antibodies at presentation and relapse in thrombotic thrombocytopenic purpura. *Thomas MR, Machin S, Crawley J Scully M. American Society of Haematology, December 2014*

Pathogenicity of anti-ADAMTS13 antibodies in thrombotic thrombocytopenic purpura. *Thomas MR, Machin S, Scully M, Crawley J. British Society of Haemostasis and Thrombosis (UK Emerging Fellows session), October 2014*

Domain specificity of anti-ADAMTS13 antibodies at presentation and relapse in TTP *Thomas MR, Machin S, Crawley , Scully M. European Haematology Association, June 2014*

Domain specificity of anti-ADAMTS13 antibodies in thrombotic thrombocytopenic purpura. *Thomas MR, Machin S, Scully M, Crawley J*. *British Society for Haematology, April 2014*. *Shortlisted for Best Oral Presentation in the Presidential session and Best Oral Presentation by an Early Stage Investigator.*

Domain specificity of anti-ADAMTS13 antibodies in thrombotic thrombocytopenic purpura. *Thomas MR, Machin S, Crawley J, Scully M*. *British Society of Haemostasis and Thrombosis, October 2012*

Posters

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TABLE OF CONTENTS

1 INTRODUCTION 18

1.1 Primary haemostasis 18
1.2 Von Willebrand Factor 19
1.2.1 Structure of VWF 19
1.2.2 Function of VWF 20
1.3 ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats) 22
1.3.1 Structure of ADAMTS13 22
1.3.2 Function of ADAMTS13 24
1.3.3 ADAMTS13-VWF interaction 24
1.3.4 Conformational activation of ADAMTS13 29
1.4 Thrombotic Thrombocytopenic Purpura 31
1.4.1 Pathophysiology 31
1.4.2 Epidemiology and subtypes 32
1.4.3 Clinical and laboratory features 32
1.4.4 ADAMTS13 assays 34
1.4.5 Management of TTP 42
1.4.6 Prognostic features in TTP 45
1.5 The immune system - tolerance and autoimmunity 47
1.5.1 Development of tolerance 47
1.5.2 Autoimmunity and autoimmune diseases 50
1.6 The autoimmune response in acquired TTP 51
1.6.1 Acquired TTP as an autoimmune disease 51
1.6.2 Factors contributing to the development of TTP 51
1.6.3 Antigen presentation in TTP 53
1.6.4 The role of T cells in TTP 54
1.6.5 The antibody response in TTP 56
1.7 Hypothesis 68
1.8 Aims 69

2 METHODOLOGY 70

2.1 Expression, purification and refolding of ADAMTS13 domain fragments in bacteria 70
2.1.1 Generation of bacterial expression vectors 70
2.1.2 Expression in Rosetta *E.coli* 75

2.1.3 Purification and refolding of bacterially expressed proteins 75
2.1.4 Analysis and characterisation of bacterially expressed proteins 78
2.2 Expression and purification of ADAMTS13 domain fragments in mammalian cells 80
2.2.1 Generation of ADAMTS13 fragment expression vectors 80
2.2.2 Expression of ADAMTS13 domain fragments 85
2.2.3 Purification of mammalian expressed proteins 87
2.2.4 Analysis and characterisation of mammalian expressed proteins 87
2.3 Plasma samples and antibody extraction 91
2.3.1 Patients 91
2.3.2 Plasma samples 92
2.3.3 ADAMTS13 activity assays 92
2.3.4 Total anti-ADAMTS13 IgG assay 93
2.3.5 IgG extraction 93
2.3.6 Quantification of total IgG 94
2.4 Determination of domain specificity of TTP patient autoantibodies 95
2.4.1 Immunoblotting using bacterially expressed material 95
2.4.2 Immunoblotting using mammalian expressed material 95
2.4.3 Immunoprecipitation 96
2.4.4 ELISA and competition assays 97
2.5 Determination of inhibitory potential of TTP patient autoantibodies 99
2.5.1 VWF 115 and VWF 106 activity assays 99
2.5.2 Fluorescence resonance energy transfer (FRETS) assay 102
2.6 Statistical analysis 102
3 Expression and purification of ADAMTS13 domain fragments and development of assays to determine the domain specificity of anti-ADAMTS13 antibodies 103
3.1 Methods 103
3.1.1 Expression, purification and refolding of ADAMTS13 domain fragments in bacteria 103
3.1.2 Expression and purification of ADAMTS13 domain fragments in mammalian cells 103
3.1.3 Determination of domain specificity of TTP patient autoantibodies 103
3.2 Results 105
3.2.1 Expression, purification and refolding of ADAMTS13 domain fragments in bacteria 105

3.2.2 Expression and purification of ADAMTS13 domain fragments in mammalian cells 113
3.2.3 Determination of domain specificity of TTP patient autoantibodies 121
3.2.4 Antibody extraction 128
3.3 Discussion 129
4 Domain specificity of TTP patient autoantibodies at presentation 133
4.1 Methods 133
4.1.1 Development of domain specificity assays 133
4.1.2 Methodology for domain specificity assays 137
4.2 Results 139
4.2.1 Patient characteristics 139
4.2.2 Domain specificity of TTP autoantibodies at presentation 143
4.2.3 Domain specificity and total anti-ADAMTS13 IgG titre 149
4.2.4 Domain specificity and disease severity 151
4.2.5 Domain specificity and relapse 155
4.3 Discussion 157
5 Pathogenic mechanisms of TTP patient autoantibodies 160
5.1 Methods 160
5.1.1 VWF115 and VWF 106 activity assays 160
5.1.2 FRETS VWF73 activity assay 160
5.1.3 Measurement of ADAMTS13 antigen levels 160
5.2 Results 160
5.2.1 VWF 115 activity assay 160
5.2.2 VWF 106 activity assay 163
5.2.3 FRETS VWF73 activity assay 164
5.2.4 ADAMTS13 antigen levels at presentation – the role of antigen clearance 169
5.2.5 Clinical correlation of ADAMTS13 antigen levels at presentation 172
5.2.6 Prognostic effect of non-inhibitory anti-ADAMTS13 antibodies alone (IC_{50}>5.6μM) at presentation 173
5.3 Discussion 174
6 Longitudinal analysis of the domain specificity of TTP patient autoantibodies 178
6.1 Methods 178

6.1.1 Response criteria 178
6.1.2 Assays 178
6.1.3 Patient selection 179
6.2 Results - patient groups 179
6.2.1 No relapse 179
6.2.2 Relapse 182
6.2.3 Inhibitory anti-ADAMTS13 IgG in clinical remission 184
6.2.4 Non-inhibitory IgG in remission 185
6.3 Discussion 186
7 DISCUSSION AND CONCLUDING REMARKS 188
BIBLIOGRAPHY 201

LIST OF FIGURES

Figure 1.1 VWF domain organisation and binding sites 19
Figure 1.2 Sites of VWF processing by ADAMTS13 20
Figure 1.3 Domain organisation of ADAMTS13 22
Figure 1.4 Structure of ADAMTS13 N-terminal domains based on the crystal structure of DTCS and homology modelling of metalloprotease domain 23
Figure 1.5 ADAMTS13-VWF interaction 25
Figure 1.6 Molecular models of the unfolding of VWF A1-A2-A3 domains . 27
Figure 1.7 Conformational activation of ADAMTS13 by VWF 30
Figure 1.8 VWF multimer gel showing presence of ULVWF multimers in TTP plasma 31
Figure 1.9 Role of B lymphocytes in autoimmune disease 49
Figure 1.10 Hypothetical model for the activation of self-reactive ADAMTS13- specific CD4+ T cells 54
Figure 1.11 Summary of results of domain specificity of anti-ADAMTS13 IgG antibodies in acquired TTP in published studies 58
Figure 1.12 Antigenic surface of spacer domain shown on the crystal structure of DTCS 60
Figure 2.1 Map of pET100/D-TOPO bacterial expression vector 70
Figure 2.2 ADAMTS13 domains expressed in *E.coli* 71
Figure 2.3 Directional TOPO© cloning (Invitrogen) 73
Figure 2.4 On-column refolding strategy 77
Figure 2.5 Mammalian expression vector pcDNA3.1/myc-His 80
Figure 2.6 ADAMTS13 fragments expressed in HEK293T cells 81
Figure 2.7 Development of an ELISA to detect domain-specific anti-ADAMTS13 antibodies 97
Figure 2.8 VWF A2 domain short substrates VWF115 and VWF106 and their cleavage by ADAMTS13 101
Figure 3.1 FPLC chromatogram of purification and refolding of the fragment Dis-TSP1 106
Figure 3.2 Coomassie stain of bacterially expressed and purified ADAMTS13 domains under reducing conditions 107

Figure 3.3 Coomassie stain of FPLC fractions from purification of bacterially expressed Dis-TSP1 domains with increasing imidazole concentrations... 109
Figure 3.4. Western blot of bacterial domains with anti-Xpress antibody in reducing conditions 110
Figure 3.5. Western blot of bacterial domains with rabbit polyclonal anti-human ADAMTS13 antibody 111
Figure 3.6 Western blot of expression of mammalian C-terminal TSP domain fragments 115
Figure 3.7 Western blot of expression of $MDTC^{555}$ in pcDNA3.1/myc-His vector 116
Figure 3.8 FPLC chromatogram of the purification of CUB1/2 117
Figure 3.9 Semi-quantitative Western blot of mammalian expressed ADAMTS13 fragments with anti-tag antibodies..... 118
Figure 3.10 Coomassie of mammalian expressed ADAMTS13 fragments MDTCS and MD..... 120
Figure 3.11 Western blot of bacterially expressed domains of ADAMTS13 probed with TTP IgG and normal IgG in non-reducing conditions..... 121
Figure 3.12 Western blot of mammalian expressed fragments of ADAMTS13 probed with TTP IgG and normal IgG in non-reducing conditions..... 123
Figure 3.13 Western blot of mammalian expressed fragments of ADAMTS13 using PVDF membrane probed with TTP IgG and normal IgG in non-reducing conditions 123
Figure 3.14 Immunoprecipitation of full length ADAMTS13 and MDTCS by TTP PEX fluid 124
Figure 3.15 Immunoprecipitation of full-length ADAMTS13 by plasma exchange fluid from TTP patients 125
Figure 3.16 Immunoprecipitation of the fragment TSP2-8 by plasma exchange fluid from TTP patients 125
Figure 4.1 Titration of MDTCS concentration required for competition assay 135
Figure 4.2 Novel assays for analysis of domain specificity of anti-ADAMTS13 IgG 136
Figure 4.3 Flowchart of the 92 acute TTP episodes..... 140

Figure 4.4 Graphs depicting anti-ADAMTS13 domain specificity ELISAs for 92 TTP patient episodes 144
Figure 4.5 Summary of domain specificity ELISA results for 92 TTP patient episodes 145
Figure 4.6 Competition ELISAs with MD, MDTC and MDTCS. 146
Figure 4.7 Detecting anti-CUB antibodies by competition ELISA 147
Figure 4.8 Summary of domain specificity of anti-ADAMTS13 antibodies at presentation of acquired TTP in the 92 patient episodes studied 148
Figure 4.9 Total anti-ADAMTS13 IgG titre at presentation by domain specificity of anti-ADAMTS13 IgG 149
Figure 4.10 Age of patients at first presentation in patients by domain specificity of anti-ADAMTS13 IgG 150
Figure 4.11 (A) Platelet count and (B) Domain specificity of anti-ADAMTS13 antibodies at presentation in patients who died or survived their first TTP episode 151
Figure 4.12 Domain specificity and clinical presentation 152
Figure 4.13 Domain specificity of anti-ADAMTS13 antibodies and platelet count at first presentation 153
Figure 4.14 Domain specificity and number of plasma exchanges to complete remission 154
Figure 4.15 Domain specificity of anti-ADAMTS13 antibodies at first presentation and in relapsed episodes 155
Figure 4.16 Kaplan Meier plots of relapse by domain specificity of anti-ADAMTS13 antibodies at first presentation 156
Figure 5.1 Inhibition of VWF115 proteolysis by ADAMTS13 162
Figure 5.2 Inhibition of VWF106 proteolysis by ADAMTS13 163
Figure 5.3 Analysis of the inhibitory potential of total IgG isolated from acquired TTP patients 166
Figure 5.4 ADAMTS13 antigen levels at presentation of TTP 169
Figure 5.5 ADAMTS13 antigen levels by domain specificity 171
Figure 6.1 Longitudinal analysis of TTP patients 181

LIST OF TABLES

Table 1.1 Characteristics of different classes of ADAMTS13 activity assays 37
Table 1.2 Studies of ADAMTS13 antigen levels at presentation of acquired TTP 39
Table 1.3 Other methods used for epitope mapping of anti-ADAMTS13 antibodies 59
Table 3.1 Bacterial ADAMTS13 domain constructs expressed, purified and quantitated in this study 104
Table 3.2 Mammalian ADAMTS13 domain constructs expressed, purified and quantitated in this study 114
Table 4.1. Demographic and clinical features at presentation 142
Table 5.1 Characteristics of fifteen patient episodes with no evidence of anti N-terminal inhibitory antibodies (IC_{50}>5.6μM) at presentation of a TTP episode (groups IV and V) 168
Table 6.1 Serial domain specificity of anti-ADAMTS13 IgG in non-relapsing patients 180
Table 6.2 Longitudinal analysis of domain specificity of anti- ADAMTS13 IgG titre, ADAMTS13 antigen and activity in relapsing acquired TTP patients.. 183

1 INTRODUCTION

1.1 Primary haemostasis

Haemostasis is a highly regulated homeostatic mechanism that ensures the maintenance of blood flow under physiological conditions but also allows rapid, localised coagulation in the event of tissue damage. The process comprises four basic mechanisms: vasoconstriction, primary haemostasis (platelet plug formation), secondary haemostasis (coagulation) and fibrinolysis.

Injury causes temporary local vasoconstriction due to contraction of vascular smooth muscle which slows blood flow and reduces blood loss. Vessel damage results in disruption of the endothelial cell monolayer and exposure of the collagen-rich extra-cellular matrix. The collagen is recognised by globular von Willebrand factor (VWF), a large adhesive glycoprotein which acts as a vascular damage sensor (1) and is necessary for initial platelet tethering and subsequent platelet adhesion (2). VWF binds collagen through its A3 domain and the tethered molecule is unfolded by local shear forces exerted by the flowing blood and elongates to expose previously hidden platelet binding sites (3).

Circulating platelets bind loosely to VWF through the receptor complex GPIb-V-IX causing the platelets to roll along VWF multimers (4), allowing them to come into contact with exposed collagen and bind it through their GPVI receptor. Collagen binding transduces intracellular signalling which increases the affinity of cell surface integrins for their ligands, causing platelet adherence, aggregation and activation (5), and leading to the formation of a primary platelet plug which temporarily blocks blood loss at the site of vessel damage. In turn, platelet activation exposes membrane phospholipids providing a surface for secondary haemostasis (coagulation), leading to the formation of a stable fibrin clot.

1.2 Von Willebrand Factor

1.2.1 Structure of VWF

VWF is synthesised as a multimer which is central to its role in haemostasis. It is synthesised only in endothelial cells (6) and megakaryocytes (7). The ~310kDa monomer pre-proVWF has a signal peptide, propeptide and multiple domains with different ligand binding sites. The domain organisation has been recently redefined by Springer's group (8) and is shown in Figure 1.1.

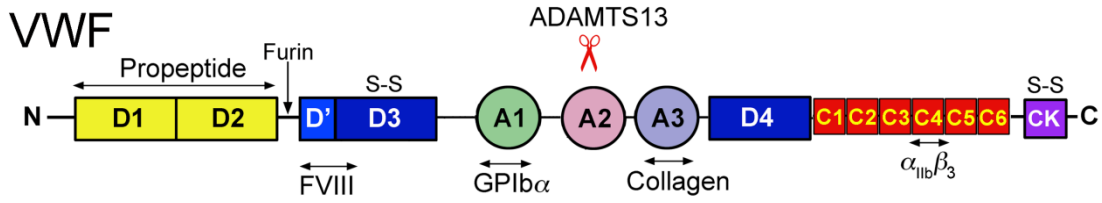


Figure 1.1 VWF domain organisation and binding sites

S-S indicates sites of intermolecular disulphide bond formation. Cleavage sites for furin and ADAMTS13 are indicated. VWF is a multi-adhesive protein and ligand binding sites (FVIII, GPIbα, collagen and $\alpha_{IIb}\beta_{IIIa}$) are shown below. Figure adapted from Zhou *et al*, 2012 (8)

The signal peptide is proteolytically cleaved in the endoplasmic reticulum (ER) (9) and VWF monomers are dimerised in the ER by intermolecular disulphide bonds between the Cys residues in the cysteine knot domain (10). The VWF dimers are transported to the Golgi apparatus where multimerisation occurs catalysed by the propeptide which forms disulphide bonds between the N-terminal D3 domains of the dimers (11). VWF is extensively glycosylated which is both essential for secretion (12) and influences its proteolysis (13, 14). Furin removes the propeptide after glycosylation (15), and the multimeric VWF and propeptide are trafficked to storage granules (Weibel-Palade bodies in endothelial cells or α-granules in platelets) (16). Release of VWF from endothelial cells occurs via basal secretion and in response to a variety of agonists, whereas VWF secretion from platelets is entirely stimulus-driven (17).

1.2.2 Function of VWF

VWF has two major functions – the recruitment of platelets to a site of vessel injury, and as a carrier protein for factor VIII (FVIII), thereby extending its half-life in plasma. The haemostatic properties of VWF are dependent on its molecular size. Larger multimers are more haemostatically reactive as they possess more ligand binding sites, but are also more conformationally sensitive to shear stress (2). The multimeric size of VWF and, therefore, its haemostatic properties are regulated by cleavage of a peptide bond in the VWF A2 domain (Tyr1605-Met1606) by the metalloprotease ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats).

A proportion of VWF released from endothelial cells remains tethered to the cell surface (18). ADAMTS13 rapidly cleaves newly secreted ultralarge VWF multimers on the endothelial surface (18), as depicted in Figure 1.2.

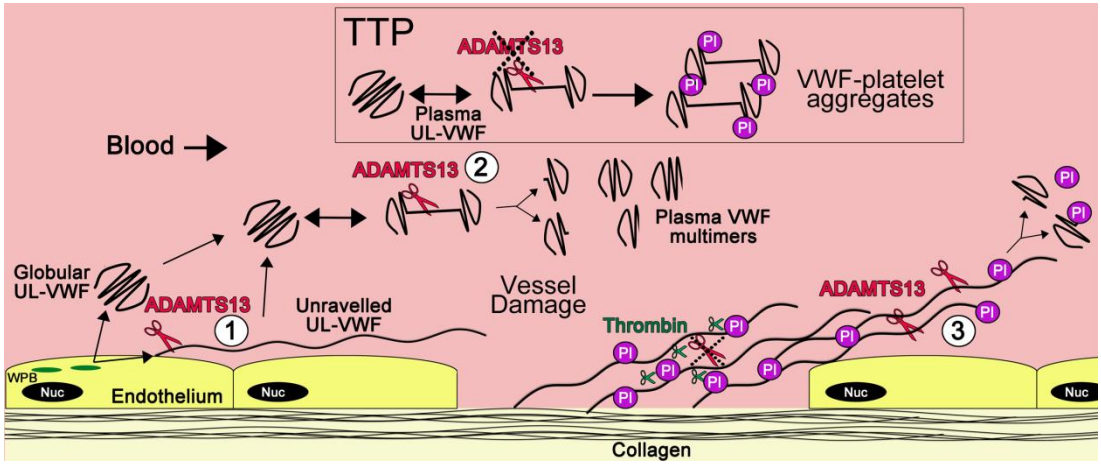


Figure 1.2 Sites of VWF processing by ADAMTS13

Figure from Crawley *et al*, 2011 (19)
1. ADAMTS13 rapidly cleaves newly secreted ULVWF multimers on the endothelial surface.
2. Unravelling of globular ULVWF in the circulation allows processing of VWF to smaller less reactive multimers. ADAMTS13 deficiency results in the loss of such plasma processing.
3. VWF-platelet strings may be proteolysed by ADADTS13 downstream of the site of injury, regulating platelet plug formation.

Once secreted or proteolytically released from the endothelium, VWF adopts a globular conformation in circulation, which is functionally quiescent with the platelet glycoprotein Ibα (GPIbα) binding site in VWF A1 domain largely hidden (3). This enables VWF to circulate without binding unnecessarily to platelets (19). Globular VWF is essentially resistant to proteolysis by ADAMTS13, with both the scissile bond and important recognition sites buried in the folded VWF A2 domain.

Globular VWF binds exposed collagen which is only revealed to the blood by vessel damage. This recognition occurs through its collagen-binding site in the A3 domain. VWF then undergoes a unique structural change where it is unfolded by shear forces acting on the tethered molecule and adopts an elongated ‘active’ conformation (3). This exposes previously hidden platelet binding sites and recruits platelets to the site of vessel injury.

VWF unfolding is thought to involve not only uncoupling of the VWF A1-A2-A3 domain cluster, but also substantial conformational changes in individual domains, particularly the A2 domain (20, 21). This unravelling exposes both the VWF A1 domain platelet binding site and the ADAMTS13 cleavage site and binding sites of the VWF A2 domain (20, 22). Thus, VWF proteolysis by ADAMTS13 is primarily dictated by substrate conformation, rather than specific enzyme activation like all the other haemostatic enzymes.

VWF may also be unravelled by higher mechanical shear forces in the microcirculation which act to stretch it and alter its conformation (Figure 1.2). Larger VWF multimers are more susceptible to mechanical shear forces and unravel more readily (22). Proteolysis of these larger species reduces VWF multimer size and hence controls haemostatic function of the plasma pool in an elegant on-demand regulatory mechanism. A defect in VWF proteolysis by ADAMTS13 is responsible for the rare life-threatening disease thrombotic thrombocytopenic purpura (TTP) that arises due to accumulation of platelet and VWF-rich microthrombi in the microcirculation (section 1.4.1) (23).

1.3 ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats)

1.3.1 Structure of ADAMTS13

ADAMTS13 is a 188 kDa protein and member of the ADAMTS family of Zn^{2+}-dependent multidomain metalloproteases. ADAMTS13 comprises 1427 amino acid residues and consists of a propeptide region, a metalloprotease (MP) domain, a disintegrin-like (Dis) domain, a thrombospondin type 1 motif (TSP1), a cysteine-rich (Cys) domain, a spacer domain, seven additional TSP1 repeats (TSP2-8) and two CUB domains, which are unique to ADAMTS13 amongst ADAMTS family members (23, 24).[i] The domain organisation of ADAMTS13 is shown in Figure 1.3.

Figure 1.3 Domain organisation of ADAMTS13

The N-terminal metalloprotease (MP) domain is followed by a disintegrin-like (Dis) domain, a thrombospondin type 1 motif (labelled 1), a cysteine-rich (Cys) domain, a spacer domain, seven additional TSP1 repeats (2-8) and two CUB domains. ADAMTS13 has a classical signal peptide which is removed on entry to the ER and a propeptide which, unlike in other metalloproteases, is not required for correct folding or to maintain enzyme latency (25).

The crystal structure of the fragment Dis-TSP1-Cys-Spacer (DTCS) was elucidated in 2009 by Akiyama and colleagues (26). The crystal structure of the other domains has not yet been determined. A model of the N-terminal domains of ADAMTS13 given by homology modelling of MP-Dis (based on crystal structures of homologous domains in ADAMTS1, 4 and 5) onto the crystal structure of ADAMTS13 DTCS is shown in Figure 1.4.

[i] CUB is an acronym for the first three proteins described which contain this domain: **C**omplement components C1r/C1s, sea urchin **U**egf protein, **B**one morphogenetic protein-1

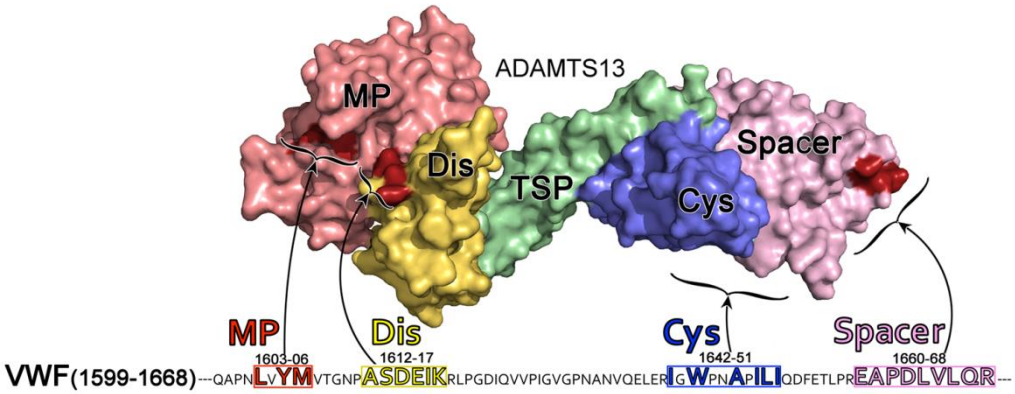


Figure 1.4 Structure of ADAMTS13 N-terminal domains based on the crystal structure of DTCS and homology modelling of metalloprotease domain

ADAMTS13 N-terminal domain structure based on the crystal structure of DTCS (26) and homology modelling of the metalloprotease domain (19). The active site, disintegrin-like, cysteine-rich and spacer domain exosites are shown in red, with their corresponding VWF exosites.

ADAMTS13 is synthesised predominantly in hepatic stellate cells (27). ADAMTS13 expression has also been detected in vascular endothelial cells (28), platelets (29) and renal podocytes (30), but the physiological relevance of its expression in other locations is unclear. ADAMTS13 is secreted into the blood as a constitutively active enzyme with a plasma concentration of approximately 1µg/ml (5nM) (31, 32). The mechanism of ADAMTS13 clearance is not known, but may be via a hepatic asialoglycoprotein receptor (33).

1.3.2 Function of ADAMTS13

ADAMTS13 functions as a VWF cleaving protease, cleaving a single peptide bond (Tyr^{1605} - Met^{1606}) located within the central VWF A2 domain (34, 35). Proteolysis is critical to control VWF multimer size and temper its haemostatic function. More recently, further functional consequences of ADAMTS13 action as a VWF cleaving protease have been described. ADAMTS13 may exert a thrombolytic effect in the microcirculation and by so doing may assist in resolving occlusive thrombi induced by $FeCl_3$ injury in venules in mice (36). ADAMTS13 has also been shown to reduce vascular inflammation, and slow the development of early atherosclerosis in mice, and to have a protective anti-inflammatory effect on ischaemia/reperfusion injury in myocardial infarction (MI) and stroke models (37-39).

1.3.3 ADAMTS13-VWF interaction

VWF-ADAMTS13 binding involves a complex and highly-specific set of exosite interactions that are dependent on the conformation of the substrate (VWF). ADAMTS13 is an unusual enzyme in that it is secreted in its active form (i.e. it has no zymogen state), and thus is always ‘on’. Unlike all other proteases involved in haemostasis, ADAMTS13 has no known specific inhibitor and is controlled by changes in its substrate induced when VWF subject to shear stress (19). Recognition of VWF by ADAMTS13 involves interactions between distinct domains of both proteins. A schematic overview of the current understanding of ADAMTS13-VWF interaction is shown in Figure 1.5.

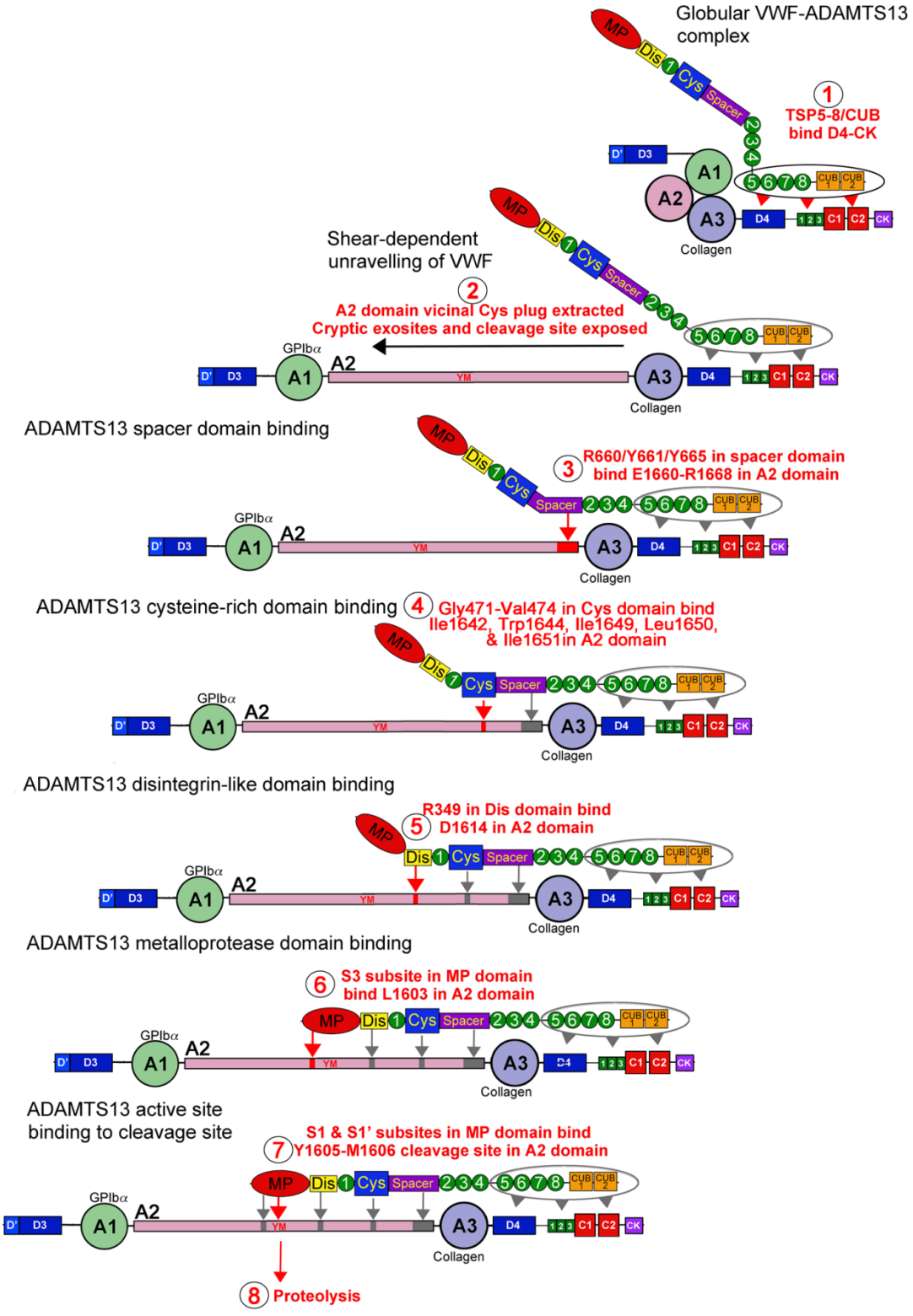


Figure 1.5 ADAMTS13-VWF interaction

Figure adapted from Crawley *et al*, 2011 (19) to include findings of de Groot *et al,* 2015 (40).

1.3.3.1 Role of ADAMTS13 C-terminal domains

The C-terminal domains of ADAMTS13 are important for ADAMTS13 recognition of globular VWF. Two studies have demonstrated that full-length ADAMTS13 recognises globular VWF, but is unable to cleave it in the absence of shear-induced unfolding. Zanardelli *et al* demonstrated that full-length ADAMTS13 could bind VWF in its globular form with a K_D of approximately 86nM, mediated by the TSP5-8 and/or CUB domains of ADAMTS13 interacting with the VWF D4-CK domains (41). Feys *et al* used immunoprecipitation of VWF-ADAMTS13 complexes in solution to derive a similar estimate of binding affinity and found that the interaction was dependent on TSP2-8 repeats (42).

Feys *et al* also demonstrated that a proportion of globular VWF and ADAMTS13 circulate as complexes with ~1 ADAMTS13 molecule per ~250 VWF molecules (42). This suggests that the larger more haemostatically active VWF multimers are the most likely globular VWF forms to be complexed with ADAMTS13 in circulation (43). Although only about 3% of plasma ADAMTS13 circulates bound to VWF, this pool may significantly contribute to thrombus regulation by co-localising ADAMTS13 and VWF to the site of vessel damage (43).

The functional significance of VWF-ADAMTS13 complexes was suggested by work by Banno *et al* who studied mice with an *Adamts13* gene that expressed a truncated form of the enzyme ($ADAMTS13^{S}$) that lacked the TSP7/8 and CUB domains (44). Although these mice had a normal plasma VWF multimeric pattern, the $Adamts13^{S/S}$ mice were more thrombogenic in high shear conditions, suggesting that loss of the C-terminal domains impaired the ability of ADAMTS13 to regulate development of the platelet plug (44).

1.3.3.2 VWF A2 domain unfolding

ADAMTS13 cannot cleave globular VWF because cryptic binding sites and the scissile bond are buried in the folded VWF A2 domain (45). The A2 domain has a rare vicinal disulphide bond between Cys^{1669} and Cys^{1670} at the C-terminus of the last α helix, which is thought to stabilise the domain folding (45). This acts as a 'molecular plug', which is pulled out when VWF encounters increased shear forces (Figure 1.6). Luken *et al* showed that when this disulphide bond is mutated, the A2 domain unfolds more readily and is, in turn, more susceptible to proteolysis by ADAMTS13 (46).

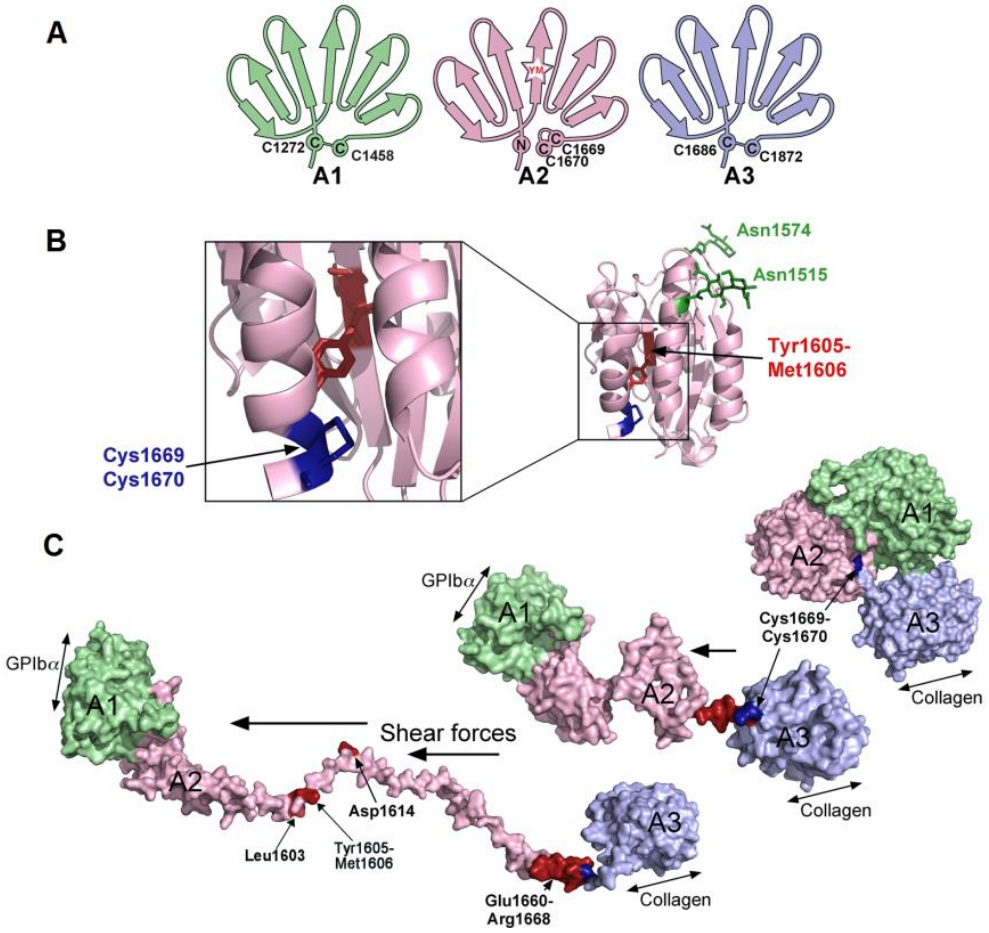


Figure 1.6 Molecular models of the unfolding of VWF A1-A2-A3 domains

A) Schematic representation of the VWF A1, A2 and A3 domains. The vicinal disulphide bond that forms the molecular plug in the A2 domain is shown. YM=Tyr1605-Met1606 scissile bond B) Structure of the VWF A2 domain highlighting the vicinal disulphide bond (blue) and ADAMTS13 cleavage site (red) hidden in the centre of the folded domain
C) Unfolding of the A1-A2-A3 domains. In globular VWF, the collagen binding site on the VWF A3 domain is exposed. Increased shear forces on VWF cause uncoupling of the A domains, extraction of the Cys1669-Cys1670 vicinal plug and unravelling of the A2 domain. This exposes the GPIbα binding site in the A1 domain, cryptic ADAMTS13 exosites and the scissile bond in the A2 domain (red). From Crawley *et al*, 2011 (19).

1.3.3.3 Spacer domain

Unfolding of the VWF A2 domain reveals cryptic exosites not exposed in the folded A2 domain or globular VWF (41, 46). Targeted mutagenesis and swaps with ADAMTS13 family members identified VWF A2 domain residues Glu^{1660}-Arg^{1668} as important for spacer domain function (47-49). The spacer domain exosite which binds the VWF A2 domain site was further characterised by work using TTP patient autoantibodies (50), discussed later in the thesis.

1.3.3.4 Cys-rich, disintegrin-like and metalloprotease domains

De Groot *et al* used engineered glycans, sequence swaps and single point mutations to identify a hydrophobic pocket in the cysteine-rich domain involving residues Gly^{471}-Val^{474} as being of critical importance for both VWF binding and proteolysis, and determined the complementary exosite on VWF (40). The same authors had previously used both deletion and substitution mutagenesis to define a functional exosite on the disintegrin-like domain involving Arg^{349} (51). This binds a complementary exosite on VWF involving Asp^{1614} in a critical but low affinity interaction which helps orientate the scissile bond towards the active site (51). Xiang *et al* identified the P3 residue VWF Leu^{1603} and complementary S3 subsite in the ADAMTS13 metalloprotease domain which act as docking sites on the N-terminal side of the Tyr^{1605}-Met^{1606} scissile bond bringing it over the active site (52). This allows the P1 and P1' residues on VWF to engage with the S1 and S1' subsite pockets of ADAMTS13 allowing proteolysis of VWF to occur (19).

1.3.4 Conformational activation of ADAMTS13

Recent work by two groups has shown that ADAMTS13 is conformationally activated by VWF (53, 54). South *et al* used kinetic analysis to show that WT ADAMTS13 had approximately 2.5-fold reduced activity compared to MDTCS or ADAMTS13 lacking the CUB1-2 domains, suggesting that the CUB domains naturally limit ADAMTS13 function (53). WT ADAMTS13 activity was enhanced ~2.5-fold by preincubation with an anti-CUB monoclonal antibody (20E9) or by VWF domain fragment D4CK. The isolated CUB1-2 domains bound MDTCS and inhibited activity by up to 2.5-fold (53). A gain-of-function (GoF) variant with mutated spacer domain (R568K/F592Y/R660K/Y661F/Y665F) was more active than WT ADAMTS13, but could not be further activated by the anti-CUB mAb or VWF D4CK and did not bind/was not inhibited by the CUB domains. Electron microscopy demonstrated a 'closed' conformation for WT ADAMTS13 but a more 'open' conformation for GoF ADAMTS13 – Figure 1.7 (53).

The group concluded that a CUB-spacer domain interaction blocks exposure of the ADAMTS13 spacer exosite and thus limits its activity. Interaction with the C-terminal domains of VWF disrupts the CUB-spacer interaction and leads to conformational activation of ADAMTS13 (53)

In parallel, Muia *et al* used small angle X-ray scattering data to indicate that the distal TSP-CUB domains interact with proximal MDTCS domains. They also used kinetic analysis to conclude that the distal TSP8-CUB domains markedly inhibit substrate cleavage, and binding of VWF (or mAb) to distal ADAMTS13 domains relieves this autoinhibition (54).

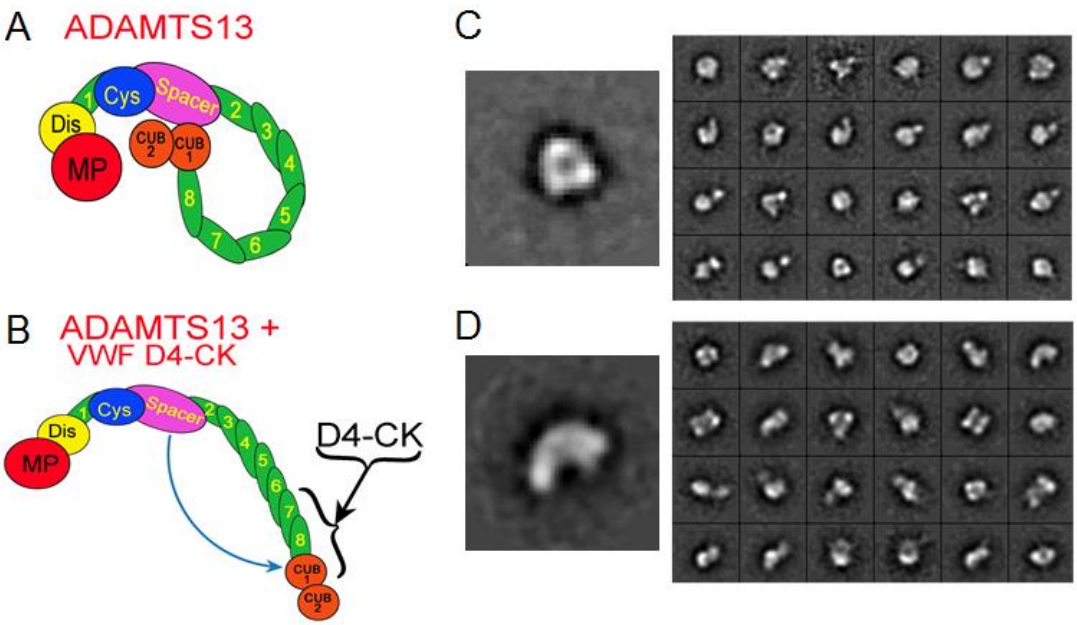


Figure 1.7 Conformational activation of ADAMTS13 by VWF

A) Schematic representation of ADAMTS13 in 'closed' conformation showing CUB-spacer domain interaction. B) Binding of VWF D4CK disrupts the CUB-spacer interaction and leads to conformational activation of ADAMTS13. C) Transmission electron microscopy of WT ADAMTS13 demonstrating the globular 'closed' conformation D) Transmission electron microscopy of GoF ADAMTS13 demonstrating the more linear 'open' conformation, resulting from disruption of the CUB-spacer interaction

1.4 Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura (TTP) is an acute life-threatening disorder first described by Moschcowitz in 1924 in a 16 year old girl who died after developing fever, haemolysis, paralysis and coma (55).

1.4.1 Pathophysiology

TTP results from a deficiency of the enzyme ADAMTS13 (23, 56). Inherited or acquired deficiency of this enzyme results in insufficient processing of ULVWF (Figure 1.8). These highly haemostatically active multimers appear to unravel under high shear during passage through the microcirculation leading to platelet aggregation and multi-organ microvascular thrombosis. This accounts for the clinical sequelae including neurological, cardiac, gastro-intestinal and renal disease. The occlusion of small vessels also causes a microangiopathic haemolytic anaemia and platelet consumption resulting in thrombocytopenia.

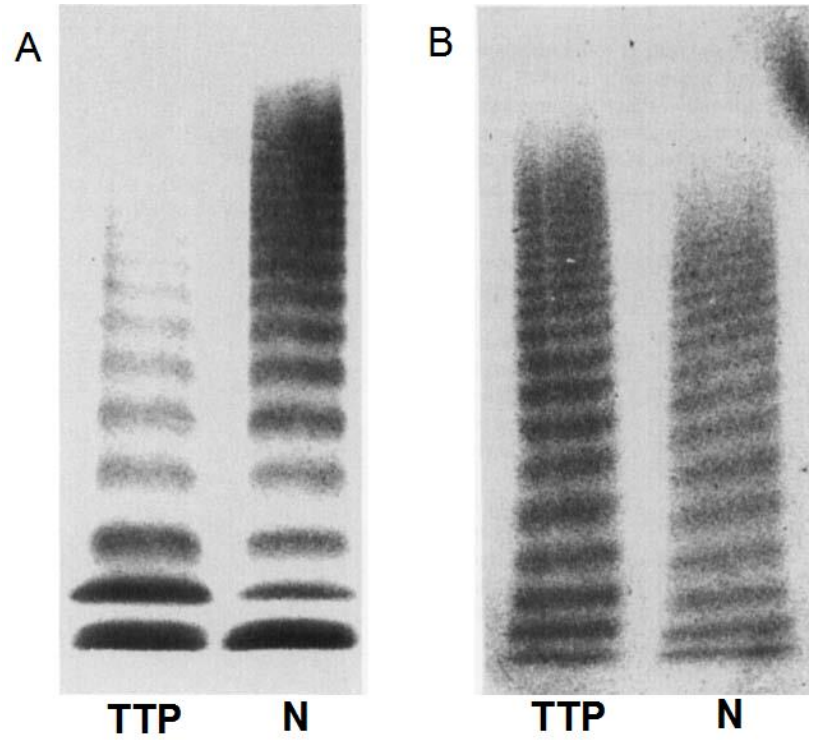


Figure 1.8 VWF multimer gel showing presence of ULVWF multimers in TTP plasma

SDS-agarose gel of VWF multimers.
A) TTP patient plasma during acute episode (platelet count 22 x10^9/l). N=normal plasma. There is a loss of normal high molecular weight multimers in the patient sample.
B) TTP patient plasma during remission (platelet count 359 x10^9/l). Ultra-large VWF multimers are present in the patient sample.

1.4.2 Epidemiology and subtypes

The majority of TTP cases are acquired idiopathic, antibody-mediated TTP with autoantibodies against ADAMTS13: such cases comprised 70% of all South East England Registry episodes (57). Secondary TTP which develops in response to a specific precipitating cause makes up about 15% cases. Identified precipitants include infections such as HIV, pregnancy, pancreatitis and certain drugs. Congenital TTP with recessive biallelic mutations in the ADAMTS13 gene comprises less than 5% cases. Mutations characterised to date are of many different types (missense, deletion, nonsense, frameshift) and are not limited to any particular part of the gene (58), and predominantly cause intracellular retention of the protein.

The annual incidence of TTP is estimated to be 6 per million in the UK (57). Demographic studies have shown a female preponderance (two- to three-fold risk compared to males) and increased incidence in Afro-Caribbeans (nine-fold increase compared with Caucasians) (59). Acquired TTP may present at any age but is more common in the 3rd and 4th decades. Congenital TTP classically presents in childhood but less severe congenital deficiency may not be unmasked until late adulthood.

1.4.3 Clinical and laboratory features

TTP is a clinical diagnosis and must be considered in any patient with microangiopathic haemolytic anaemia and low platelets in the absence of another cause (60). Clinical features are variable and the classical pentad of microangiopathic haemolytic anemia, thrombocytopenia, fever, neurological involvement and renal failure is rarely seen. The differential diagnosis of TTP is wide and includes atypical haemolytic uraemic syndrome (aHUS); disseminated intravascular coagulation (DIC); autoimmune disease *e.g.* systemic lupus erythematosus (SLE); severe hypertension; infections such as endocarditis; catastrophic antiphospholipid syndrome (CAPS) and thrombotic microangiopathies associated with malignancy or bone marrow

transplantation, but these conditions have a different pathophysiological basis and require a different therapeutic approach.

TTP is a life-threatening condition: in a recent study 15 out of 40 patients required ITU admission and 15% cases were intubated and ventilated (61). Neurological impairment is present in up to 80% of cases and may be fluctuating. Neurological features include headache, behavioural changes, transient ischaemic attacks, seizures or coma (62). Renal involvement is variable, ranging from proteinuria and microhaematuria to reversible impairment of renal function, but acute renal failure is rarely seen and suggests an alternative diagnosis. Cardiac features may include infarction, arrhythmias, heart failure, hypotension and sudden cardiac arrest. Gastrointestinal features such as abdominal pain, nausea, vomiting and diarrhoea occur in about a third of cases (57) and patients may present with symptoms of thrombocytopenia.

Autopsy studies confirm multiorgan involvement, with platelet and VWF-rich microthrombi (63) (in contrast to the fibrin deposits seen in haemolytic-uraemic syndrome, or an inflammatory component in disseminated intravascular coagulopathy or autoimmune disease).

The blood film shows red cell fragmentation, polychromasia, anaemia and low platelets (below $150x10^9$/l but usually $<50x10^9$/l). The reticulocyte count is increased and bilirubin raised due to haemolysis. The clotting screen is typically normal. Lactate dehydrogenase (LDH) is disproportionately increased relative to the degree of haemolysis, due to associated tissue ischaemia. It is well recognised that presentation is often more severe in *de novo* cases compared to relapses with more serious neurological presentation, lower haemoglobin (Hb) and higher LDH (57, 64), probably because relapses tend to present earlier as patients are monitored and have a lower threshold for seeking medical attention.

1.4.4 ADAMTS13 assays

1.4.4.1 ADAMTS13 activity assays

A large number of assays to measure ADAMTS13 activity either in the research or clinical setting have been described. The assays are based on the cleavage of full-length VWF multimers or VWF peptides that span the cleavage site and important binding sites, and the direct or indirect detection of the cleavage products.

1.4.4.1.1 Activity assays using full length VWF

In the original gel electrophoresis assay developed by Furlan *et al*, purified VWF with denaturants is incubated with citrated plasma for approximately 24 hours and low percentage agarose gel electrophoresis and Western blotting are used to determine the proteolysis of purified VWF multimers (34). Disappearance of high molecular weight multimers and appearance of cleavage products can be seen if proteolysis has occurred. Tsai *et al* developed an assay allowing direct visualisation of the 170 and 140kDa cleavage products of the 250kDa VWF subunits on denatured SDS-PAGE and Western blotting (35), eliminating the possibility of non-specific degradation of the multimers.

The collagen binding assay, first described by Gerritsen *et al*, is based on the preferential binding of high molecular weight VWF multimers to type III collagen (65). ADAMTS13 activity (proteolysis of VWF) is inversely proportional to the amount of collagen-bound VWF. This assay was used in clinical laboratories as it is relatively straightforward to perform, can accommodate many samples simultaneously and is quite robust in the detection of severe ADAMTS13 deficiency (66). However, it is time consuming requiring a 16-24 hour incubation in the presence of denaturants.

1.4.4.1.2 Cleavage of short substrates

Rapid activity assays have been developed more recently which use short VWF substrates that span the scissile bond and important ADAMTS13 binding sites. As these are domain fragments, they do not adopt a folded or closed conformation and are efficiently proteolysed without chaotropic agents as the scissile bond is always available The VWF A2 domain fragments commonly used are VWF73 (D1596-R1668) and VWF115 (E1554-R1668).

The VWF73 fluorescence resonance energy transfer (FRETS) assay involves a substrate with a fluorophore and quencher molecule on either side of the scissile bond (67). If the bond between Y1605 and M1606 is cleaved by ADAMTS13, the energy transfer quenching the fluorescence does not occur and fluorescence is emitted which is proportional to the ADAMTS13 activity.

ELISA-based methods have also been developed to measure ADAMTS13 activity using epitope-tagged VWF fragments as substrates (68-70). The substrate is immobilised on a microtitre plate via an antibody to one tag. After incubation with plasma samples, an antibody directed at another tag or the cleavage site detects residual substrate, meaning ADAMTS13 activity is inversely proportional to the residual substrate.

Both FRETS and ELISA-based assays are used in clinical laboratories and show good concordance with the traditional methods using multimeric VWF. An international multicentre study showed that assays based on multimeric VWF displayed good performance characteristics, whereas those based on VWF peptides were excellent (71). In the research setting, VWF73 or VWF115 cleavage products have also been separated and quantified by high performance liquid chromatography (HPLC) (72) or mass spectrometry (73), allowing the study of ADAMTS13 enzyme kinetics.

1.4.4.1.3 Flow-based assays

Assays have been developed to measure ADAMTS13 activity under flow conditions. In the simplest, flow is generated by vortexing the reaction mix (~3000rpm) which unfolds the VWF multimers allowing cleavage (74) but the cleavage products are detected by traditional gel electrophoresis or CBA. Test systems have been developed capable of assessing plasma ADAMTS13 activity in vitro under flow (18, 75). Shenkman *et al* used a cone and plate(let) analyser to evaluate the ability of TTP plasma to increase platelet deposition on a polystyrene surface (75) but this assay has not been widely used. In the assay developed by Dong *et al*, the formation of VWF strings is studied under flow after stimulation of endothelial cells with histamine. The strings are visualised indirectly by viewing platelet binding to these strings using phase-contrast video microscopy. In the presence of active ADAMTS13, VWF is cleaved and the strings disappear (18). This assay was evaluated in the multicentre comparative study which showed that although more physiological both in terms of substrate and flow, it was only reliable in discriminating ADAMTS13 levels higher or lower than 20% without measuring a precise value (71). The assay is difficult to standardise and not suitable for routine clinical practice.

The characteristics of the different classes of ADAMTS13 activity assay are summarised in Table 1.1.

Assay	Examples	Advantages	Disadvantages
Static assays Full length VWF	Collagen binding assay Multimer analysis	Multimeric VWF Sensitive (3-6% A13 activity) Reproducible	Denaturants artificially expose scissile bond & ?affect A13 Time consuming
Static assays VWF peptides	FRETS VWF73 VWF115 assay	More sensitive (1-3% A13 activity) Reproducible Rapid Kinetic analysis	Non-physiological substrates
Vortex assay	Multimer analysis	Multimeric VWF Physical unfolding Simple	Undefined shear mechanism VWF not anchored
Flow assay	Stimulated HUVECS, cleavage of platelet-VWF strings	Multimeric VWF Laminar shear flow Most representative of *in vivo* conditions	Technically demanding Time consuming Cannot give precise value

Table 1.1 Characteristics of different classes of ADAMTS13 activity assays

1.4.4.2 Anti-ADAMTS13 antibody assays

1.4.4.2.1 Functional assays

All of the functional assays for ADAMTS13 activity are capable of detecting inhibitory autoantibodies with a range of sensitivities (66). Neutralising anti-ADAMTS13 antibodies can be titrated *in vitro* using classic mixing studies of heat-inactivated patient and normal plasmas as 1:1 dilution or several dilutions in a Bethesda-type assay. However, although potentially useful, Bethesda assays are far from optimised and generally lack sensitivity (76). The sensitivity of functional assays for identification of anti-ADAMTS13 antibodies ranges from 44 to 90%, even in patients with ADAMTS13 activity <5% (77-79) and results from different functional assays may not be concordant (80). This is because no one assay reflects the *in vivo* situation and the different activity assays have differing non-physiological aspects *e.g.* non-physiological substrate; requirement for a denaturant.

1.4.4.2.2 Immunological assays

Immunological assays detect both inhibitory and non-inhibitory anti-ADAMTS13 antibodies. The initial ELISA described for detection of anti-ADAMTS13 antibodies bound purified recombinant ADAMTS13 to a microtitre plate via anti-His antibodies (81), but subsequent assays have used direct coating of ADAMTS13 to the plate (82). Commercial immunoassays are also available. ELISA appears more sensitive than functional assays for the identification of anti-ADAMTS13 IgG (83, 84). However, the specificity may be lower as in one study 4% of healthy individuals and 13% patients with systemic lupus erythematosus (SLE) had evidence of anti-ADAMTS13 IgG despite normal ADAMTS13 activity (81). ELISA methods vary in the dilution of plasma used, the nature of the ADAMTS13 antigen coated on the plates and the method of detection and these variables could affect the sensitivity and cut-off value for positive results (76).

Western blotting as a method of detecting anti-ADAMTS13 antibodies was described by Peyvandi *et al* (85). Western blotting may be more sensitive

than ELISA but is not a quantitative assay (76). It is more laborious, time-consuming and difficult to use for large numbers of clinical samples.

1.4.4.3 ADAMTS-13 antigen assays

Immunoassays using different monoclonal and polyclonal antibodies have been developed to quantify plasma ADAMTS13 antigen levels (31, 32, 86). A summary of studies of ADAMTS13 antigen levels in acquired TTP patients at presentation published to date are reported is shown in Table 1.2.

Study	**Number of acquired TTP patients**	**Mean / median A13 antigen levels**	**Range**	**% patients with low A13 antigen (<5% antigen)**
Feys *et al* 2006 (32)	11	24%	SD 30%	82%
Rieger *et al* 2006 (31)	33	13%	<6-68%	100%
Shelat *et al* 2006 (87)	21	51%	SD 9%	-
Starke *et al* 2007 (88)	6	6%	0.7-295%	83% (66%)
Yagi *et al* 2007 (89)	30	1.1%	SD 1.6%	100%
Liu *et al* 2006 (90)	11	10%	SD 8%	100%
Yang *et al* 2011 (91)	40	10%	<2-72%	90% (23%)
Ferrari *et al* 2014 (92)	68	-	<1.5-34%	100% (24% undetectable)

Table 1.2 Studies of ADAMTS13 antigen levels at presentation of acquired TTP

LLN=lower limit of normal range, SD=standard deviation, - =cannot determine from published data

All of the reports published to date have shown mean / median ADAMTS13 antigen levels to be reduced at presentation in idiopathic TTP. However, there have been marked discrepancies in the degree of reduction found. Shelat *et al* determined ADAMTS13 antigen levels in 21 patients and found mean antigen levels to be about half of normal using a commercial immunoassay (87), whereas Yagi *et al* found mean levels of only 1% normal in a highly sensitive novel ELISA (89). Similarly, studies differed over whether patients may have normal ADAMTS13 antigen levels at TTP presentation or not, with some studies finding a proportion of patients with normal levels (and one patient described with supra-physiological levels at presentation (88)). This wide range in antigen results at TTP presentation has led some researchers to conclude that antigen assays are not clinically helpful in diagnosing TTP in the absence of ADAMTS13 activity assays (76).

These differences are likely due in part to definition of disease. Shelat *et al* defined idiopathic TTP patients as MAHA with thrombocytopenia with no apparent cause, and the cohort had a wide range of ADAMTS13 activity levels [62% patients had severe deficiency (<10% activity), 29% patients moderate deficiency (10-50% activity) and 9% had normal activity] and included 19% patients with no anti-ADAMTS13 IgG or inhibitor (87). They found that antigen levels were lower in idiopathic TTP patients with inhibitory autoantibodies than those with non-inhibitory IgG or no IgG/inhibitor, but this finding is significantly limited by the inclusion of patients with moderately reduced / normal ADAMTS13 activity or no autoantibody in their cohort (87).

The nature of the antigen assay used is also likely play a role in the wide range of ADAMTS13 antigen levels found by different groups i.e. whether it detects ADAMTS13 antigen complexed with autoantibodies, or only free antigen.

1.4.4.4 Clinical role of ADAMTS13 activity assays

The reported frequency of severe ADAMTS13 deficiency in TTP varies from 18-100% (77-79, 93-96). Why is there such discrepancy? The main issues remain case definition and patient selection. There is no consensus definition of TTP and without a standardised definition of disease, cohorts of patients will differ meaning results cannot be easily compared between studies (97).

Some authors equate idiopathic TTP with severe ADAMTS13 deficiency (98), whilst others argue that such an approach might lead to cases being missed who would benefit from plasma exchange (PEX) (99). Cases have been described where patients have normal ADAMTS13 activity by static assays, but evidence of ADAMTS13 deficiency in flow assays, which may more closely represent the *in vivo* situation (100). In some subtypes of secondary TTP e.g. pancreatitis, ADAMTS13 activity in static assays is usually normal but patients benefit from plasma exchange (101). It may be that in the future, classifications of TTP include defined subtypes with different clinical features and different levels of ADAMTS13 activity (97).

Severe ADAMTS13 deficiency defines a group of patients that tend to have idiopathic disease, require more PEX to remission, tend to relapse and need additional immunosuppression but have lower mortality (59, 79) Another study found that severe ADAMTS13 deficiency defines a subset of patients that are characterised by various autoimmune manifestations, lower platelet count and mild renal involvement (96). However, the diagnosis of TTP remains a clinical one. Although the presence of severe ADAMTS13 deficiency is characteristic of TTP, it is neither sensitive nor specific enough to determine the decision to commence or withhold PEX (102). ADAMTS13 assays help confirm the diagnosis, monitor the course of the disease and need for possible extra therapy (62).

1.4.5 Management of TTP

1.4.5.1 Plasma exchange (PEX)

Prompt diagnosis and treatment is the key to survival in TTP; without therapy mortality is approximately 90%; with PEX mortality is reduced to 10-20% (103). Plasma is a source of the missing / dysfunctional enzyme ADAMTS13, and PEX using cell separators allows large volumes of plasma to be given and assists in the removal of autoantibodies against ADAMTS13 in the case of acquired TTP. PEX has been shown to be superior to plasma infusion at the end of the first treatment cycle and at six months (response rate 47% and 78% *vs*. 25% and 49%) (103). Plasma therapy does not address the underlying autoimmune nature of acquired idiopathic TTP, but patients are responsive to therapy and able to wean from plasma exchange because the autoimmune response to ADAMTS13 may not be intense and often spontaneously wanes after a few weeks (104). In fatal cases, patients may have high levels of inhibitors that are not amenable to PEX (98).

1.4.5.2 Steroids

As most idiopathic TTP is antibody-mediated, immunosuppression is used to help attain remission and/or prevent relapse. Steroids, such as pulsed intravenous methylprednisolone or high dose oral therapy are widely used in the treatment of TTP (62). Higher dose pulsed steroids have been shown to reduce the percentage of TTP patients that fail to achieve complete remission (105), but there has been no randomised controlled trial comparing the addition of steroids to PEX alone.

1.4.5.3 Rituximab

Rituximab is a chimeric IgG_1 monoclonal anti-CD20 antibody which results in the depletion of antibody-producing B cells. CD20 is expressed on B lymphocytes from the pre-B cell to the pre-plasma cell stage. The precise nature of action is unknown, but B cell depletion is believed to occur through antibody-dependent cell-mediated cytotoxicity (ADCC) (106) followed by complement-mediated lysis and apoptosis of the targeted cells (107-109). In TTP, a reduction in the anti-ADAMTS13 antibody titre can usually be

detected following therapy, which occurs within days of rituximab administration (82).

Initial evidence for the effectiveness and safety of rituximab in immune-mediated TTP came from studies of its use in refractory/relapsed patients (82, 110). More recently, a phase II UK study has shown benefit in using rituximab as part of first line therapy at presentation of TTP, where it appears to reduce the risk of relapse (61), and there is emerging data that early administration during acute episodes may reduce time to remission (111). A French study of refractory patients also found patients treated with rituximab had shorter overall treatment duration and reduced one year relapse rate than historical controls (112). Rituximab may also be used prophylactically to prevent relapse in patients with a fall in ADAMTS13 activity (111).

Rituximab has been used at a dose of 375mg/m^2 weekly for 4-6 weeks in acute TTP. Ideally PEX should be withheld for at least four hours after a rituximab infusion, as there is evidence that it is removed by plasma exchange (82, 113). Giving rituximab more frequently, e.g. every 3-4 days, may overcome removal by PEX (113), and lower doses (as used in other non-malignant autoimmune conditions) may be sufficient in the non-acute situation.

Relapses may occur in some patients after rituximab therapy following reconstitution of the B cell compartment (61). Peripheral B cell return usually starts 6-9 months after treatment, although this is variable (114). However, unlike some autoimmune diseases such as rheumatoid arthritis (RA), relapse in TTP does not coincide with B cell return (82). Germinal centre B cells have been shown to be resistant to the effects of rituximab in murine models, due to poor tissue penetration or local protective effects (109). In addition, plasmablasts and plasma cells are able to escape rituximab by downregulation of CD20 expression (115). A study of the effect of anti-CD20 treatment on B cell development in the bone marrow of patients with RA showed that naïve and unswitched memory cells were efficiently depleted after rituximab, but the number of plasma cells remained unchanged (116).

1.4.5.4 Other therapies

Other immunosuppressants such as mycophenylate mofetil, ciclosporin A, tacrolimus may be considered as second line therapy (62). Splenectomy may rarely be considered in the non-acute period of idiopathic TTP, but has only limited proven benefit (62). In a retrospective case series of 33 patients splenectomised for relapsed/refractory disease, the ten year relapse-free survival was 70% (117).

Newer treatment modalities include agents which block VWF-glycoprotein Ib binding. Inhibition of VWF-glycoprotein Ib with a monoclonal antibody prevented and reversed symptoms of TTP in baboons (118). The anti-VWF nanobody caplacizumab has completed a phase II clinical trial in acquired TTP (TITAN trial) (119). Treated patients had a shorter time to platelet normalisation and reduced exacerbations during treatment (119). Importantly, there was an acceptable safety profile with regards increased tendency to mild/moderate bleeding. However, it must be remembered that whilst such agents may reduce microthrombus formation and hence organ damage, they do not affect the underlying immune pathology of acquired TTP.

In vitro spiking studies with recombinant ADAMTS13 have shown that it has the potential to override anti-ADAMTS13 antibodies leading to an improvement in ADAMTS13 activity (120), and rADAMTS13 was able to correct TTP features in a mouse model of TTP (121). Case reports have suggested that N-acetylcysteine may have a role in therapy, acting to decrease the size of VWF by reducing the disulphide bonds of VWF multimers (122) or by reducing the VWF A1 domain residue 1278-1458 disulphide bond which is crucial for binding to platelet GPIbα (123) (124).

Case reports also exist in the literature suggesting a benefit of the proteasome inhibitor, bortezomib, for refractory TTP (125, 126). Bortezomib inhibits the 20S ribosomal subunit, increasing availability of NFκB-inhibitory protein, IκB (127). Via this mechanism, it eliminates autoreactive B-

lymphocytes, plasma cells, and may inhibit autoantigen-presenting dendritic cells (128, 129).

1.4.6 Prognostic features in TTP

TTP remains a life-threatening disease with a 10-20% mortality rate, which has not improved significantly since the introduction of PEX therapy. Exacerbation within 30 days of remission occurs up to 45% of patients and recurrences (after 30 days) have been reported in up to 50% cases (78, 130-133). We currently have only limited ability to identify those individuals with a more severe disease phenotype who are most at risk of death, or to determine which patients are more likely to relapse.

1.4.6.1 Predicting mortality

A number of clinical factors are associated with a poorer prognosis. Severe neurological or cardiac involvement at presentation is associated with a worse outcome (57). A predictive model for death in idiopathic TTP with severe ADAMTS13 deficiency has been developed by the French TMA reference centre based on cerebral involvement, age and LDH level (134). Another group suggested that age>40, Hb<9 and fever at presentation are predictive of mortality at 6 months (135).

Several studies have examined the potential utility of ADAMTS13 assays to predict disease severity. Patients with severe ADAMTS13 deficiency (<10%) have lower mortality than those with activity>10% (78, 79). This is likely to reflect the higher mortality of TMA secondary to underlying conditions such as malignancy or bone marrow transplantation, which is often unresponsive to plasma exchange and where ADAMTS13 activity is not severely reduced.

Severe depletion of ADAMTS13 antigen during acute disease has been associated with increased mortality in a small study, and antigen level at initial clinical recovery was higher in patients who achieved a sustained remission (91).

There is conflicting evidence on the relevance of anti-ADAMTS13 antibodies to clinical outcome. Patents with anti-ADAMTS13 antibodies at presentation appeared to respond less well to PEX (78, 95, 136). Some studies have reported poorer responses to PEX in patients with higher inhibitor titres (78, 136-138), but others have not (57, 79, 83). The discrepancy in these results is likely to be due in part to the small study sizes, but may also reflect the assays used to detect the antibodies and/or different therapeutic strategies. The relationship between total anti-ADAMTS13 IgG titre and prognosis is not clear and there is no absolute level that dictates a worse prognosis. However, one study found that higher anti-ADAMTS13 IgG (>67%) in conjunction with a raised troponin T was associated with a significantly worse prognosis (139).

1.4.6.2 Predicting relapse

In a prospective cohort study, Ferrari *et al* found that high levels of inhibitory IgG at presentation was associated with the persistence of undetectable ADAMTS13 activity in remission, which was in turn predictive of relapses within 18 months (83). In a larger but retrospective study of 109 patients, those with ADAMTS13 activity <10% or an inhibitor or anti-ADAMTS13 IgG had a three-fold increase in relapse during the first year of follow-up, whilst if activity<10% and IgG antibody were present, the risk increased 3.6 fold (85). Severely reduced ADAMTS13 activity during remission has also been confirmed as predictive of relapse (83, 85, 140), and there is an increased risk of relapse in patients with inhibitors detectable during clinical remission (78, 79, 83).

1.5 The immune system - tolerance and autoimmunity

1.5.1 Development of tolerance

A crucial requirement of the normal immune system is the ability to react to foreign antigens expressed by pathogens whilst ignoring self-antigens – immunological tolerance. Immunological tolerance to autoantigens may be induced when developing lymphocytes encounter these antigens in the central lymphoid organs (central tolerance), or when mature lymphocytes encounter self-antigens in the peripheral tissues (peripheral tolerance).

The principal mechanisms of central tolerance in T cells are cell death, and for CD4+ cells, the generation of regulatory T cells. T cells undergo negative selection in the thymus where immature lymphocytes that interact strongly with self-peptides that are expressed ectopically in the medullary region of the thymus receive a 'death' signal that triggers apoptosis (141). Some immature CD4+ T cells that recognise self-antigens in the thymus do not die but develop into regulatory T cells and enter peripheral tissues. What determines whether a thymic T cell that recognises a self-antigen will die or become a regulatory T cell is not known (142).

Central tolerance is the major mechanism to determine the overall T cell number in the body. However, thymic deletion of destructive T cell populations is incomplete (143) and peripheral tolerance is important for preventing T cell responses to self-antigens that are present mainly in peripheral tissues and not in the thymus. Extrinsic mechanisms of peripheral tolerance involve T reg cells and suppressive cytokines such as IL-10, TGF-β and antigen presenting cells (APC). Intrinsic mechanisms include T cell anergy, phenotypic alteration of T cells and apoptosis (143).

Self-antigens may fail to elicit autoantibody responses because of lack of T cell help, due to tolerance in helper T cells. There are, however, specific mechanisms of B cell tolerance. A key determinant of B cell central tolerance is the strength of B cell receptor (BCR) signalling (144). A strong BCR signal due to binding with high affinity to an autoantigen in the bone marrow may

cause an immature B cell to reactivate its Ig gene recombination machinery and begin to express a new light chain. This process of changing receptor specificity is called receptor editing (145). It is estimated that 25-50% of mature B cells in a normal individual may have undergone receptor editing during their maturation (142). If editing fails, self-reacting B cells undergo clonal deletion (146). Intermediate binding affinity to autoantigen will permit B cells to survive and progress to the periphery (147).

Peripheral B cell tolerance comes from functional inactivation, whereby mature B lymphocytes that encounter high concentrations of self-antigens in peripheral lymphoid tissue become anergic and cannot respond again to that antigen (148). In the last decade, a new subset of B cells has been identified in murine models as regulatory B cells (B reg cells) due to their suppressive capacity which is thought to be due to secretion of IL-10 (149, 150). However there is still limited data about their function and they remain relatively poorly characterised in humans. Figure 1.9 shows the role of B cells in autoimmune disease.

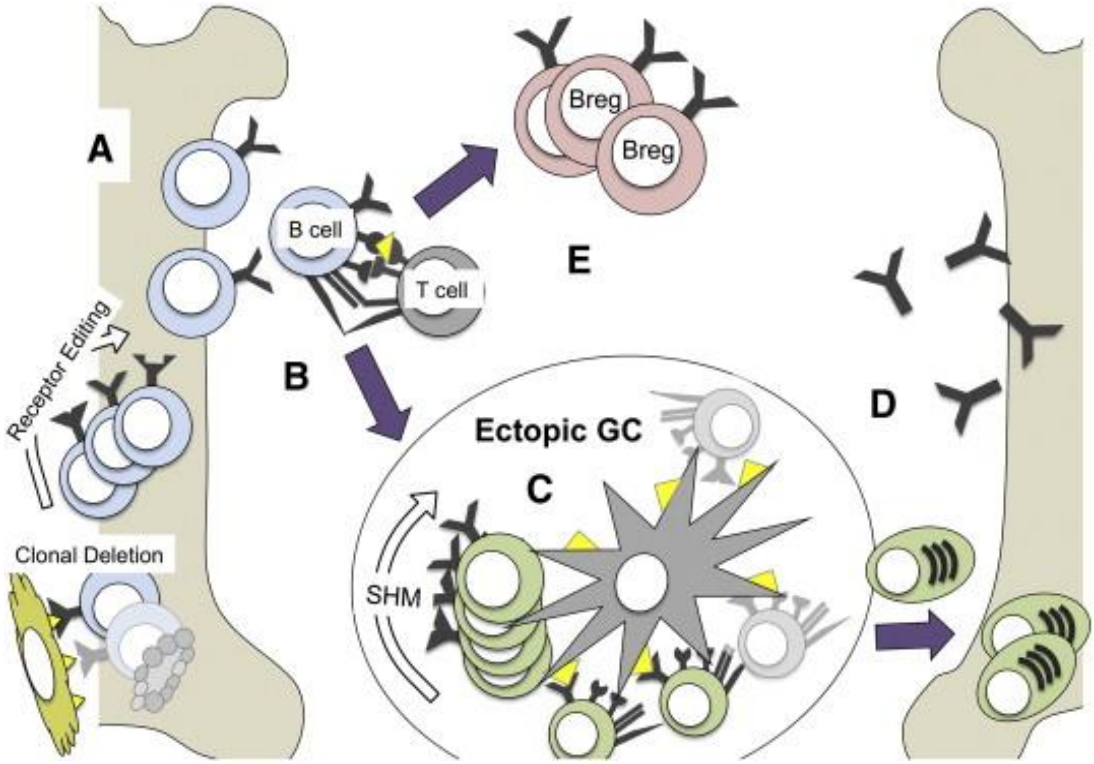


Figure 1.9 Role of B lymphocytes in autoimmune disease

A) Autoreactive B cells that escape central tolerance mechanisms in the bone marrow are not sufficient to cause overt autoimmune disease and other mechanisms are necessary. B) B cells are efficient antigen presenting cells that activate T cells. T cells in turn activate B cells enabling them to start germinal centre (GC) reactions. C) In the germinal centre, B cells undergo somatic hypermutation (SHM) and class switch recombination amplifying the autoimmune response and shaping the pathogenic autoimmune memory (D).
E) B cells may also control autoimmune disease via regulatory B cells.
From Salinas *et al* 2013 (144).

1.5.2 Autoimmunity and autoimmune diseases

Autoimmunity, defined as the presence of autoreactive B or T lymphocytes in the periphery, is a common and probably even physiological condition (144). It is caused by the fact that central tolerance mechanisms are not perfect, and allow a limited number of autoreactive cells to survive. There is evidence that low-affinity reactivity to self-antigens is required for survival of T and probably B lymphocytes in the peripheral immune system (151).

However, autoimmunity does not lead automatically to autoimmune disease (144). Autoantibodies may be found in healthy individuals and their prevalence may increase with age e.g. anti-nuclear antibodies (ANA) and rheumatoid factor (RF), or be a transient phenomenon possibly related to infections e.g. antiphospholipid antibodies. However, clinical autoimmune disease can also be preceded by the presence of autoantibodies, as shown by two studies in rheumatoid arthritis where approximately half of the patients were positive for RF or anti-citrullinated protein antibodies up to ten years before the onset of symptoms (152, 153). The progression from autoimmunity to autoimmune disease is not only determined by the degree of central tolerance leakage, but also by failure of peripheral tolerance checkpoints (144, 147).

It is estimated that autoimmune disorders collectively affect 3-10% of the general population (154). Multiple factors are thought to contribute to the development of autoimmune diseases, including the inheritance of susceptibility genes and environmental triggers such as infection and tissue injury.

1.6 The autoimmune response in acquired TTP

1.6.1 Acquired TTP as an autoimmune disease

Acquired TTP demonstrates some classic features of an autoimmune disease, such as the female preponderance and the tendency to cluster with other autoimmune conditions in a given individual or family. Depending on the patient cohort, 10-33% of patients presenting with acute acquired TTP with severe ADAMTS13 deficiency have another overt autoimmune disorder (138, 155). These may be systemic autoimmune disorders such as SLE, seen in 6.5% of a recent German cohort, or organ-specific diseases (a remarkable 23% of the cohort had Hashimoto's thyroiditis) (156).

1.6.2 Factors contributing to the development of TTP

1.6.2.1 Genetic factors

Evidence for genetic susceptibility in some individuals comes from studies of HLA associations in TTP. Three studies have shown an over-representation of the HLA-DRB1*11 allele in patients with acquired TTP and severe ADAMTS13 deficiency, with an incidence of 44-62% compared to a reported prevalence of 12-25% in healthy controls (157-159). HLA-DRB1*04 was underrepresented and HLA-DRB1*04/ DR*53 seemed to protect from a recurrent disease course. A pilot genome-wide association study (GWAS) of acquired antibody-mediated TTP in 44 Caucasian TTP patients found multiple SNPs in the HLA-II region which were significantly associated with TTP (160). Further associations were found with genes important in B cell development and function, and a large-scale GWAS study by the same group is underway.

1.6.2.2 Environmental factors

Environmental triggers are important in the development of TTP. HIV is well recognised as a cause of TTP (161), but many other infectious agents have been implicated including influenza A, parvovirus and Brucella (162-164). The large variety of microorganisms implicated suggests that direct molecular mimicry is unlikely as a mechanism. Triggering of the innate immune system by microbes via pattern recognition receptors (PRR) results

in upregulation of MHC class II and co-stimulatory molecules like CD40 and promotes the development of autoimmune lymphocytes (165). Pos *et al* have suggested that this may play a role at the onset of acquired TTP by lowering the threshold for activation of intermediate-affinity ADAMTS13-specific T cells that have escaped negative selection in the thymus (166). The inflammatory response may also lead to the release of DNA and histones, potentially precipitating the disease (167).

TTP may occur during pregnancy and the post-partum period. In the South East England Registry, 5% episodes were triggered by pregnancy (57). This may be due to alteration in substrate-enzyme balance precipitated by the increase in VWF and decrease in ADAMTS13 that occur in pregnancy (168)**.** However, oestrogen has also been shown to affect the differentiation and function of antigen presenting cells (169), and TTP may also be triggered by the combined oral contraceptive pill (57).

Drugs may also trigger autoimmunity. A variety of agents have been described which precipitate a TTP-like syndrome, but the majority of these actually cause a non-immune mediated TMA. Only quinine and ticlopidine have been shown to act via an immune mechanism, and quinine is associated with antibodies against platelets rather than ADAMTS13 (170). However, ticlopidine therapy increases the risk of developing anti-ADAMTS13 inhibitory autoantibodies by 200- to 300-fold (171) (172). Despite initial suspicions, clopidogrel has not been shown to increase the development of ADAMTS13 inhibitors (173).

Survival and maturation of autoreactive B cells at various stages depends on survival signals such as B-cell activating factor (BAFF). Plasma BAFF levels were elevated at presentation in one study of 66 patients with idiopathic antibody-mediated TTP (174). It is unclear whether increased BAFF is a primary cause of autoimmunity or whether autoimmunity is related to increases in pro-inflammatory cytokines such as type 1 interferons known to promote BAFF production (175) and which are upregulated in acute TTP (176). Interestingly, BAFF levels were not elevated in the cases of HIV-TTP

investigated which all had anti-ADAMTS13 IgG, suggesting the increase seen in idiopathic disease is not merely marker of tissue damage (174).

1.6.3 Antigen presentation in TTP

ADAMTS13 is efficiently internalised by antigen-presenting cells (APC) via the macrophage mannose receptor (177). A recent study has shown that dendritic cells exposed to ADAMTS13 presented peptides derived from several ADAMTS13 domains, and peptides derived from the CUB2 domain were presented with the highest efficiency (178). Dendritic cells from donors with an HLA-DRB1*11 allele exposed to a higher concentration of ADAMTS13 presented only differently processed versions of the same CUB2 peptide, which contains the predicted DRB1*11-binding sequence FINVAPHAR (178). The authors hypothesised that functional presentation of CUB2-derived peptides on HLA-DRB1*11 contributes to the onset of acquired TTP by stimulating low-affinity self-reactive CD4+ T cells (Figure 1.10).

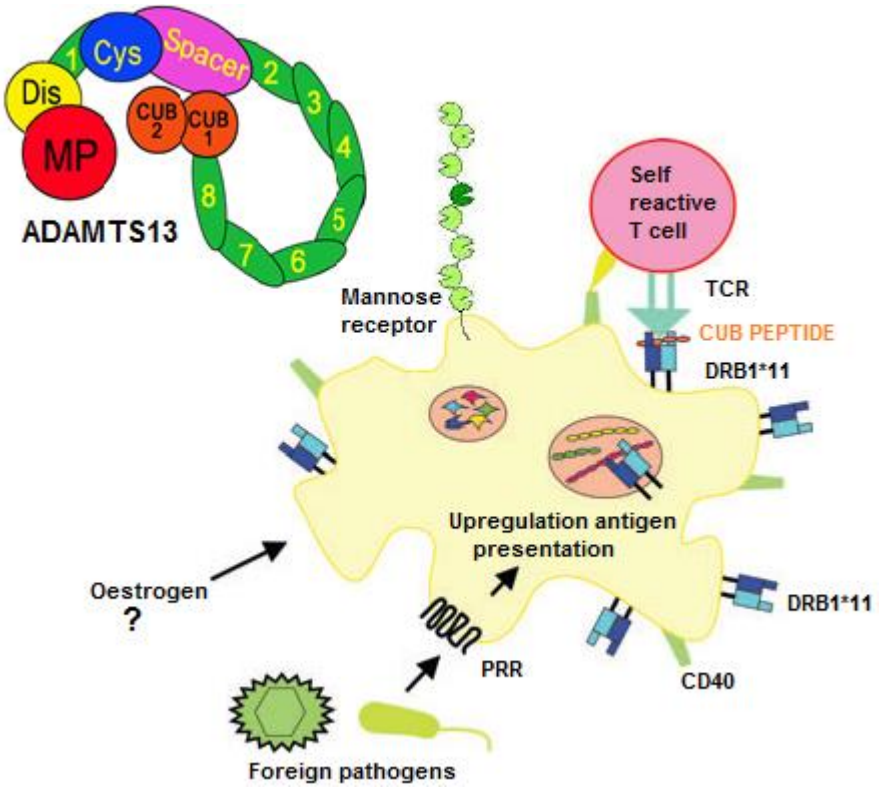


Figure 1.10 Hypothetical model for the activation of self-reactive ADAMTS13- specific CD4+ T cells

Triggering of the innate immune system by micro-organisms via pattern recognition receptors (PPR) results in upregulation of MHC class II and co-stimulatory molecules like CD40. ADAMTS13 is internalised by APC via the macrophage mannose receptor (177). CUB-2 derived peptides are preferentially presented in an HLA-DRB1*11 dependent manner (178). This may result in the activation of intermediate-affinity ADAMTS1-specific T cells that have escaped negative selection in the thymus. Oestrogens have been shown to affect the functional properties of APC. Adapted and modified from Pos *et al*, 2011(166).

1.6.4 The role of T cells in TTP

The association between HLA-DRB1*11 and acquired idiopathic TTP suggests that antigen-specific CD4+ cells contribute to anti-ADAMTS13 autoantibody formation. Additional evidence for the involvement of T cells in the pathophysiology of TTP comes from a clinical observation during therapeutic plasma exchange (166). After initial improvement, patients often experience a drop in the platelet count around day 7-10, a phenomenon that may be associated with an increase in ADAMTS13 inhibitor titres and is referred to as inhibitor boosting or exacerbation (179, 180). This phenomenon occurred less frequently in patients who received the T-cell immunosuppressant ciclosporin A (181).

The contribution of T regulatory cells to TTP pathophysiology has been evaluated by two groups. McDonald *et al* studied 50 patients with acquired TTP at different timepoints, and found no difference in T reg numbers at TTP presentation or during remission (182). Mariani *et al* reported increased numbers of CD4+/CD25+ T cells during remission in patients with a history of TTP when compared to healthy controls, and found that those with a history of recurrent disease had a lower percentage of CD4+/CD25+ cells than patients who had only had a single episode of TTP (183). However, CD25 positivity alone does not define cells with a regulatory function, and when the same authors investigated CD4+CD25+^{bright} cells and FoxP3+ cells, these were not altered in TTP cases. No group to date has performed functional analysis of these CD4+CD25+^{bright} cells and FoxP3+ cells which could reveal a qualitative change in Treg function, as has been described in some other autoimmune diseases (184, 185).

1.6.5 The antibody response in TTP

The autoantibody-mediated response appears central to most cases of acute idiopathic TTP. Autoantibodies directed towards ADAMTS13 are present in the plasma of the majority of acquired TTP patients with ADAMTS13 activity <5% (138).

1.6.5.1 Isotype and subclasses of anti-ADAMTS13 antibodies

Anti-ADAMTS13 antibodies are comprised predominantly of immunoglobulin class G (IgG), although IgM and IgA have been reported in a limited number of patients (83-85). The clinical significance of non-IgG antibodies is unclear. The main IgG subclasses of anti-ADAMTS13 antibodies are IgG_4 (69-90%) and IgG_1 (52-73%) (50, 84, 186). Lower levels of IgG_2 are present (50, 84). Levels of IgG_3 varied in different studies with Ferrari *et al* and Pos *et al* finding lower titres (average 20%) but Bettoni *et al* and McDonald *et al* finding high IgG_3 titres during acute TTP episodes (50, 84, 186, 187).

In one study, both mortality and number of PEX to remission increased with increasing number of IgG subclasses present (186), and IgG_2 was associated with mortality and cardiac disease. However, another study found that high levels of IgG_1 with low / undetectable IgG_4 were associated with an adverse outcome (84). The same group found high levels of IgG_4 to be associated with increased risk of relapse. All relapsed cases had IgG_4 antibodies and IgG_4 levels were inversely correlated with IgG_1 (84). This may be explained by subclass switching from IgG_1 to IgG_4, possibly resulting from continuous antigenic stimulation (166).

However, the significance of an elevated IgG_4 titre is unclear, since IgG_4 cannot activate complement (in contrast to IgG_1 antibodies which activate the classical complement pathway by binding FcγRs). High titre IgG_4 may activate T reg cells secreting IL-10, thereby stimulating the TH2 response and downregulating the TH1 response leading to a dysregulated humoral immune response as is seen in autoimmune pancreatitis (188, 189). In general, a broader isotype/subtype usage of antibodies not only indicates a

more extensive B cell activation but, more importantly, implies that different effector functions can cooperate to enhance autoimmune disease (144).

1.6.5.2 Immune complexes

The extent to which anti ADAMTS13 antibodies circulate free or in a complex with ADAMTS13 (thus forming immune complexes, IC) and how this changes over time has been studied by one group using both co-immunoprecipitation of ADAMTS13 with immunoglobulins, and an ELISA technique (92, 190). They analysed a cohort of 68 patients with acquired TTP at presentation and found that 100% had free IgG antibodies and 95% had ADAMTS-13 specific IC.

In the 28 patients studied in remission, 75% had free antibodies (mainly IgG) and a remarkable 93% had persisting immune complexes. The IC mainly comprised IgG_4 antibodies, and whilst ADAMTS13 inhibitor, anti-ADAMTS13 antibody titre and levels of $IgG_{1\text{-}3}$ IC all decreased in remission, IgG_4 immune complexes persisted over years even in patients who had received rituximab and showed no sign of relapse (92). The clinical relevance of these IgG_4 complexes in remission is unclear, as IgG_4 is unable to fix complement and tends to form small-sized ICs, which would have limited effector functions. There was an inverse correlation between free and complexed anti-ADAMTS13 antibodies during the acute phase in the four patients studied longitudinally, but this relationship did not hold in remission (92, 190).

Lotta *et al* measured total ADAMTS13 IC with another novel ELISA in TTP patients with anti-ADAMTS13 IgG by WB and ADAMTS13 activity <10% at presentation, and found IC in seven out of 15 acute presentations and 10/21 patients in remission (191). Seven out of 19 patients with negative anti-A13 WB and activity <45% at presentation had circulating ADAMTS13 IC, and this was confirmed by immunoprecipitation. ADAMTS IC were not associated with ADAMTS13 activity, antigen or anti-ADADMTS13 IgG in this cohort. However, in the 15 patients studied with acute TTP, increasing levels of

ADAMTS13 IC were associated with increased number of PEX to remission (191).

1.6.5.3 Domain specificity of anti-ADAMTS13 antibodies

1.6.5.3.1 Spacer domain

Work on epitope mapping of antibodies in patients with acquired TTP has identified antibodies to the spacer domain in virtually all the patients studied to date (50, 192-196). The investigators used a range of different materials and methods – see Figure 1.11 and Table 1.3.

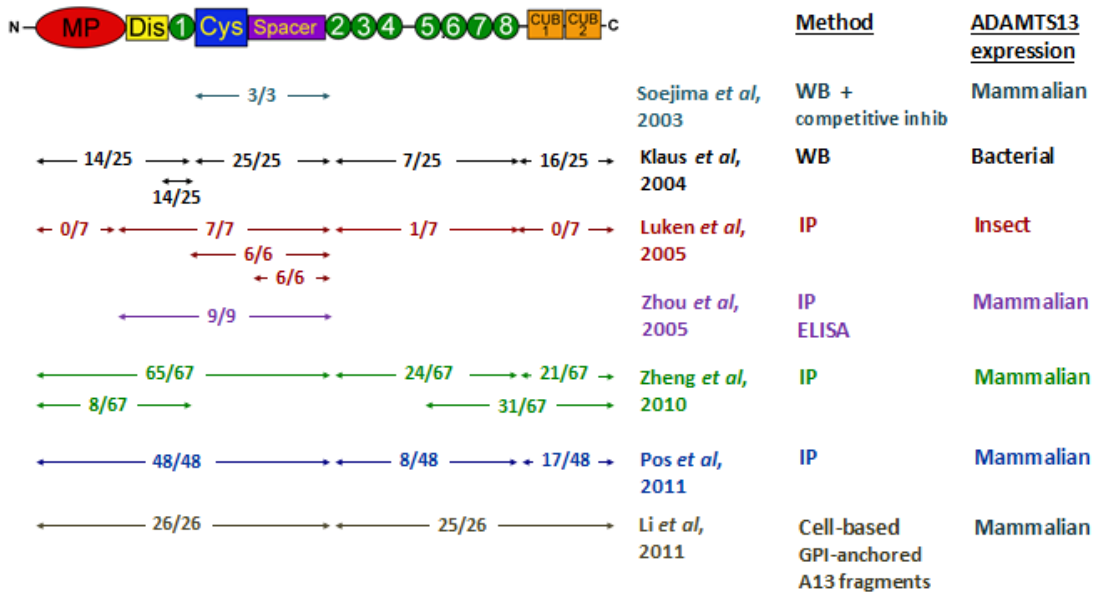


Figure 1.11 Summary of results of domain specificity of anti-ADAMTS13 IgG antibodies in acquired TTP in published studies

Number of patients positive for antibodies against domains/numbers tested shown beneath

Study	Method	ADAMTS13 protein expression	No. of patients	Domain	Result
Luken *et al, 2006 (194)*	Ig V gene phage-display library → mAb IP	Insect	1 (remission)	S DT	3 mAb 1 mAb
Yamaguchi *et al, 2011 (197)*	ADAMTS13 phage library immobilised & in solution	Bacterial	13 (2 failed)	Peptides from Signal/pro M D T C S 2-8 CUB	 2/11 5/11 2/11 1/11 2/11 7/11 7/11 1/11

Table 1.3 Other methods used for epitope mapping of anti-ADAMTS13 antibodies

M=metalloprotease domain, D=disintegrin-like domain, T=TSP1 domain, DT=disintegrin/TSP1 domain fragment, C=cys-rich domain, S=spacer domain, 2-8=TSP2-8 domains, CUB=CUB domains

Further work identified two amino acid regions in the spacer domain containing residues necessary for the binding of anti-ADAMTS13 autoantibodies (198). These two regions were not exclusively required for antibody binding, as several other regions also contributed to the antigenicity of the spacer domain. The majority of anti-ADAMTS13 antibodies in patients with acquired TTP in a subsequent study targeted a single epitope comprising $Arg^{660,}$ $Tyr^{661,}$ and Tyr^{665} (RYY) on the outer surface of the spacer domain – Figure 1.12 (199). Further work showed that residues Arg^{568} and Phe^{592} also contribute to this antigenic surface (50), and epitope analysis of ADAMTS13 autoantibodies by a Japanese group using a phage library expressing various ADAMTS13 peptides found the peptide sequence Gly^{662}-Val^{687} in multiple clones in 5 out of 11 patients (197).

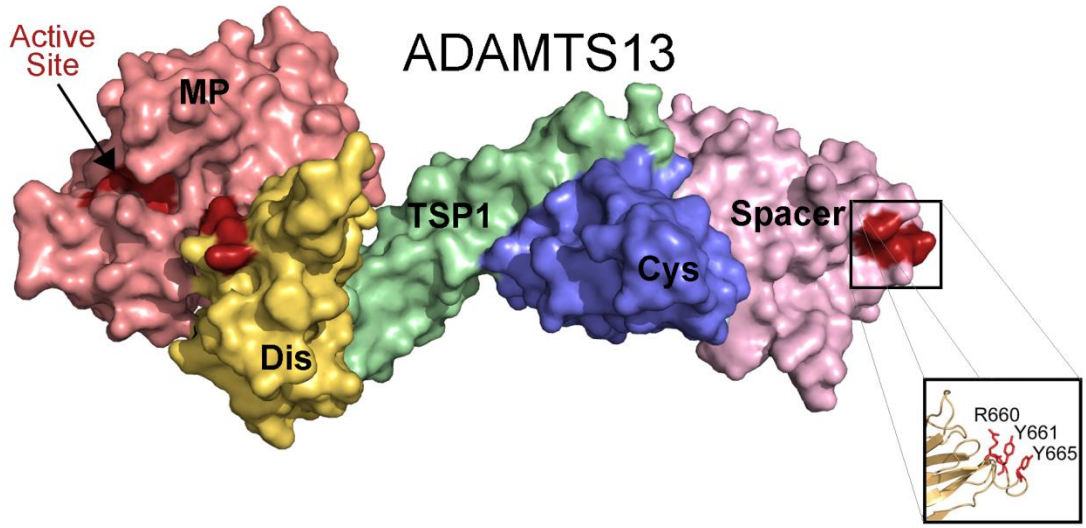


Figure 1.12 Antigenic surface of spacer domain shown on the crystal structure of DTCS

Residues R660, Y661 and Y665 align as a cluster on the surface of ADAMTS13 MDTCS. Adapted from Pos *et al*, 2010 (199)

Deuterium-hydrogen exchange coupled with mass spectrometry has been used by one group recently to identify the autoantibody binding site of a human monoclonal antibody fragment (single-chain Fv, scFv) isolated by phage display from a TTP patient (200). They demonstrated that the scFv bound residues Arg^{636}, Leu^{637}, Arg^{639} and Leu^{640} in the spacer domain and termed this 'exosite 4'. The mAb fragment also bound RYY in exosite 3, as well as Lys^{608} upstream. Site-directed mutagenesis of these residues abolished/significantly reduced the antibody fragment binding but also abolished/ reduced ADAMTS13 activity in a FRETS assay and in proteolysis of multimeric VWF, suggesting this epitope is also part of the VWF binding site (200).

These results are consistent with studies demonstrating the critical role of the ADAMTS13 spacer domain in the binding and proteolysis of VWF (48, 49, 201). As discussed previously, the spacer domain binds a cryptic VWF A2 domain exosite that is revealed upon unfolding of globular VWF (202). Autoantibodies binding these residues are likely to interfere with binding of ADAMTS13 to unfolded VWF A2 domain and limit VWF processing (199). Whether the spacer domain is particularly immunogenic, or resembles epitopes found in microbial pathogens is not known.

1.6.5.3.2 Antibodies targeting other N terminal domains

Despite the frequency of autoantibodies against the spacer domain, patients may have additional antibodies that recognise other domains (Figure 1.11) demonstrating the polyclonal nature of the autoimmune response.

More than half of the patients in one study using bacterially expressed material had antibodies detected against the first TSP1 domain (192). There was some discrepancy between this and other studies which found no reactivity with the disintegrin-TSP1 domains (193), and only 12% of patients' samples reacting with a fragment comprising the metalloprotease-disintegrin-TSP1 domains (MDT) (196). The role of the TSP1 region is not clear but it may facilitate interactions either directly with VWF (49), or possibly with cells via CD36 (203). Although antibodies against the MP and Dis domains are less common, given the essential role of these domains in VWF proteolysis (51, 204), it is expected that autoantibodies against these regions would most likely be inhibitory.

The exact function and frequency of antibodies against the cys-rich domain is also unclear. Several groups studied the cys-rich spacer fragment (either directly or by subtraction) and found antibodies against this region in the majority of patients investigated (192, 193, 195, 196). However further analysis by Luken *et al* showed the 6 patients in their study had anti-spacer antibodies. They were not able to study the cys-rich domain in isolation as it was retained intracellularly (193) but in subsequent work 6/6 patients did not bind DTCSpacer1 (a hybrid construct with the spacer domain from ADAMTS1) suggesting no autoantibody formation against the cys-region domain in this group (198). Epitope analysis using a phage library expressing various peptides of ADAMTS13 found 2 out of 13 patients had IgG which recognised peptides from the cys-rich domain (205), although this approach only allows detection of short linear sequences.

1.6.5.3.3 Antibodies targeting the C terminal domains

Antibodies to the distal region of the ADAMTS13 protein may interfere with substrate recognition or lead to ADAMTS13 depletion by the formation of antibody-antigen complexes, but there is conflicting evidence in the literature on their prevalence. In an early study using a Western blotting technique with bacterially expressed material, two-thirds of patients had antibodies against the CUB domains (192). A prevalence of approximately one-third was found in two studies using an immunoprecipitation technique with mammalian expressed material (50, 196), whilst a smaller study found no anti-CUB antibodies in the seven patients tested using immunoprecipitation with ADAMTS13 fragments expressed in insect cells (193).

The prevalence of anti-TSP2-8 antibodies at presentation has been variously reported as 14-37% (50, 192, 193, 196). One study found the presence of IgG antibodies against TSP2-8 and/or CUB was inversely correlated with patient platelet counts on admission (196). A handful of patients have been described with IgG that predominantly targets C-terminal domains with no or weak reactivity towards the N-terminal fragment MDTCS (196, 206). A recent study using a novel flow cytometric methodology found IgGs from 25/26 patients with acquired TTP bound to cells expressing a GPI-anchored C-terminal fragment TSP2-8 plus CUB (207).

Thus, previous studies of the domain specificity of anti-ADAMTS13 antibodies have used a range of different protein expression methods and assay techniques. Early studies used bacterially expressed material which whilst allowing synthesis of large quantities of protein, does not allow for normal glycosylation and may not result in material being correctly folded / displaying physiological epitopes. Expression of proteins in mammalian cells is more time-consuming with much lower yields, but is more likely to result in correctly folded protein. Immunoblotting is potentially straightforward, gives a good overview of the domains affected by an autoimmune response, but may not identify conformation-specific epitopes. In contrast, immunoprecipitation allows antibody-antigen binding in solution, meaning conformation-specific epitopes are not disrupted, but is labour-intensive for large numbers of samples and protein fragments. Peptide libraries offer good coverage of the protein in short peptides but again cannot detect conformational epitopes

From the available studies to date, the spacer domain appears to be the major antibody binding domain in patients with idiopathic TTP, although antibodies with specificity for other regions including the C-terminal domains have been identified in subsets of TTP patients.

Interestingly, whereas the dominant B cell epitope lies in the spacer domain, the major T cell epitope appears to be in the CUB domain (178). Although T cell and B cell epitopes are often close or even overlap, they can also be derived from spatially distant regions of a protein, because the presentation of T cell and B cell epitopes to the immune system is fundamentally different (208). CD4+ T cell epitopes consist of short contiguous peptide fragments bound to MHC class II on antigen presenting cells which are recognised by the TCR. In contrast, B cell epitopes are 3-dimensional surfaces of folded proteins which are recognised by antibodies or the receptors of memory cells, and are often comprised of non-contiguous amino acid sequences (208). However, given the recent data on the ‘closed’ conformation of ADAMTS13 in circulation mediated by a CUB-spacer domain interaction (53), the major B cell and T cell epitopes may indeed be in close proximity.

1.6.5.4 Non-neutralising antibodies

The use of Western blotting or immunoprecipitation techniques gives a good overview of the regions and domains affected by an autoimmune response, and allows major immunogenic regions to be identified (192). It does not, however, reveal the extent to which the different antibodies contribute to the IgG levels identified from routine ELISA assays, and which are inhibitory or non-inhibitory. In some TTP patients, non-neutralising IgG antibodies can occur that do not inhibit protease activity *in vitro* but may compromise ADAMTS function *in vivo* by influencing plasma half-life (209). Studies have found that 10-15% of the autoantibodies found in patients with acquired TTP are non-neutralising (83, 84). These studies used an ELISA to detect anti-ADAMTS13 IgG in TTP patients and a Bethesda-type assay using a full-length VWF substrate ADAMTS13 activity assay to detect inhibitors. It should be remembered that no one ADAMTS13 activity assay reflects the *in vivo* situation in TTP, and the requirement for a denaturant in the activity assay may alter the proportion of antibodies found to be inhibitory.

A recent study investigated the anti-ADAMTS13 IgG found in up to 5% of healthy individuals (81, 210). They isolated anti-ADAMTS13 autoantibodies in a 2-step chromatographic purification procedure (ADAMTS13 affinity matrix and protein G) from three acquired TTP patients and a pool of 45 randomly selected healthy donors (134). The anti-ADAMTS13 autoantibodies present in healthy individuals were non-neutralising in the FRETS-VWF73 activity assay, and showed low affinity towards ADAMTS13 using Biacore (in contrast to the high affinity inhibitory IgG in the three patients).

Epitope mapping of the anti-ADAMTS13 IgG from the normal donors using immunoprecipitation of mammalian ADAMTS13 fragments showed a similar pattern to the TTP patients, in that that it interacted with full length ADAMTS13, MDT and MDTCS but recognised the C terminal domains more weakly. However, unlike the TTP IgG which recognised MDTCS more strongly than MDT, the IgG from normal donor bound more strongly to MDT than MDTCS (134). The group also used peptide arrays for epitope mapping

and demonstrated that the anti-ADAMTS13 IgG in healthy individuals shares linear epitopes with TTP patient IgG, with shared recognition of peptides from the metalloprotease, disintegrin-like and spacer domains, and also TSP8 and CUB (134). However, the usefulness of the peptide array approach is called into question by the fact that no hit was observed for the peptide including the spacer residues Arg^{660}, Tyr^{661} and Tyr^{665} (RYY), which is considered the main target for autoantibodies from many acquired TTP patients (199). However, the group speculated that low affinity non-inhibitory anti–ADAMTS13 antibodies occurring in some healthy individuals might provide a template for the emergence of high affinity pathogenic antibodies in acquired TTP.

To date, the precise role and domain specificity of non-inhibitory anti-ADAMTS13 antibodies in TTP patients have not been defined. Antibodies directed against the C terminal domains of ADAMTS13 which block VWF binding might modulate enzyme function beyond what is measured routinely.

1.6.5.5 The role of VH1-69

Gene analysis of the variable heavy chain of anti-ADAMTS13 antibodies revealed predominant usage of the *VH1-69* germline gene (211). Pos *et al* hypothesised that “shape complementarity” between *VH1-69* encoded variable domain residues and exposed exosites in the spacer domain explains the frequent usage of the *VH1-69* gene segment in anti-spacer antibodies (166). The heavy chain complementarity determining region 2 (HCDR2) of the VH1-69 germline contains a unique hydrophobic “Ile-Ile-Pro-Ile-Phe” motif (211) which may interact with hydrophobic residues Tyr^{661} and Tyr^{665} present on the antigenic surface of the spacer domain (26, 199).

1.6.5.6 Longitudinal analysis of the anti-ADAMTS13 humoral response

There has been very limited work on the longitudinal immune response in TTP to date. One study in a single patient studied the evolution of the anti-ADAMTS13 immune response (100). The patient had a normal ADAMTS13 activity by static and flow-based ADAMTS13 activity assays at presentation and experienced five relapses over eight years during which he developed a severe ADAMTS13 deficiency, first by the flow-based assay and subsequently in the static assays.

Epitope mapping was performed by immunoprecipitation using the fragments PMDTCS-13 (N-terminal domains of ADAMTS13 from propeptide to spacer), PMDTCS-1 (a hybrid construct with the spacer domain from ADAMTS1), TSP2-8 and CUB1-2 expressed in insect cells. The principal antigenic epitope in all the acute TTP episodes was in the spacer domain since antibodies from each episode recognised PMDTCS-13 but reacted weakly with PMDTCS-1. There were weak anti-CUB antibodies in all episodes but no evidence of anti TSP2-8 antibodies. There was no evidence of epitope spreading to other domains during the evolution of the immune response in this study.

A possible explanation of the observed change in properties of the anti-ADAMTS13 antibodies in this case from non-inhibitory to inhibitory might be affinity maturation by somatic hypermutation of the patient's immunoglobulin genes (100). B cells producing non-neutralising antibodies might be precursor cells from which inhibitory anti-ADAMTS13 B cells can then evolve by somatic hypermutation later in the disease. Another possible explanation might be IgG subclass switching (189). In this patient, the demonstration of ADAMTS13 inhibition in activity assays coincided with a rising titre of anti-ADAMTS13 antibodies, thus another explanation might be that very low levels of such antibodies might be sufficient to lead to an acute episode of TTP but not be sufficient to make *in vitro* assays demonstrate severe ADAMTS13 deficiency (99).

However, this study was of single atypical patient. The patient had HIV-TTP and thus had major immunological defects in the T-cell as well as B-cell compartments. He was non-compliant with highly active anti-retroviral therapy (HAART), had recurrent *Staphylococcal aureus* infections and responded unusually quickly to plasma exchange in the first episode (requiring only five PEX to remission with no exacerbation on stopping). This case is therefore not generalisable to idiopathic acquired TTP.

In another longitudinal study of a single patient, the rising inhibitor titre was due to both an increase in anti-ADAMTS13 IgG concentration and increased inhibitory activity of the antibodies (212). There was also a shift in the IgG subclasses from IgG_1 to IgG_2. Previous studies on the same patient showed that throughout the disease course her antibodies interacted with full length ADAMTS13, but not with fragments truncated upstream of the spacer domain, suggesting the presence of anti-N terminal antibodies alone (213).

Thus, IgG antibodies play a central role in the pathogenesis of most cases of acute TTP but the immunological response in TTP has not been studied in detail longitudinally nor the relative contribution of inhibitory / non-inhibitory antibodies against different domains determined.

1.7 Hypothesis

TTP remains a life-threatening disease with a 10-20% mortality rate, which has not improved significantly since the introduction of PEX therapy. Relapses have been reported in up to 50% cases. We currently have only limited ability to identify those individuals with a more severe disease phenotype who are most at risk of death, or to determine which patients are more likely to relapse. It is currently unclear which are the primary pathogenic species of antibody. Thorough investigation of the antibody repertoire in TTP patients performed both at presentation and longitudinally may improve our ability to prognosticate in acquired TTP.

TTP is unlike many autoimmune disorders inasmuch as the target is a single soluble plasma protein. This is unusual as many autoimmune targets are cellular and involve complement activation on the surface of those cells. The basis of the hypothesis was that different domains of ADAMTS13 are variably functionally important, and therefore antibodies against different domains may also have different pathogenic potential.

I therefore hypothesise that:

1. Autoantibodies against different ADAMTS13 domains inhibit ADAMTS13 to different extents and therefore contribute variably to the pathogenesis of TTP.
2. The identification and characterisation of the repertoire of antibodies in longitudinally collected samples in a number of patients with acute idiopathic TTP, provides a means to identify those patients most likely to achieve remission and those at higher risk of relapse. This in turn, may allow a means of monitoring TTP patients during treatment and possibly tailoring therapy accordingly.

1.8 Aims

The main aim of this study was to identify a better way to risk stratify patients with acquired TTP, in order to identify individuals who might die from the disease as opposed to those who would respond well to treatment. It is clear from existing work that the autoimmune response in acquired TTP is polyclonal. However, it is not known whether or not antibodies directed against different domains of ADAMTS13 are variably pathogenic. It is also unclear how the domain specificity of the antibodies changes in response to treatment and whether re-emergence of antibodies against different domains may be indicative of/predictive of relapse.

To test my hypothesis with specific reference to these unknowns, the thesis is divided into chapters with aims as follows:

1. To express recombinant ADAMTS13 domain fragments in both bacterial and mammalian expression systems to use in domain specificity studies (chapter 3).
2. To develop novel assays to determine the domain specificity of anti-ADAMTS13 antibodies (chapter 3).
3. To determine the domain specificity of anti-ADAMTS13 antibodies at presentation of acquired TTP and study the clinical correlates of this (chapter 4).
4. To explore the contribution of different pathogenic mechanisms in acquired TTP (chapter 5).
5. To study the humoral response in TTP longitudinally through therapy, remission and relapse (chapter 6).

2 METHODOLOGY

2.1 Expression, purification and refolding of ADAMTS13 domain fragments in bacteria

2.1.1 Generation of bacterial expression vectors

2.1.1.1 Bacterial ADAMTS13 domain fragment vectors generated

Certain bacterial expression vectors (pET100 (Invitrogen) - fusing a polyhistidine and Xpress epitope tag to the amino-terminus of different ADAMTS13 domain coding regions - Figure 2.1) had previously been generated in our lab from earlier work in the group. Available expression vectors were MP, Dis-TSP1, Spacer, TSP2-4, TSP5-8 and CUB1/2 (Figure 2.2).

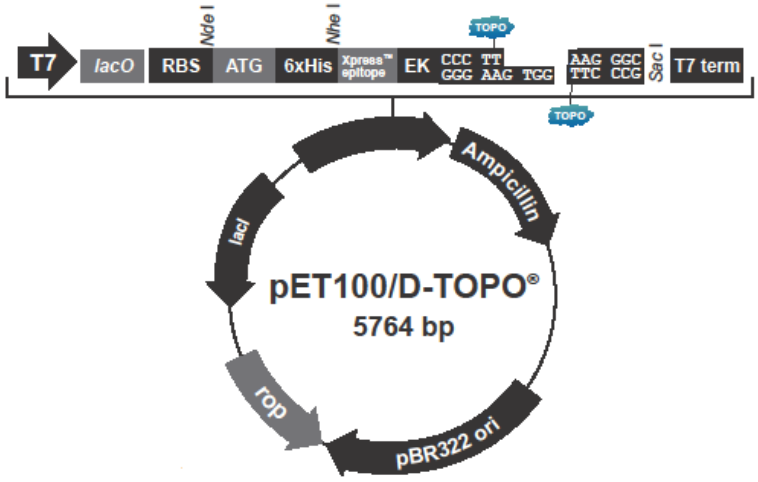


Figure 2.1 Map of pET100/D-TOPO bacterial expression vector

Ampicillin = ampicillin resistance gene; Xpress ^{TM} epitope=tag for detection; 6xHis = polyhistidine tag which aids in purification.

The pET100 vector has a T7/lac promoter for high level isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible expression of the gene of interest in *E. coli* expression, and directional TOPO© cloning site for directional cloning of blunt-ended PCR products. It has a cleavable detection tag (Xpress), 6xHis tag for purification and antibiotic resistance marker for selection in *E.coli*. High level expression is achieved because the T7 RNA polymerase is more processive than native *E.coli* polymerase and is dedicated to the transcription of the gene of interest (Invitrogen).

I generated vectors for the remaining domain fragments (TSP1 and Cys – Figure 2.2) by PCR amplification using KOD Xtreme™ Hot Start DNA polymerase (Novagen) and full-length ADAMTS13 plasmid DNA as a template, as detailed in the following sections.

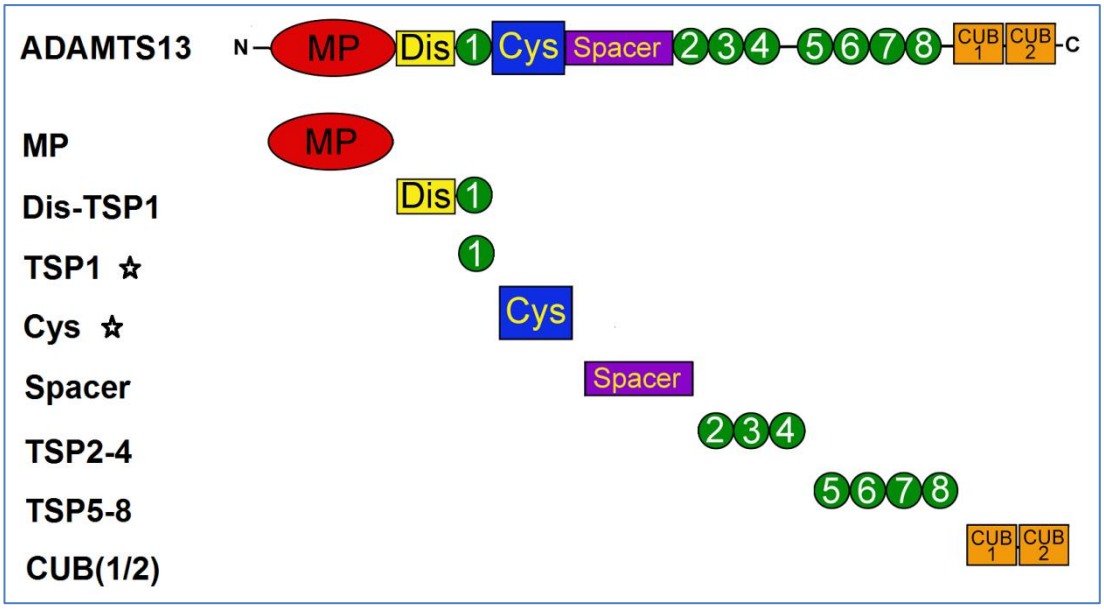


Figure 2.2 ADAMTS13 domains expressed in *E.coli*

Bacterial expression vectors MP, Dis-TSP1, Spacer, TSP2-4, TSP5-8 and CUB(1/2) were available from earlier work. The remaining domain fragments (TSP1, Cys) were generated by PCR amplification and cloned into the pET100 vector. ☆ = novel vector

2.1.1.2 Generation of ADAMTS13 domain fragments by PCR

Blunt-end PCR products encoding for the domain fragments TSP1 and Cys were generated by PCR amplification using KOD Xtreme™ Hot Start DNA polymerase (Novagen) and full-length ADAMTS13 plasmid DNA as a template. Primers were designed according to the guidelines in the KOD HotStart DNA polymerase protocol (Novagen). The four base pair sequences (CACC) necessary for directional cloning were included on the 5' end of the forward primer. All primers were manufactured and HPLC purified by Thermo Fisher, Germany. PCR reactions were performed using 1µl KOD Xtreme™ Hot Start DNA polymerase (1U/µl); 1.5µl of sense (5') primer and 1.5µl of anti-sense (3') primer (10µM each); 10ng of plasmid DNA template; 25µl of 2x reaction buffer, 10µl of dNTP's (2mM each) and PCR grade water to a final volume of 50µl. The basic amplification conditions were as follows: polymerase activation (94°c, 2min); 25 cycles of denaturation (98°c, 10 sec), annealing (lowest primer Tm°c, 30 sec) and extension (68°c, 1 min per kb) with a final extension at 68°c for 7 minutes.

2.1.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to size and determine the success of PCR in generating a product of the expected size as a single discrete band. 1-1.2% agarose gels (w/v) were prepared in TBE buffer (89mM Tris-HCL, 89mM boric acid and 2mM EDTA) containing 5µg/ml SYBR™ safe DNA gel stain (Invitrogen). Plasmid DNA or a 1 kb ladder (NEB) was mixed with 6xDNA loading buffer (40% w/v sucrose and 0.09% bromophenol blue), loaded and run on the agarose gel at 50v for 30 minutes. DNA was visualised using a Safe Imager™ blue light transilluminator (Invitrogen), and the amount of the PCR product estimated by comparison to the ladder.

2.1.1.4 DNA extraction from an agarose gel

DNA bands of the predicted size containing fragments of interest were purified using the QIAQuick Spin gel extraction kit according to manufacturer's instructions (QIAgen). Briefly, gel slices containing DNA were re-suspended in three volumes of solubilisation buffer and incubated at 50°c

for ten minutes. One volume of isopropanol was added and the sample applied to column containing a silica membrane for selective adsorption of plasmid DNA in high-salt, low pH buffer. After centrifugation, the column was washed with an ethanol-containing buffer and the DNA eluted with 50µl low-salt buffer of higher pH.

2.1.1.5 Directional TOPO cloning

Once a PCR product of the correct size was obtained, the cDNA fragment was cloned by directional TOPO© cloning into the pET100/D-TOPO vector (Invitrogen) according to manufacturer's protocol. This PET vector has topoisomerase covalently attached to its end allowing the PCR fragment to be readily ligated into the linearised vector (Figure 2.3).

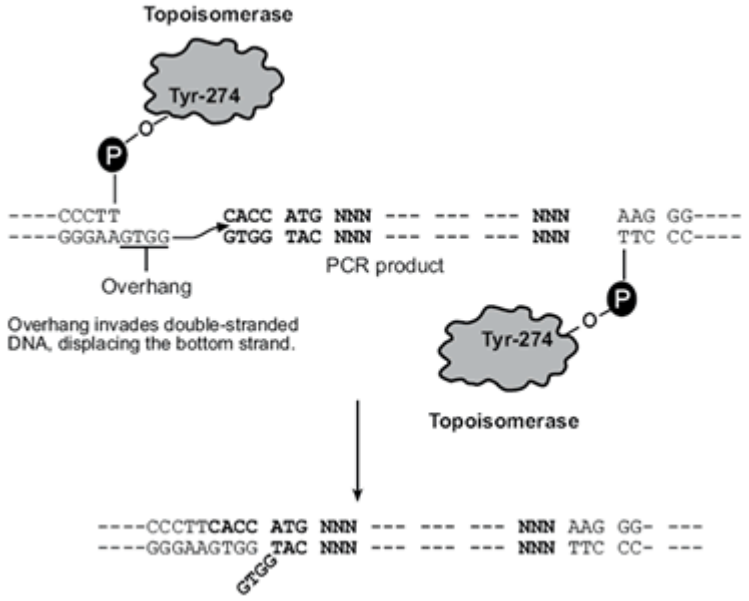


Figure 2.3 Directional TOPO© cloning (Invitrogen)

Directional joining of dsDNA using TOPO©-charged vector with a four nucleotide overhang. PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases and stabilises the PCR product in the correct orientation.

A 1:1 molar ratio of PCR product to vector was used in a salt solution and the reaction mix incubated for 5 minutes at room temperature (RT). The ligation product was then placed on ice and immediately used to transform competent *Escherichia coli* (*E.coli*).

2.1.1.6 Transformation of competent cells

Competent cells (One shot TOP10 cells, Invitrogen; NEB Turbo cells, New England Biolabs or XL-10 Gold cells, Stratagene) were transformed with the ADAMTS13 vector according to manufacturer's instructions. 3µl of the TOPO cloning reaction was added to a vial of chemically competent *E. coli.* The tube was incubated on ice for 30 minutes, then the cells were heat-pulsed in a water-bath at 42°c for 30 seconds, before being placed back on ice for an additional 2-5 minutes. Transformed bacteria were grown in 250-950µl of preheated S.O.C. medium at 37°c for 1 hour with shaking. Bacteria were subsequently plated out on a pre-warmed LB-agar (Invitrogen) selection plate containing 100µg/ml ampicillin and incubated at 37°c overnight. Ampicillin-resistant colonies were picked and grown in 5ml LB-broth (Invitrogen) with 100µg/ml ampicillin overnight at 37°c with shaking.

2.1.1.7 Generation of glycerol stocks

700µl of the bacterial culture from section 2.1.1.6 was mixed with 300µl of autoclaved 50% glycerol solution. This glycerol stock was vortexed and stored at -80°c, and could be partially thawed if additional transformed bacteria were required at a later date.

2.1.1.8 Plasmid miniprep and sequencing

Plasmid DNA was extracted from the small volume bacterial culture using the Qiagen Miniprep kit according to manufacturer's instructions. Briefly, the culture was centrifuged for 20 minutes at 4000rpm and the supernatant decanted. The cell pellet was resuspended in 250µl resuspension buffer containing RNase and mixed with 250µl alkaline lysis buffer. After five minutes, 350µl of neutralisation buffer was added and precipitated proteins, cell debris and chromosomal DMA were spun down by centrifugation at 13000rpm for 20 minutes. Supernatant was passed over a column containing a silica membrane for the selective adsorption of plasmid DNA in high-salt buffer. The column was washed with an ethanol-containing buffer, then plasmid DNA was eluted with 50µl low-salt buffer. All new vectors were verified by sequencing prior to use using T7 or T7 reverse primers. DNA was sequenced at the MRC CSC Genomics Core Laboratory.

2.1.2 Expression in Rosetta *E.coli*

All recombinant ADAMTS13 domain fragments were expressed at very high levels in Rosetta DE3 *E.coli* (Novagen) (72). The Rosetta cells are genetically modified to allow encoding of eukaryotic tRNA species that are rare in bacteria, thereby enhancing eukaryotic protein synthesis. DE3 indicates that the cells carry a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter, and are therefore suitable for protein production from target genes cloned in pET vectors by induction with IPTG.

Competent Rosetta DE3 cells were transformed with 1ng of each construct DNA. Briefly, DNA was added to 20µl cells and mixed gently. After incubation on ice, the cells were heat-shocked at 42°c for 30 seconds then immediately transferred to ice. Transformed bacteria were grown in 80µl of preheated S.O.C. medium at 37°c for 1 hour with shaking, then each transformation reaction was plated onto a pre-warmed selection plate and incubated overnight at 37°c.

Cultures of transformed Rosetta *E.coli* in 2xYT broth (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH7.0) were grown at 37°c with shaking until in Log-phase growth (OD_{600} 0.6-1.0 or approximately 2-6 hours). Recombinant protein expression was induced by the addition of 1M IPTG to a final concentration of 1mM. Cells were cultured for a further 12-18 hours at 37˚C/250rpm. Bacteria were harvested by centrifugation at 5000xg for 30 minutes at 4°c; the supernatant was discarded and the cell pellet stored at -80°c.

2.1.3 Purification and refolding of bacterially expressed proteins

2.1.3.1 Inclusion body preparation

The high level of ADAMTS13 domain expression in the bacteria results in insoluble protein aggregates known as inclusion bodies. Inclusion bodies containing the recombinant domain fragments were prepared using BugBuster reagent (Novagen), rLysozyme and Benzonase Nuclease

(Novagen), according to manufacturer's instructions. The resulting pellet of purified inclusion bodies was stored at -80°c.

2.1.3.2 A13 domain purification and refolding by metal ion affinity chromatography

ADAMTS13 domain fragments were purified from inclusion body preparations under denaturing conditions (8M urea) by fast pressure liquid chromatography (FPLC) in a single step using a Ni^{2+}-chelating HiTrap column coupled to an ÄKTA FPLC (GE Healthcare) with Unicorn 5.1 software. This was combined with a novel on-column refolding strategy in an attempt to produce soluble, correctly folded protein.

A Ni^{2+} chelating HiTrap column (GE Healthcare) was charged with nickel(II) sulphate hexahydrate. The column was equilibrated with solubilisation/ loading buffer (8M urea, 500mM NaCl, 20mM Tris pH 8.0, 30mM imidazole). Inclusion bodies were resuspended from the pellet using 5ml solubilisation/loading buffer and 10mM dithiothreitol (DTT) with vortexing and 10-20 minute incubation. Samples were diluted with solubilisation/loading buffer to a volume of 40ml (thus reducing DTT concentration to <2mM) and incubated with mixing for 20 minutes, before centrifuging to remove bacterial debris. The supernatant was filtered through a 0.45µm syringe filter before being loaded onto the Ni^{2+}-chelating HiTrap column. The column was then washed with solubilisation/loading buffer.

To optimise on-column refolding, buffers containing a glutathione redox pair (500mM NaCl, 20mM Tris pH 8.0, 1mM reduced glutathione, 0.1mM oxidised glutathione), glycerol and sucrose (500mM NaCl, 20mM Tris pH 8.0, 15% (v/v) glycerol and 8% (w/v) sucrose) were sequentially passed over the column (Figure 2.4).

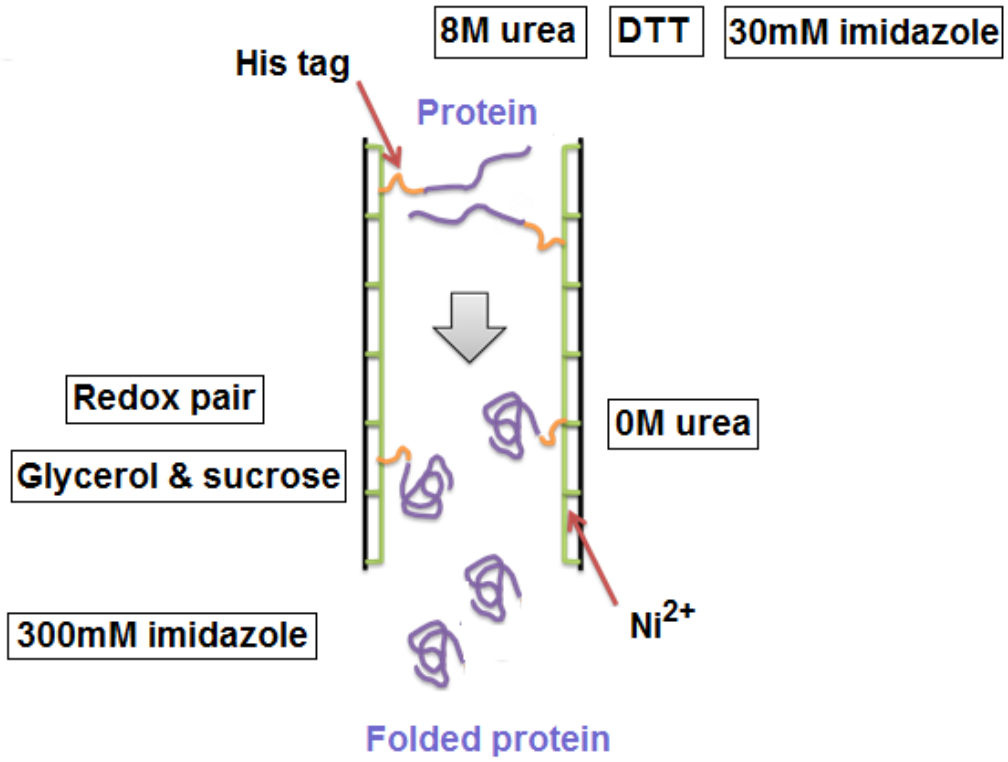


Figure 2.4 On-column refolding strategy

The unfolded protein is loaded onto the column in a denaturing buffer with low imidazole concentration to reduce non-specific protein binding. The protein binds via the metal ion binding His-tag. Sequential buffer change removes the urea in the presence of agents to optimise correct refolding on column, then the folded protein is eluted with a higher concentration of imidazole.

The His-tagged proteins were eluted by a gradient over 5-10 minutes into ice-cold elution buffer containing a higher concentration of imidazole (500mM NaCl, 20mM Tris pH 8.0, 300mM imidazole, 15% (v/v) glycerol and 8% (w/v) sucrose). This aimed to gradually increase the protein concentration in the eluted fractions and thus reduce the risk of aggregation, and also to reduce the amount of non-specific bacterial proteins by gradually increasing the imidazole. The column was stripped with stripping buffer (500mM NaCl, 20mM Tris pH 8.0, 50mM EDTA) to remove any aggregated protein from the column matrix and remove the Ni^{2+} ions.

2.1.4 Analysis and characterisation of bacterially expressed proteins

2.1.4.1 SDS-PAGE/Coomassie

SDS-PAGE under reducing (15% β-mercaptoethanol, β-ME) and non-reducing conditions were performed to ascertain which fraction from FPLC contained the desired protein. Samples were heated at 95°c for 5-10 minutes and electrophoresis was performed on 12% precast NuPAGE Novex Bis-Tris gels (Invitrogen) at 200V for 30-40 minutes. Gels were washed for 3x5 minutes in ddH_20, then either stained for one hour using Imperial stain (Thermo Scientific) and destained in ddH_20 for 1-2 hours; or used for Western blot analysis (section 2.1.4.3).

Recombinant proteins were dialysed into 20mM Tris (pH8.0), 500mM NaCl, 5% sucrose, 5% glycerol (except for the stripped spacer domain which was dialysed into 20mM Tris pH8.0, 500mM NaCl, 4M urea).

2.1.4.2 Quantification of total protein (BCA)

Proteins were then quantified by the Pierce© bicinchoninic (BCA) total protein assay (Thermo Scientific) according to manufacturer's instructions. This method is based on the reduction of Cu^{2+} to Cu^{1+} by proteins in alkaline conditions. The reaction product of BCA and Cu^{1+} is purple and the change in light absorbance is proportional to the protein concentration. Dilutions of bovine serum albumin (BSA) of known concentration were used to generate a standard curve and a number of dilutions of each sample were prepared. 25µl of each standard or sample were applied in duplicate to the wells of a microtitre plate (Sterilin, Thermo Scientific). 200µl of the working reagent was added to each well and the plate incubated for 30 minutes at 37°c on a plate shaker. The absorbance was measured at 560nm using a spectrophotometer (uQuant, Biotek).

2.1.4.3 Western blot

Western blot with anti-Xpress epitope monoclonal antibody (Invitrogen) was also used to quantitate the ADAMTS13 domains relative to each other. This was normalised for each protein to ensure approximately equal loading (in molar terms) of each domain fragment for subsequent analysis of patient antibodies.

Western blots were performed by transferring protein separated by SDS-PAGE to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). Proteins were transferred to the membrane in a transfer buffer (25mM Tris base, 190mM glycine, 20% methanol). The transfer was performed at 33V for 60 minutes. Membranes were blocked with 5% milk/ phosphate buffered saline (PBS, 10mM phosphate buffer, 2.7mM potassium chloride, 137mM sodium chloride) for one hour and ADAMTS13 and fragments were detected using anti-Xpress monoclonal antibody (Invitrogen diluted 1 in 5000 in blocking buffer). Following a 3x5 minute wash with PBS/0.1% Tween (PBST); membranes were incubated with peroxidase conjugated polyclonal goat anti-mouse antibody (Dako, 1 in 5000 in blocking buffer). The membrane was washed for 4x5 minutes and developed with chemiluminescent horseradish peroxidase substrate Immobilon (Millipore) and Amersham hyperfilm ECL (GE Healthcare).

Western blotting was also performed using a polyclonal anti-human ADAMTS13 antibody raised in rabbit to determine whether the recombinant domains were recognised by a more physiological detection antibody. The protocol was as above but using rabbit polyclonal anti-ADAMTS13 (1 in 7000, in-house) as the primary antibody, and peroxidase conjugated goat anti-rabbit antibody (1 in 50 000, Dako) as the secondary antibody.

2.2 Expression and purification of ADAMTS13 domain fragments in mammalian cells

2.2.1 Generation of ADAMTS13 fragment expression vectors

2.2.1.1 Mammalian ADAMTS13 expression vector and ADAMTS13 domain fragment vectors generated

A mammalian expression vector (pcDNA3.1/myc-His 5.5kb Invitrogen containing full-length wild-type ADAMTS13 complementary deoxyribonucleic acid (cDNA) was available from previous studies (72, 214-216). pcDNA3.1 is a mammalian expression vector with multiple cloning sites preceding the C-terminal myc epitope and 6xHis tag, an ampicillin resistance gene and a cytomegalovirus (CMV) promoter to drive high level transcription in mammalian cells (Figure 2.5).

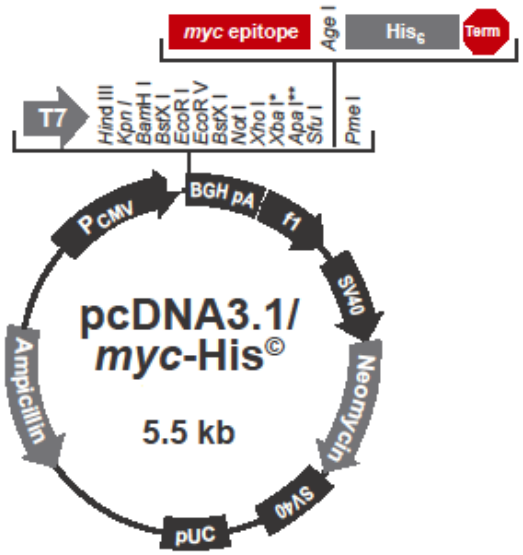


Figure 2.5 Mammalian expression vector pcDNA3.1/myc-His

From Invitrogen. Ampicillin = ampicillin resistance gene; P_{CMV} = cytomegalovirus promoter to initiate gene expression; Neomcin = neomycin resistance gene; 6xHis = polyhistidine tag which aids in purification.

The vectors that express the truncated ADAMTS13 variants MP-Dis, MDTCS and TSP2-4 were also available from previous work from the group. I aimed to generate domain fragments that spanned ADAMTS13 to provide material to use in subsequent domain specificity assays. I therefore used the vector for full-length ADAMTS13 and the existing truncated variants to generate novel domain fragments as detailed in the following sections (Figure 2.6). TCS and MDTC were generated by PCR using pcDNA3.1/myc-His MDTCS as a template. Vectors expressing the ADAMTS13 fragments TSR2-8 and TSR5-8 had been previously generated, but contained errors associated with PCR-based amplification and I performed site directed mutagenesis to correct these.

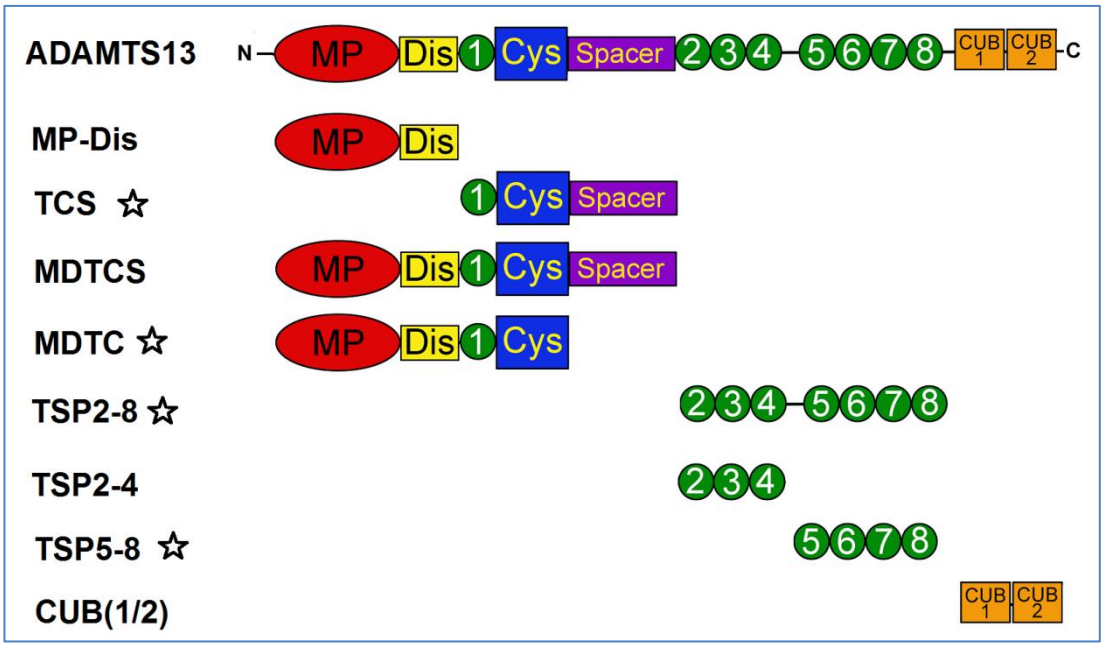


Figure 2.6 ADAMTS13 fragments expressed in HEK293T cells

A mammalian expression vector (pcDNA3.1/myc-His) containing full-length ADAMTS13 cDNA was available from previous studies. Vectors expressng the ADAMTS13 fragments MP-Dis, MDTCS and TSP2-4 were also available. I generated vectors for fragments TCS, MDTC, TS2-8 and TSP5-8. ☆ = novel vector

2.2.1.2 Site directed mutagenesis

Vectors expressing the ADAMTS13 fragments TSR2-8 and TSR5-8 had been previously generated by the group but contained errors associated with PCR-amplification. Site directed mutagenesis was performed to correct these. Single point mutations were introduced using primers which contained the desired mutation and annealed to the corresponding sequence on opposite strands of the plasmid.

Primers were designed according to the guidelines in the KOD HotStart DNA polymerase protocol (Novagen). A higher T_M is required for site directed mutagenesis due to the longer specific primers. All primers were manufactured and HPLC purified by Thermo Fisher, Germany. PCR reactions were performed using 1U KOD HotStart DNA polymerase, 1.5µl of sense (5') primer and 1.5µl of anti-sense (3') primer (10µM each); 10ng of plasmid DNA template; 25µl of 2x reaction buffer, 10µl of dNTP's (2mM each), 1µl of dimethylsulfoxide (DMSO) solution which acts as a denaturant, and PCR grade water to a final volume of 50ul.

The basic amplification conditions were as follows: polymerase activation (95°c, 2min); 18 cycles of denaturation (95°c, 15 sec); annealing (60°c, 30 sec) and extension (68°c, 1 min per kb) with a final extension at 68°c for 7 minutes. The PCR products were treated with *Dpn*I endonuclease at 37°c for one hour. *Dpn*I is specific for methylated DNA and thus digests the template but not the PCR products. Competent cells were transformed with 5µl of the PCR reaction as described in section 2.1.1.6. If no colonies were obtained, the PCR product was checked by agarose gel electrophoresis as described in section 2.1.1.3 and the PCR or transformation repeated with modifications to the reaction conditions.

2.2.1.3 Generation of ADAMTS13 truncation mutants by PCR

Domain fragments TCS and MDTC were generated by PCR using pcDNA3.1/myc-His MDTCS as a template, by amplifying the template vector excluding the domains that were to be excised (thus splicing them out) and reannealing the vector. PCR reactions were performed as in section 2.1.1.2 but without the addition of DMSO. The basic amplification conditions were as follows: polymerase activation (94°c, 2 min); 22 cycles of denaturation (98°c, 10 sec); annealing (57°c, 30 sec) and extension (68°c, 1 min per kb) with a final extension at 68°c for 7 minutes. The PCR products were treated with *Dpn*I at 37°c for one hour and run on a 1% agarose gel to verify size and yield (section 2.1.1.3), before gel extraction and sequencing.

2.2.1.4 DNA phosphorylation and ligation

For the truncation variants, *Dpn*I treated PCR products were phosphorylated and ligated to reform circular plasmids. The linearised pcDNA3.1/TCS or pcDNA3.1/MDTC vector was phosphorylated with T4 polynucleotide kinase (Ambion) for 37°c for at least one hour. The reaction was then heated at 65°c for 10 minutes to deactivate the phosphorylase and T4 ligase used to reanneal the vector, with 0.2U of T4 ligase in a 50µl reaction incubated at 4°c overnight. Competent cells were transformed with 5µl of the ligated vector as described in section 2.1.1.6. If no colonies were obtained, the PCR product was checked by agarose gel electrophoresis as described in section 2.1.1.3, and PCR repeated with modifications to the reaction conditions.

Repeated attempts to generate TCS were made with no success, so an *Asc*I digestion step was introduced after the ligation. The metalloprotease domain of ADAMTS13 has a unique restriction site for *Asc*I and this was used to remove PCR products containing MP.

2.2.1.5 Transformation of competent cells, isolation of plasmids and sequencing

Competent cells were transformed with the mutated/truncated ADAMTS13 vectors as described in section 2.1.1.6. Plasmid DNA was isolated from colonies by endo-free plasmid Minipreps (Qiagen) as described in section 2.1.1.8. All new vectors were verified by sequencing prior to use using the T7 forward primer and rBGH reverse primer. DNA was sequenced at the MRC CSC Genomics Core Laboratory.

2.2.1.6 Large scale plasmid preparation and quantification

Bacteria containing the desired ADAMTS13 vector were cultured on a large scale (200ml – 2500ml) to obtain sufficient amounts of plasmid (up to 10mg) for large scale transfection of mammalian cells. DNA was extracted by Maxiprep /Megaprep/Gigaprep (Qiagen) depending on amount required, as per manufacturer's instructions. These methods use similar principles to those outlined in section 2.1.1.8 with minor differences in protocol as follows.

After lysis of the bacteria, cell debris and precipitated chromosomal DNA were removed by filter rather than centrifugation. The column was equilibrated with a low salt buffer to allow plasmid binding, washed with an ethanol-containing buffer and plasmid DNA eluted using a high salt buffer. An isopropanol precipitation was performed to concentrate and desalt the DNA. The DNA was pelleted by centrifugation at 5000rpm for 90+minutes. The DNA pellet was washed with 70% ethanol, air dried and resuspended in endotoxin-free buffer TE (Qiagen).

The DNA was quantitated with a NanoDrop spectrophotometer by measuring the absorbance at 260nm. DNA concentrations were verified using the Quant-iT^{TM} dsDNA assay measuring fluorescence on the Qubit^{TM} fluorometer according to manufacturer's instructions (Invitrogen).

2.2.2 Expression of ADAMTS13 domain fragments

2.2.2.1 Mammalian cell culture

HEK293T cells which do not normally express ADAMTS13 were used for transient transfection. The stable cell line HEK293 ADAMTS13 was already available from previous work in the group and the stable cell line HEK293 TSP2-8 was kindly gifted by J. Voorberg. Cells were cultured at 37°c, 5% CO_2, in complete media: minimal essential media (MEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Biosera), 2mM L-glutamine (Invitrogen), 50 000U penicillin / 50 000µg streptomycin and 1x non-essential amino acids (NEAA, Invitrogen) and were generally grown in T175 or T175 triple flasks. When confluent, cells were washed with PBS (5ml/T175 flask) then split 1:4 using 1ml trypsin /T175 flask to detach cells, and grown to confluence again in 3-4 days.

2.2.2.2 Transient transfection of HEK293T cells

ADAMTS13 vectors were transiently transfected into 70-80% confluent HEK293T cells. To enable purification of adequate amounts of each ADAMTS13 truncation, up to 18x T175-triple flasks/vector of HEK293T cells were transfected. The cells were transfected using a DNA concentration of up to 3µg/ml and linear polyethylenimine (PEI, Polysciences Inc) with a DNA/PEI ratio of 1:2.25. 111µg of DNA was used per triple flask with a working volume of 75ml / triple flask. Briefly, for one T175-triple flask, 250µl of 1mg/ml linear PEI was diluted into 3.75ml of 0.15M autoclaved NaCl. The resulting PEI solution was added dropwise to 3.75ml of 0.15M autoclaved NaCl containing 111 µg of DNA and the DNA/PEI complex incubated at RT for 20 minutes. The DNA/PEI complex was added to 67.5ml OptiMEM® reduced-serum media (Invitrogen). Each T175-triple flask was washed with PBS and cells were cultured in the 75ml OptiMEM® reduced-serum media (Invitrogen) for 3-4 days before harvesting.

2.2.2.3 Cryopreservation of mammalian cells

HEK293T cells and ADAMTS13 and TSP2-8 stable cell lines were cryopreserved in liquid nitrogen for future use. Once cells reached confluence in a T175 flask, they were washed with PBS, trypsinised with 1ml of trypsin and transferred to a 50ml tube. Trypsin was neutralised by adding 45ml complete media and the cells were centrifuged at 1200rpm for 5-10 minutes. The supernatant was discarded and cells were resuspended in 1.5ml complete medium. 1.5ml of freezing medium (cold complete medium with 10% DMSO) was added dropwise with gentle swirling. Cells were aliquoted in 1ml fractions in cryovials and stored in an isopropanol-containing cryofreezing container (Nalgene) at -80°c overnight prior being transferred to liquid nitrogen.

To recover cryopreserved cells, vials were removed from the liquid nitrogen to ice then thawed at 37°c until only a small amount of ice remained. The cells were transferred to a 50ml tube and 40ml of cold complete medium added. The cells were then spun down for 5min at 1200rpm and the pellet resuspended in 12.5ml warm complete medium, thus removing the DMSO. The cells were seeded into a T75 flask.

2.2.2.4 Harvesting of ADAMTS13 and fragments

Both stable and transiently transfected cells were cultured in T175 triple flasks containing 75ml of OptiMEM®. After 3-4 days, conditioned media was harvested, centrifuged and filtered to remove cell debris, and concentrated up to 40-fold using tangential flow filtration coupled to a 10kDa MWCO unit (Amicon). Cells were harvested by adding protein loading buffer and using a cell scraper.

Conditioned media samples for use in activity assays were not purified, but were further concentrated by Amicon Ultra centrifugal filter devices (Millipore), aliquoted and stored at -80°C. Conditioned media samples for use in domain specificity assays were dialysed into 20mM Tris pH8.0,

500mM Tris, 20mM imidazole, then purified by metal ion affinity chromatography (section 2.2.3.1).

2.2.3 Purification of mammalian expressed proteins

2.2.3.1 ADAMTS13 purification by metal ion affinity chromatography

ADAMTS13 and ADAMTS13 truncations were purified using Ni^{2+}-chelating chromatography as previously described (51, 204). Fast protein liquid chromatography (FPLC) was performed using an ÄKTA purifier (GE Healthcare) and the Unicorn 5.1 software and a Ni^{2+}-chelating column. The method used was similar to that in section 2.1.3.2 for bacterially-expressed proteins but without a refolding gradient.

Briefly, a Ni^{2+} chelating HiTrap column (GE Healthcare) was charged with nickel(II) sulphate hexahydrate. The column was equilibrated with 20mM Tris-HCL (pH7.8), 500mM NaCl, 20mM imidazole. Conditioned medium containing full-length ADAMTS13 or an ADAMTS13 fragment was passed over the column, allowing the construct to bind via the metal ion binding His-tag. The column was washed with 20mM Tris-HCL, 500mM NaCl, 20mM imidazole and then the protein was eluted using a buffer containing 500mM imidazole. Peak fractions were dialysed into PBS.

2.2.4 Analysis and characterisation of mammalian expressed proteins

2.2.4.1 Western blotting

The conditioned medium and cell pellets were analysed by Western blotting to estimate the ADAMTS13 fragment expression levels, secretion and intracellular retention. Western blots were performed as described in section 2.1.4.3. Later Western blots used a PVDF membrane (Immobilon®-P, Millipore)**.** This required pre-wetting in 100% methanol for 15 seconds then ddH_2O for 2 minutes, and equilibrating for at least 5 minutes in the transfer buffer before use**.** ADAMTS13 and ADAMTS13 fragments were detected using anti-His (C terminal)-HRP (1 in 5000, Invitrogen) or anti-myc-HRP (1 in

5000, Invitrogen). The construct MD does not have a myc tag as this was removed for earlier work, thus only anti anti-His (C terminal)-HRP could be used. Fragments MDTC and TSP2-8 have V5 epitopes and anti-V5-HRP was used for detection of these (1 in 2000, Invitrogen).

Western blotting was also performed using a polyclonal anti-human ADAMTS13 antibody raised in rabbit to determine whether the recombinant domains were recognised by a more physiological detection antibody as described in section 2.1.4.3 .

Semi-quantitative Western blotting using anti-tag antibodies was used to estimate the concentration of ADAMTS13 fragments relative to each other and to a standard (full-length ADAMTS13 of known concentration, as determined by ELISA - section 2.2.4.3).

2.2.4.2 SDS-PAGE/Coomassie

SDS-PAGE under non-reducing conditions with Coomassie staining was performed to assess the purity of the different ADAMTS13 fragments expressed at sufficient concentration to be visualised on a gel, as described in section 2.1.4.1.

2.2.4.3 ADAMTS13 antigen ELISA

Full-length ADAMTS13 was quantitated using an ELISA previously developed by the group (86), where ADAMTS13 is captured by a polyclonal rabbit anti-ADAMTS13 antibody (anti-TSP2-4 depleted) and detected by biotinylated anti-TSP2-4 antibody. Briefly, a 96 well plate was coated with rabbit polyclonal anti-ADAMTS13 antibodies (anti-TSP2-4 depleted) at a concentration of 5µg/ml in 50mM sodium carbonate (pH 9.6) overnight at 4°c. Normal human plasma (Technoclone, Austria) was used to generate a standard curve. The plate was washed with PBST after each incubation step. After blocking for one hour (PBS/2% BSA), dilutions of control plasma and test samples were added and incubated for 2 hours at RT. Bound

ADAMTS13 was detected by biotinylated anti-TSP2-4 antibody (0.2µg/ml, 1 hour) followed by streptavidin-HRP (1 in 1000, GE Healthcare or 1 in 500 Sigma) for 1 hour. Finally, plates were washed and incubated with ortho-phenylene-diamine (OPD, Sigmafast) for colour development, which was stopped with 2.5M H_2SO_4. Absorption at 492nm was read using a spectrophotometer.

2.2.4.4 ADAMTS13 vs. MDTCS ELISA

A novel ELISA was developed to determine the relative molar concentration of the N-terminal fragment MDTCS compared to full length ADAMTS13. A monoclonal antibody directed against the metalloprotease domain (3H9, gifted by H. Feys (217)) was used to capture the antigen, and anti-myc-HRP (Invitrogen) used for detection via the myc tag. Purified myc-tagged full-length ADAMTS13 (previously quantitated by the ADAMTS13 antigen ELISA described in section 2.2.4.3) used as the standard.

A Maxisorb plate was coated with 5µg/ml of anti-metalloprotease mAb in 50mM sodium carbonate (pH 9.6) overnight at 4°c. The plate was washed three times with PBST after each incubation step. After blocking for two hours with PBS/2.5% BSA, dilutions of the standard ADAMTS13 and MDTCS of unknown concentration in PBS/1%BSA were applied to the plate and incubated for 2 hours at RT on a plate shaker. Bound ADAMTS13 and MDTCS were detected using HRP-conjugated anti-myc antibody (1 in 1000, Invitrogen) in PBS/1%BSA incubated for 2 hours at RT on a shaker. Finally, plates were washed and incubated with 170µl OPD for colour development which was stopped with 2.5M H_2SO_4. Absorption at 492nm was read using a spectrophotometer. A standard curve of 0-20nM of ADAMTS13 was fitted and samples were diluted to ensure that they fell on the linear part of the curve.

2.2.4.5 ADAMTS13 vs. MDTC ELISA

A similar novel ELISA was developed to measure the concentration of the V5-tagged construct MDTC. The method was as described in section 2.2.4.4 but the standard was purified V5-tagged ADAMTS13 quantitated by the ELISA in section 2.2.4.3 (kindly gifted by Dr Brenda Luken), and the detection antibody was anti-V5-HRP (Invitrogen).

2.2.4.6 Quantification of total protein

For proteins which appeared pure by Coomassie staining, total protein concentration was measured by reading the absorbance at 280nm in a NanoDrop 2000 spectrophotometer (Thermo Scientific). The Beer-Lambert law for concentration $c=A/\varepsilon L$ was used with an extinction coefficient calculated for each fragment.

2.3 Plasma samples and antibody extraction

2.3.1 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.

TTP patients were diagnosed clinically based on the combination of microangiopathic haemolytic anaemia with thrombocytopenia and end-organ damage with no other known cause (62). 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 (one sample was taken after a plasma infusion). All patients had severe deficiency in plasma ADAMTS13 activity (<10%), with the exception of one patient with 12% activity, and one with 27% activity, (taken after plasma infusion had been given), and were positive for anti-ADAMTS13 IgG (65, 67, 82, 218).

Patients were selected based on their medium/high anti-ADAMTS13 titre (i.e. >15% using the anti-ADAMTS13 IgG ELISA –section 2.3.4 (82)). Through exclusion of patients with low-titre anti-ADAMTS13 antibodies, this cohort of patients was consequently enriched for those patients that relapsed 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.

2.3.2 Plasma samples

2.3.2.1 Sample collection and preparation

Blood for ADAMTS13 assays was collected by peripheral cannulation using a 21G needle into a Vacutainer® system (Beckton Dickinson) containing 0.5ml 0.105M sodium citrate. Platelet-poor plasma was separated from citrated blood by centrifugation within four hours of collection. Samples were spun at 2000g for 15 minutes at RT and the top two-thirds of plasma carefully removed and transferred to a polypropylene tube. After a further centrifugation at 2000g for 15 minutes, the platelet poor plasma was aliquotted into 2ml polypropylene tubes or 1.5ml Eppendorf tubes and stored at -80°C. Plasma samples sent from other hospital were double spun at site and transferred frozen on dry ice.

2.3.2.2 Samples tested

Samples from patients with acquired TTP containing high titres of anti-ADAMTS13 IgG were used for optimisation of assays. Plasma exchange (PEX) fluid from the first plasma exchange was also available from selected patients at presentation and offered a larger volume for preliminary work. Pooled normal human plasma from 16 healthy volunteers with no detectable ADAMTS13 inhibitor was used as a negative control for all antibody assays.

2.3.3 ADAMTS13 activity assays

Plasma ADAMTS13 activity was measured by collagen binding assay, as described by Gerritsen with modifications, until May 2010 (65) (218). ADAMTS13 activity was expressed as a percentage relative to the activity in pooled normal plasma (PNP) (normal range 55%-126%; lower limit of detection 5%). ADAMTS13 activity was measured by a fluorescence resonance energy transfer (FRET) assay from May 2010 onwards (67).

ADAMTS13 activity was again expressed as a percentage relative to the activity in PNP (normal range 60%-123%; lower limit of detection 5%).

2.3.4 Total anti-ADAMTS13 IgG assay

Total plasma anti-ADAMTS13 IgG was measured using an ELISA technique as previously described (82). Briefly, microtitre plates were coated with purified recombinant full-length ADAMTS13 (Baxter Bioscience), patient plasma was added and incubated for one hour and any bound anti-ADAMTS13 IgG was detected using anti-human globulin. A standard curve was prepared by diluting an index reference plasma containing high levels of anti-ADAMTS13 IgG in PBS/BSA to achieve 100%, 80%, 40%, 20%, 10%, 5% and 0% concentrations. The normal range was <6.1%, calculated as the 95th percentile of 49 healthy controls.

2.3.5 IgG extraction

2.3.5.1 Protein G IgG extraction

Total IgG was isolated from plasma (or PEX) taken from each patient at diagnosis using protein G spin columns according to manufacturer's instructions (GE Healthcare). 500µl of plasma was applied to the equilibrated column, washed with binding buffer and total IgG eluted using a low pH elution buffer and immediately neutralised upon elution using neutralisation buffer. The protocol resulted in an approximately 2.6 fold dilution of the IgG. Eluted fractions were dialysed into 20mM Tris, 150mM NaCl, pH 7.6 prior to use in VWF 115 activity assays.

2.3.5.2 Melon gel IgG extraction

A Melon^{TM} gel IgG purification system was also used. This uses physiological pH to prevent adverse effects upon isolated IgG and a mild purification buffer suitable for use in activity assays (Thermo Scientific). The melon gel binds non-antibody plasma proteins, such as albumin and transferrin, which are often present in high abundance, allowing antibody to flow through. Total IgG was isolated from plasma (or PEX) taken from each patient at diagnosis

using melon gel spin columns according to manufacturer's instructions. To avoid dilution of the IgG, samples were buffer exchanged into the melon gel purification buffer using Zeba™ desalting spin columns (Pierce) before applying to the melon gel.

2.3.6 Quantification of total IgG

2.3.6.1 Quantification of IgG using a NanoDrop spectrophotometer

To quantitate the total IgG in the samples, the absorbance at 280nm was read using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Unknown protein concentrations are calculated using the mass extinction coefficient of 13.7 at 280nm for a 1% (10mg/ml) IgG solution. The spectrophotometer was first blanked using the appropriate buffer (melon gel purification buffer or TBS). A 2µl sample volume was used to establish adequate liquid column formation, and good replicates were achieved.

2.3.6.2 Human IgG ELISA

Total IgG was also quantitated using a human IgG quantitation ELISA according to manufacturer's instructions (Bethyl Laboratories). A 96 well plate was coated with goat anti-human IgG-Fc antibody (1:100 dilution in 0.05M carbonate-bicarbonate pH9.6) for 1 hour at RT. The plate was washed 5 times between each incubation step with 50mM Tris, 0.14M NaCl, 0.05% Tween 20, pH8.0. After blocking with 50mM Tris, 0.14M NaCl, 1% BSA, pH8.0, diluted standards (human reference serum 0-500ng/ml IgG) or samples (1 in 2500- 1 in 1280000) were applied to the plate and incubated at RT for 1 hour. Bound IgG was detected using HRP-conjugated goat anti-human IgG-Fc antibody. After washing, the plate was incubated with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) for colour development which was stopped with 0.18M H_2SO_4. Absorption at 450nm was read using a spectrophotometer.

2.4 Determination of domain specificity of TTP patient autoantibodies

2.4.1 Immunoblotting using bacterially expressed material

To ascertain which domains the anti-ADAMTS13 autoantibodies from each TTP patient recognise, immunoblotting techniques using bacterially expressed material were first used. The bacterially expressed and purified ADAMTS13 domain fragments (up to 500ng) were electrophoresed on a 12% precast NuPAGE Novex Bis-Tris gel (Invitrogen), and then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) – see section 2.1.4.3. Full length ADAMTS13 expressed in mammalian cells was used as a positive control.

Detection of these fragments was performed by incubating plasma or protein G purified IgG from TTP patients (section 2.3.5.1) diluted 1 in 25 in PBS/3% milk/3% bovine serum albumin (BSA, Sigma Aldrich) with the membrane. After washing with PBST, the domains recognised by each patient's antibodies were visualised using an anti-human IgG-HRP conjugate (1 in 3000, AbCam) and Immobilon (Millipore) detection reagents. A monoclonal human-derived anti-spacer antibody II-1 (211) was also used to determine whether the physiological epitope comprising $Arg^{660,}$ $Tyr^{661,}$ and Tyr^{665} (RYY) was available in the spacer material.

2.4.2 Immunoblotting using mammalian expressed material

Western blots were performed by transferring purified recombinant full length ADAMTS13 and fragments separated by non-reducing SDS-PAGE to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). Later Western blots used a PVDF membrane (Immobilon) – see section 2.2.4.1**.**

Membranes were blocked with PBS/5% milk for one hour. Detection of the fragments was performed by incubating protein G purified IgG from TTP patients (section 2.3.5.1) diluted 1 in 25 in PBS/5% milk with the membrane overnight at 4°C. After washing with PBST, the fragments recognised by

each patient's antibodies were visualised using an anti-human IgG-HRP conjugate (1 in 3000, AbCam). The membrane was washed for 4x5 minutes and developed with ECL chemiluminescent horseradish peroxidase substrate Immobilon (Millipore) and Amersham hyperfilm ECL (GE Healthcare).

2.4.3 Immunoprecipitation

Immunoprecipitation allows antibody-antigen binding in solution, meaning conformation-specific epitopes are not disrupted. This strategy had also been used successfully by other groups studying the autoimmune response in TTP (193, 196). Antibodies from 170µl of plasma or PEX were allowed to bind 50µl protein G (Protein G Mag Sepharose, GE Healthcare) in Tris-buffered saline (TBS- 50mM Tris, 150mM NaCl pH7.5), 1%BSA and 0.6% protease inhibitor complex (Sigma-Aldrich) for at least one hour. Pooled normal human plasma was used as a negative control. After washing with TBS/0.1% Tween (TBST), purified mammalian expressed ADAMTS13 fragments (up to 1µg in a total volume of 300µl) were added to antibody-loaded sepharose and incubated in TBS/1%BSA with slow end-over-end mixing overnight.

After washing five times with TBST (with the bead solution transferred to a fresh tube during last wash), bound proteins were eluted with 50µl 2.5% acetic acid and electrophoresed in reducing or non-reducing conditions on a 4-12% Bis-Tris acrylamide gel and transferred to a PVDF membrane. Mammalian expressed full length ADAMTS13 or MDTCS was included as a positive control. Blots were probed with anti-myc HRP (1 in 1000, Invitrogen) or anti-V5 HRP (1 in 1000, Invitrogen) for V5 tagged constructs.

2.4.4 ELISA and competition assays

2.4.4.1 Initial ELISA development

A variety of strategies were employed to develop an ELISA for detection of domain-specific antibodies as such an approach could potentially allow high-throughput with screening of multiple samples against different fragments of ADAMTS13, without the requirement for labour-intensive multiple immunoprecipitations and detection by Western blot, and would require less plasma.

Various strategies were tried including using anti-His tag antibodies (Figure 2.7A) or polyclonal rabbit anti-human ADAMTS13 to capture the ADAMTS13 fragments (Figure 2.7C) with detection of bound anti-ADAMTS13 antibodies with anti-human IgG-HRP. Coating the plate with purified IgG from TTP patients was also attempted (Figure 2.7B) with bound proteins detected with anti-tag antibody.

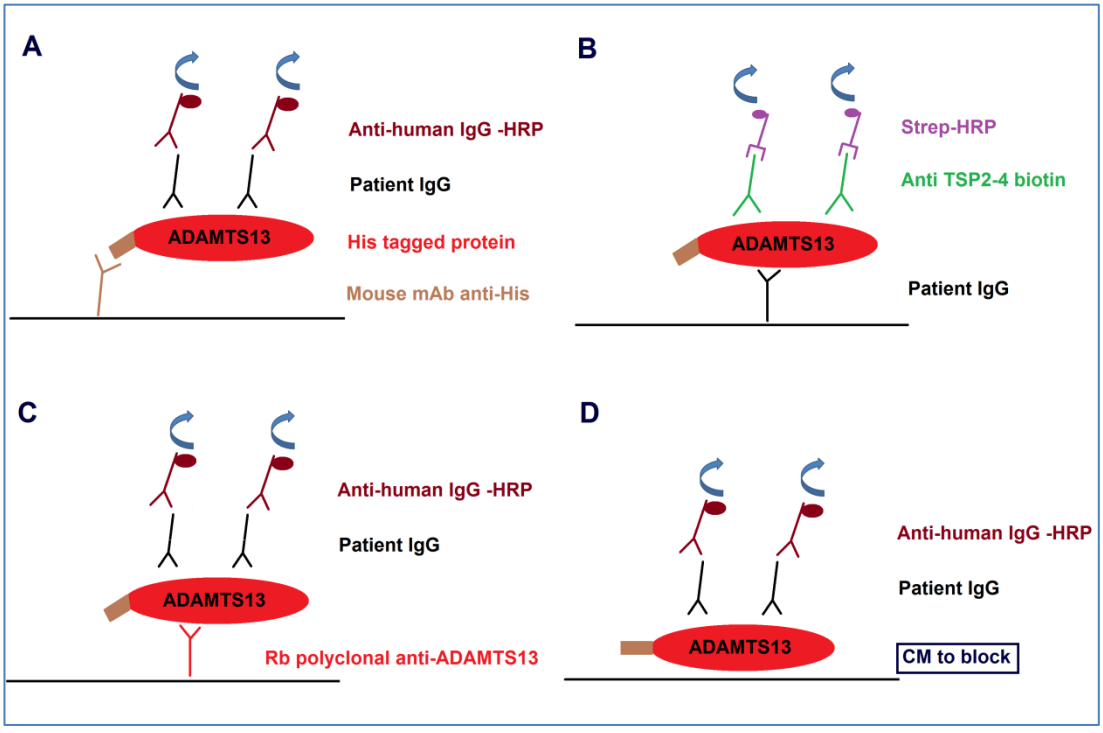


Figure 2.7 Development of an ELISA to detect domain-specific anti-ADAMTS13 antibodies

Strep-HRP=streptavidin HRP; CM= conditioned media

The most successful strategy was to coat the plate directly with full length ADAMTS13 or fragments (Figure 2.7D), and this assay was developed further. However, direct coating did not work for some of the ADAMTS13 fragments, presumably because the proteins bound to the plate through their antigenic surfaces. This was circumvented by using both a direct ELISA for some fragments (TSP2-8, section 4.1.2.2) and spiking other fragments into an ELISA detecting antibodies against full-length ADAMTS13 (section 4.1.2.1).

2.5 Determination of inhibitory potential of TTP patient autoantibodies

2.5.1 VWF 115 and VWF 106 activity assays

2.5.1.1 Expression of VWF115

VWF115 is a 115 amino acid VWF A2 domain fragment (Glu^{1554}-Arg^{1668} Figure 2.8). This short substrate had previously been cloned by our group into the bacterial expression pET100 (Invitrogen), which adds a polyhistidine tag and the Xpress epitope to the N-terminus of the fragment (72). VWF115 was expressed at high level in Rosetta DE3 *E.coli* cells (Novagen). Competent DE3 cells were transformed with ~1ng of the vector as described in section 2.1.1.6. A bacterial colony was used to inoculate 12ml of LB broth containing 100ug/ml ampicillin. Cultures were grown at 37°C with shaking until in Log-phase growth (OD_{600} 0.6-1.0 or approximately 3-4 hours). The culture medium was stored overnight at 4°C. The culture media was spun to collect the bacterial pellet which was then resuspended in 5 ml of fresh medium and used to inoculate 1L 2xYT broth. The cultures were grown at 37°C with shaking to an OD_{600} of 0.6-1.0 (2-6 hours).

Recombinant protein expression was induced by the addition of IPTG to a final concentration of 1mM and left for 16 hours. Bacterial cells were harvested by centrifugation and the pellet stored at -80°C. Inclusion bodies were harvested using BugBuster reagent (Novagen) as per manufacturer's instructions (section 2.1.3.1).

2.5.1.2 Purification of VWF115

Inclusion bodies were solubilised in a buffer containing 20mM Tris (pH 7.8), 500mM NaCl, 20mM imidazole and 8M urea. VWF115 was then purified using a nickel HiTrap column coupled to an ÄKTA FPLC system (GE Healthcare). A linear gradient from the solubilisation buffer to a refolding buffer (20mM Tris pH 7.8, 500mM NaCl, 40mM imidazole) over one hour was used to remove the urea and refold the protein. The VWF115 was eluted from the column with a buffer containing 500mM imidazole and the substrate was then thoroughly dialysed against 20mM Tris-HCL, 150mM NaCl (pH7.8)

using a 3-6kDa cut-off membrane. The purity of the protein was confirmed by SDS-PAGE with Coomassie staining (section 2.1.4.1) and it was quantitated using a BCA assay (Thermo Scientific, section 2.1.4.2).

2.5.1.3 VWF115 activity assay

The ability of the patient autoantibodies to inhibit ADAMTS13 function was assessed using an established activity assay (51, 72, 204, 216). IgG for use in VWF 115/106 assays was dialysed into 20mM Tris, 150mM NaCl, pH 7.6 prior to use. Briefly, 2nM ADAMTS13 in concentrated conditioned medium (expressed and quantified as previously described in sections 2.5.1.1-2) in 20mM Tris pH 7.6, 150mM NaCl was preincubated for one hour with 5mM $CaCl_{2,}$ both with and without 17μM total IgG isolated from TTP or control plasmas. 5μM purified VWF115 was added to start the reaction and incubated at 37°C. At 0 to 60 mins, subsamples were stopped with EDTA (50mM final concentration). EDTA stops the proteolysis of VWF115 by chelating Zn^{2+} and Ca^{2+} ions. Samples were stored at -80°C until analysis by SDS-PAGE and Coomassie staining.

2.5.1.4 VWF106 activity assay

VWF106 (Glu^{1554}-Arg^{1659}) is a short VWF A2 domain fragment which lacks the nine C-terminal amino acids of VWF115 (Figure 2.8). VWF106 was available from previous work in our lab and was kindly gifted by Dr Brenda Luken. 3.5nM ADAMTS13 in concentrated conditioned medium in 20mM Tris pH 7.6, 150mM NaCl was preincubated at 37˚C with 5mM $CaCl_2$ and 29μM total IgG for one hour. Purified VWF106 (5 μM) was added to start the activity assay and various time point aliquots were removed and stopped with EDTA (50mM final concentration). A higher concentration of enzyme was used and reaction times extended to two hours, because the cleavage of VWF106 is less efficient than that of VWF115. Samples were stored at -80°c until analysis by SDS-PAGE and Coomassie staining.

2.5.1.5 SDS-PAGE analysis of VWF115 and VWF106 proteolysis

Samples from the assays described in sections 2.5.1.3-4 were loaded on a 4-12% Bis-Tris gel and run for 30 minutes at 200V. Gels were stained with Coomassie stain (Imperial protein stain, Pierce) allowing visualisation of uncleaved VWF115 (17kDa) and the cleavage products (10kDa and 7kDa). Similarly, VWF106 (16kDa) and its cleavage products (10kDa and 6kDa) could be visualised.

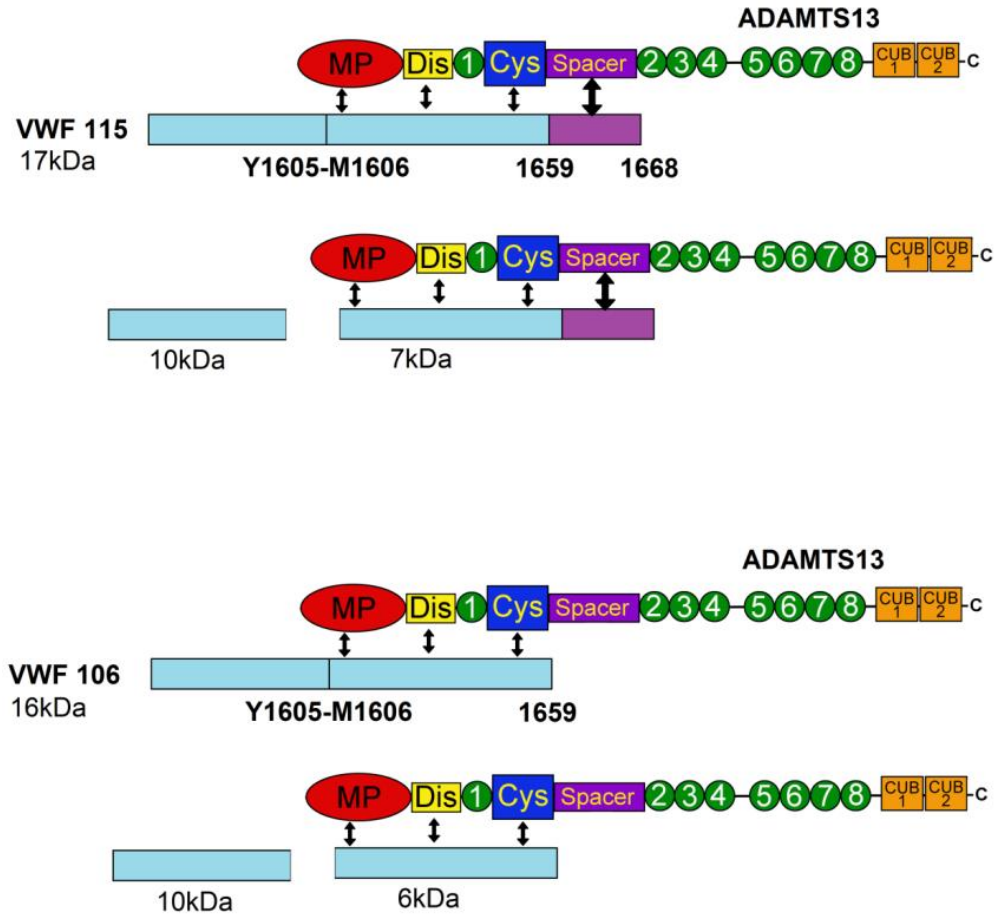


Figure 2.8 VWF A2 domain short substrates VWF115 and VWF106 and their cleavage by ADAMTS13

VWF115 is a 17kDa VWF A2 domain fragment which contains the scissile bond and binding exosites for the metalloprotease, disintegrin-like, cysteine-rich and spacer domains of ADAMTS13. VWF106 is identical to VWF115, but lacks nine residues from its C-terminus that are critical to ADAMTS13 spacer domain binding.

2.5.2 Fluorescence resonance energy transfer (FRETS) assay

MDTCS (in concentrated conditioned medium, final concentration 0.125nM) in 5mM Bis Tris, 25mM $CaCl_2$, 0.005% Tween, pH 6.0 was preincubated at 37°C for 30 minutes with 0-5.6µM total IgG from TTP or healthy plasmas, to allow calcium binding and antibody-antigen interaction. 1µM FRETS-VWF73 substrate (Peptide International) was added and fluorescence was measured over one hour to monitor substrate proteolysis (Fluorostar Omega). After titration of each IgG preparation, assays were repeated at three IgG concentrations spanning the IC_{50} for each sample. This was performed both with and without preincubation with 10nM purified MDTC, to ascertain the proportion of inhibition attributable to anti-spacer domain antibodies.

2.6 Statistical analysis

Statistical analysis of the results was performed using SPSS Statistics and GraphPad Prism version 6 software. For continuous variables, differences between patients with varying patterns of domain specificity were evaluated by Mann Whitney U test or the Kruskal Wallis test, as the data sets were not normally distributed.

For discrete variables, differences were evaluated using the $\chi 2$ test or Fisher's exact test for smaller numbers. 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 and 95% confidence intervals, which were used as an estimate of the likelihood of mortality. A probability (p) value of <0.05 was taken as statistically significant.

3 Expression and purification of ADAMTS13 domain fragments and development of assays to determine the domain specificity of anti-ADAMTS13 antibodies

The first aim of this project was to express isolated domains and domain fragments of ADAMTS13 to allow the development of novel assays to determine the domain specificity of anti-ADAMTS13 antibodies in acquired TTP. Eukaryotic expression systems are most likely to give correctly folded protein but only in limited quantity. Bacterial expression systems allow rapid synthesis of large quantities (up to mg amounts of protein). Based on this, I started with a panel of individual domains or small domain fragments synthesised in bacteria due to the ease of expression.

3.1 Methods

3.1.1 Expression, purification and refolding of ADAMTS13 domain fragments in bacteria

See section 2.1 for methodology

3.1.2 Expression and purification of ADAMTS13 domain fragments in mammalian cells

See section 2.2

3.1.3 Determination of domain specificity of TTP patient autoantibodies

3.1.3.1 Immunoblotting using bacterially expressed material

See section 2.4.1

3.1.3.2 Immunolotting using mammalian expressed material

See section 2.4.2

3.1.3.3 Immunoprecipitation

See section 2.4.3

3.1.3.4 Initial ELISA development

See section 2.4.4

Construct	Construct	Novel vector	Predicted MW (kDa)	Expressed in Rosetta DE3	His tag purified	Soluble protein	Quantity of purified protein (mg)	Issues
MP	MP	No	27	Yes	Yes	Yes	635	
DisT	Dis 1	No	17	Yes	Yes	Yes	104	
TSP1	1	Yes	10	Yes	Yes	Yes	66	
Cys	Cys	Yes	17	Yes	Yes	Yes	186	
Spac	Spacer	No	19	Yes	Yes	No	816	Aggregated Not recognised by spacer mAb II-1
TSP2-4	2 3 4	No	23	Yes	Yes	Yes	795	
TSP5-8	5 6 7 8	No	30	Yes	Yes	Yes	217	
CUB1/2	CUB 1 CUB 2	No	36	Yes	Yes	Yes	25	

Table 3.1 Bacterial ADAMTS13 domain constructs expressed, purified and quantitated in this study

Domain organisation of full-length ADAMTS13. Predicted molecular weights of bacterially expressed ADAMTS13 domains including His tag and Xpress epitope are given. Total amounts of purified protein were determined by the BCA assay.

3.2 Results

3.2.1 Expression, purification and refolding of ADAMTS13 domain fragments in bacteria

The ADAMTS13 domain fragments expressed in bacteria and subsequently purified, refolded and quantitated in this study are summarised in Table 3.1.

3.2.1.1 Generation of bacterial ADAMTS13 domain vectors

I successfully generated the domain fragments TSP1 and Cys by PCR amplification using full-length ADAMTS13 plasmid DNA as a template, and cloned them into the pET100/D-TOPO vector. Previously generated bacterial vectors that express recombinant MP, Dis-TSP1, Spacer, TSP2-4, TSP5-8 and CUB1/2 were available from earlier work in the group. I verified all existing and novel vectors by sequencing prior to protein expression.

3.2.1.2 Expression of ADAMTS13 domains in bacteria

I went on to express all of the domain fragments at high levels in Rosetta DE3 *E.coli*. The ADAMTS13 fragments synthesised spanned all domains of the enzyme. The predicted molecular weights of the various fragments including tag are shown in Table 3.1 (bacterially expressed domains lack glycans).

3.2.1.3 ADAMTS13 domain purification and refolding by FPLC

I subsequently partially purified all the bacterially expressed ADAMTS13 domain fragments using a Ni^{2+}-chelating HiTrap column coupled to an ÄKTA FPLC. A novel on-column refolding strategy yielded soluble protein in the elution fraction for all domains except for the spacer domain, which aggregated on the column and had to be stripped off using a denaturing buffer containing 8M urea.

A representative FPLC chromatogram is shown in Figure 3.1 with the milli-absorbance units (mAU) at 280nm displayed against the volume of liquid flowing through the column.

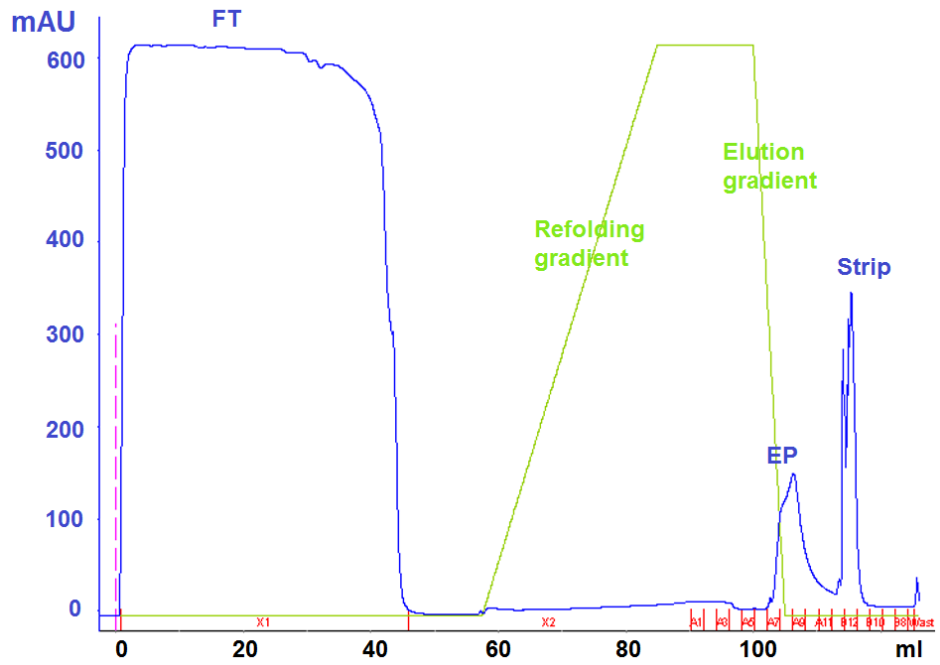


Figure 3.1 FPLC chromatogram of purification and refolding of the fragment Dis-TSP1

The 280nm absorbance (blue line) is plotted against the elution volume over time.
Buffer gradients are shown in green. The unfolded protein is loaded onto the column in a denaturing buffer with low imidazole concentration to reduce non-specific protein binding. The Dis-TSP1 protein binds via the metal ion binding His-tag, and the other non-tagged bacterial proteins appear in the flow through (FT) as an absorbance peak containing large amount of protein (approx. 600mAU) Sequential buffer change gradually removes the urea in the presence of agents to optimise correct refolding (glutathione redox pair, glycerol and sucrose) - refolding gradient shown in green. The folded protein is eluted by a gradient over 10 minutes into ice-cold elution buffer containing a higher concentration of imidazole (elution gradient shown in green). This aims to gradually increase the protein concentration in the eluted fractions and thus reduce the risk of aggregation. The smaller elution peak (EP) is seen, which should contain the refolded protein of interest. The column is stripped with EDTA-containing stripping buffer (Strip = stripping peak) to remove any aggregated protein from the column matrix and remove the Ni^{2+} ions. Fractions of the flow-through, elution and stripping peaks are then analysed by SDS-PAGE to determine which contains the protein of interest.

3.2.1.4 Analysis and characterisation of bacterially expressed proteins

SDS-PAGE under reducing and non-reducing conditions were performed for each ADAMTS13 domain fragment purified to ascertain which fraction from FPLC (flow through, elution peak or stripping peak) contained the desired protein (e.g. Figure 3.3 for Dis-TSP1). The protein was present in the elution peak for all the ADAMTS13 fragments, except the spacer domain which had to be stripped off the column and was present in the stripping peak.

Coomassie staining under reducing (Figure 3.2) and non-reducing conditions of the bacterially expressed material showed proteins of expected molecular weight. (Silver staining was not required as sensitivity was not an issue). Coomassie staining revealed bands of approximately 28kDa for the metalloprotease domain (MP), 17kDa for the domain fragment Dis-TSP1 (DisT), 10kDa for the TSP1 domain (TSP) and 17kDa for the cysteine-rich domain (Cys). Bands were also seen of approximately 19kDa for the spacer domain (Spac), 23kDa for TSP2-4, 30kDa for TSP5-8 (5-8) and 36kDa for the CUB domain fragment (CUB).

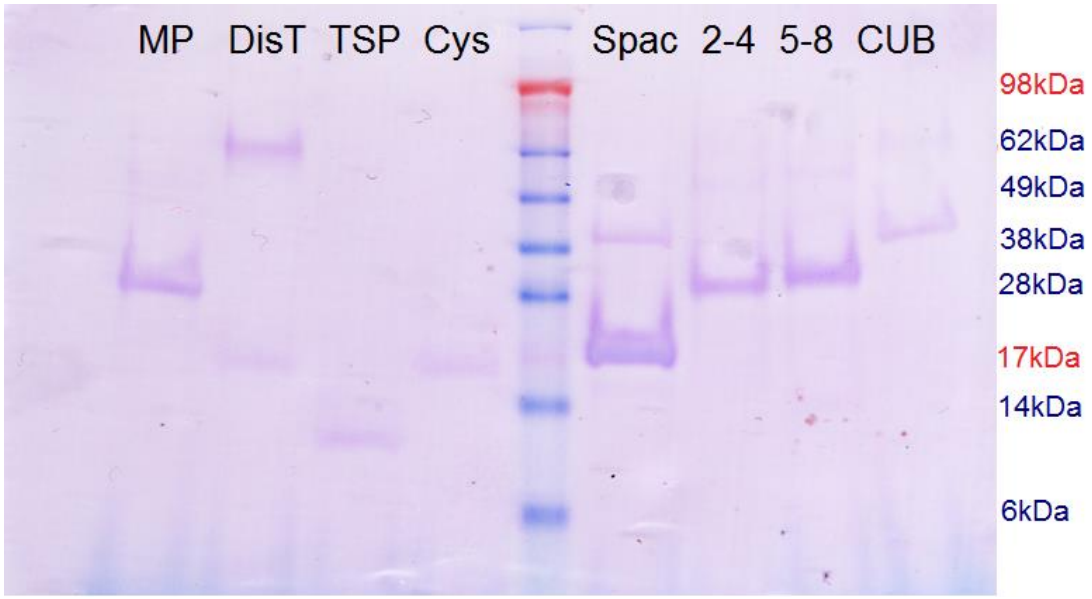


Figure 3.2 Coomassie stain of bacterially expressed and purified ADAMTS13 domains under reducing conditions

MP= metalloprotease domain , DisT =Dis-TSP1, TSP= TSP1 domain, Cys=cysteine-rich domain, Spac=spacer domain, 2-4=TSP2-4, 5-8=TSP5-8, CUB=CUB domains1/2 .

Additional bands of higher molecular weight proteins were seen in non-reducing conditions for several domains, reflecting multimeric material due to intermolecular disulphide bond formation. These higher molecular weight bands were also seen for the Dis-TSP1 and spacer domains under reducing conditions (Figure 3.2), either because reducing conditions did not persist for long enough or more likely due to protein aggregation taking place, despite the presence of SDS.

Based on the results seen in the previous gel with evidence of multimeric material, I attempted to reduce multimerisation by gradually increasing the imidazole concentration when eluting the bacterially expressed ADAMTS13 domains from the Ni^{2+}-chelating column. For this, I eluted using a linear gradient over ten minutes. With this strategy, the imidazole concentration and hence His-tagged protein concentrations are lower initially, reducing the risk of aggregation. The strategy resulted in reduced multimerisation of the eluted proteins (Figure 3.3). This is demonstrated by a single band representing monomeric material in the first fraction of the elution peak (EP1) with lower imidazole concentrations, in both non-reducing and reducing conditions.

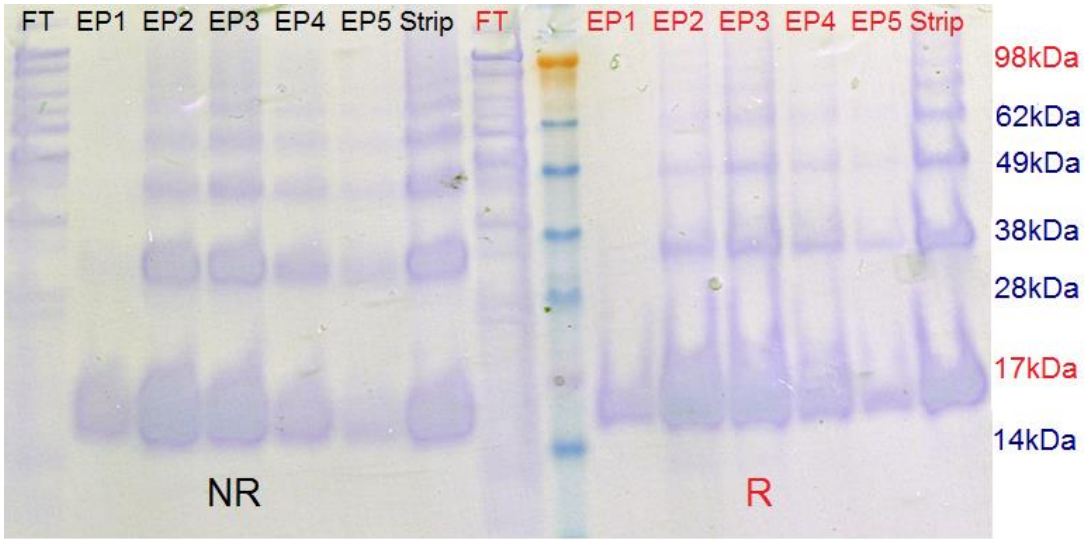


Figure 3.3 Coomassie stain of FPLC fractions from purification of bacterially expressed Dis-TSP1 domains with increasing imidazole concentrations

FT=flowthrough, EP=elution peak, Strip=stripped fraction
NR=non-reducing conditions, R=reducing conditions
No protein of the correct size is seen in the flow through, which represents non-specfic bacterial proteins. The 17kDa DisTSP1 is seen in the elution peak with a single band representing monomeric material in the first fraction of the elution peak (EP1) with lower imidazole concentrations, in both non-reducing and reducing conditions. DisTSP1 is seen in the stripped peak but there are also higher order multimers/aggregates.

Total amounts of purified protein of the different bacterially expressed ADAMTS13 domains as determined by the BCA assay are shown in Table 3.1. Western blot analysis using an anti-Xpress tag antibody (specific for the Xpress epitope tag on the amino-terminus of the different constructs) confirmed that the purified ADAMTS13 domains were isolated as bands of the expected molecular weight, but with evidence of multimerisation (Figure 3.4), similar to the Coomassie. The Western blot revealed bands of approximately 28kDa for the metalloprotease domain, 17kDa for the domain fragment Dis-TSP1, and 17kDa for the cysteine-rich domain. Bands were also seen of approximately 19kDa for the spacer domain, 23kDa for TSP2-4, 30kDa for TSP5-8 and 36kDa for the CUB domain fragment. The TSP1 domain has a predicted molecular weight of 10kDa but only a higher molecular weight band could be seen in the Western blot, suggesting reducing conditions did not persist for long enough or that protein aggregation was taking place, despite the presence of SDS.

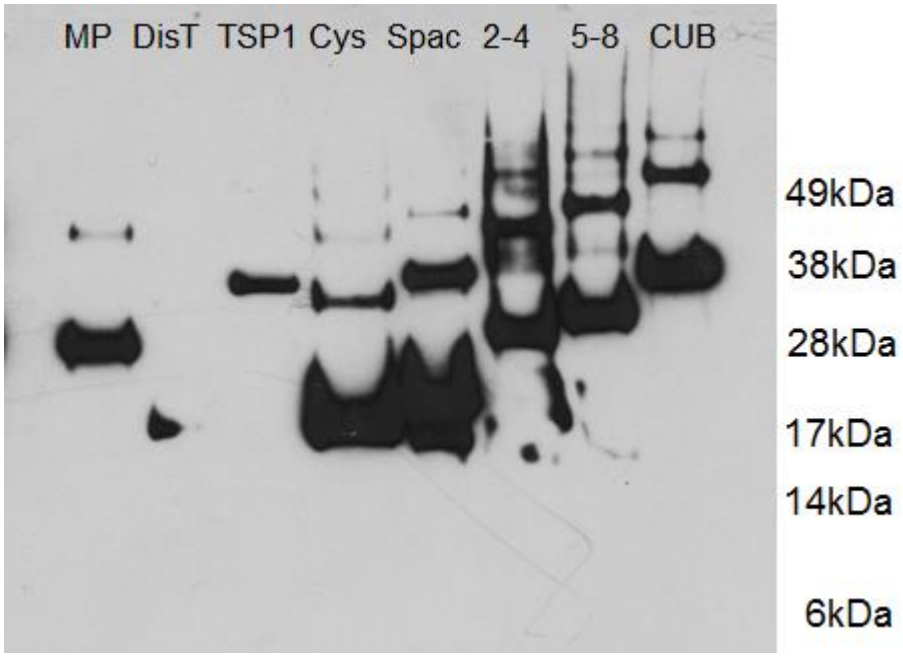


Figure 3.4. Western blot of bacterial domains with anti-Xpress antibody in reducing conditions

Bands are seen of approximately 28kDa for the metalloprotease domain (MP), 17kDa for the domain fragment Dis-TSP1 (DisT), and 17kDa for the cysteine-rich domain (Cys). Bands were also seen of approximately 19kDa for the spacer domain (Spac), 23kDa for TSP2-4 (2-4), 30kDa for TSP5-8 (5-8) and 36kDa for the CUB domain fragment. Only a higher molecular weight band could be seen for TSP1.

To examine whether the bacterially expressed ADAMTS13 domains were recognised by a more physiological detection antibody, Western blotting was also performed using a polyclonal anti-human ADAMTS13 antibody raised in rabbit. The majority of the bacterially expressed ADAMTS13 domains were recognised by the rabbit polyclonal anti-human ADAMTS13 (Figure 3.5).

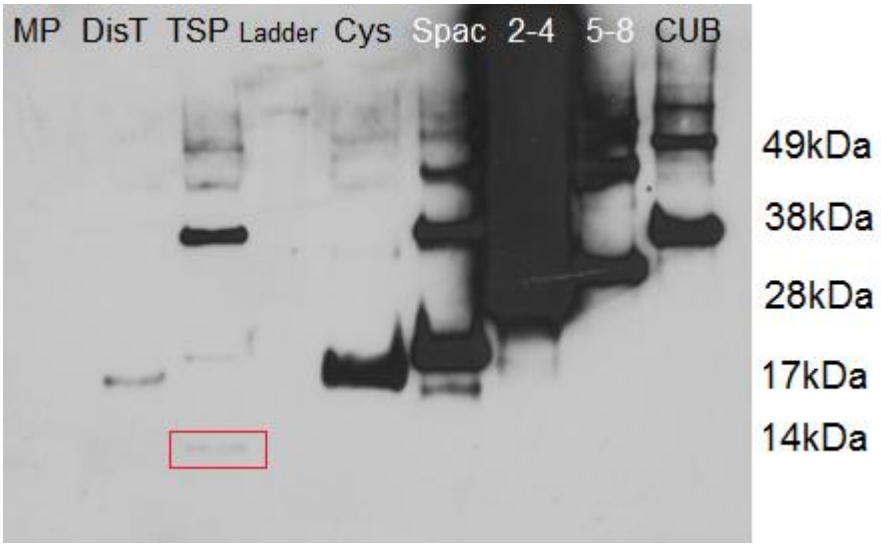


Figure 3.5. Western blot of bacterial domains with rabbit polyclonal anti-human ADAMTS13 antibody

Bands are seen of approximately 17kDa for the domain fragment Dis-TSP1 (DisT), 10kDa for TSP1 domain (boxed in red) and 17kDa for the cysteine-rich domain (Cys). Bands were also seen of approximately 19kDa for the spacer domain (Spac), 23kDa for TSP2-4 (2-4), 30kDa for TSP5-8 (5-8) and 36kDa for the CUB domain fragment. MP=metalloprotease domain.

Interestingly, there was no reactivity against the recombinant metalloprotease domain (Figure 3.5). This was consistent with results of previous affinity isolation of anti-MP antibodies from the rabbit polyclonal antibody which demonstrated little anti-MP activity - data not shown. The antibody was strongly reactive against TSP2-4 (also correlating with results of previous affinity isolation from the rabbit polyclonal antibody which demonstrated a high titre of anti-TSP2-4 - data not shown). A band is now seen at the expected molecular weight of 10kDa for the TSP1 domain (boxed in red), but most the material is of higher molecular weight. Again in this Western blot, some higher molecular weight multimers persisted despite reducing conditions, indicative of some aggregation.

Based on these studies, the ADAMTS13 domains expressed in bacteria were all of the expected molecular weight, but there were issues with aggregation and some incorrect disulphide bond formation. Despite this, the bacterially expressed ADAMTS13 domains were still recognised by antibodies which recognise mammalian full length ADAMTS13 suggesting they could be of use in determining the domain specificity of anti-ADAMTS13 IgG by Western blotting. I therefore proceeded to immunoblotting using bacterially expressed material (section 3.2.3.1).

3.2.2 Expression and purification of ADAMTS13 domain fragments in mammalian cells

The easiest way to ensure that proteins adopt their native fold is to express them in a mammalian expression system. After problems developing an immunoblotting strategy using bacterially expressed material (section 3.2.3.1), I switched to mammalian cell expression to produce ADAMTS13 domain fragments. The ADAMTS13 domain fragments expressed in mammalian cells and subsequently purified, refolded and quantitated in this study are summarised in Table 3.2.

3.2.2.1 Generation of ADAMTS13 fragment vectors

A mammalian expression vector (pcDNA3.1/myc-His) containing full-length ADAMTS13 cDNA was available from previous studies. I successfully generated novel vectors for domain fragments TCS and MDTC by PCR using pcDNA3.1/myc-His MDTCS as a template. Previously generated vectors expressing the ADAMTS13 fragments TSR2-8 and TSR5-8 contained errors associated with PCR-based amplification. The vector for TSR2-8 required insertion of T at position 1135, and TSR5-8 required deletion of CA after the signal peptide and I corrected these by site directed mutagenesis. The vectors that express the truncated ADAMTS13 variants MP-Dis, MDTCS and TSP2-4 were available from previous work in the group (see also Figure 2.6 for list of novel and existing vectors). I verified all novel and existing vectors by sequencing prior to expression work.

3.2.2.2 Expression of ADAMTS13 fragments

Full-length ADAMTS13 and ADAMTS13 fragments MDTCS, TSP2-8 and CUB1-2 were successfully expressed by large scale transient transfection of HEK23T cells. HEK293T cells were specifically chosen as I did not want to use a cell line that expressed ADAMTS13 endogenously (e.g. liver cells), as it would have the potential to contaminate the ADAMTS13 fragment I was expressing. Endothelial cells are not viable expression host cells due to difficult growing and transfection protocols. HEK cells have been used routinely and ADAMTS13 from these cells has been robustly and biochemically characterised.

Construct	Construct	Novel vector	MW (kDa)	Expressed in HEK293T	Issues	Solutions attempted (see text for details)	His tag purified	Tag	Quantitation method	Final conc (nM)
ADAMTS13		No	188	Yes	-	-	Yes	Myc	ELISA	3850
MD		No	40	Yes	-	-	Yes	[§]	WB Nanodrop	3000
TCS		Yes	55	No	Not expressed	Repeat transfections Resequenced	No	Myc	-	-
MDTC		Yes	80	No	MDTC Gly[560] not expressed	Cropped to Cys[555] Resequenced. V5-tagged vector used*	Yes	V5	ELISA WB	150
MDTCS		No	95	Yes	-	-	Yes	Myc	ELISA WB Nanodrop	750
TSP2-4		No	25	Yes	Wrong size (50kDa)	Vector resequenced. Reducing gels ?dimerising. Deglycosylated.	Yes	Myc	-	-
TSP5-8		Yes	32	No	Retained intracellularly	-	No	Myc	-	-
TSP2-8		Yes	70	Yes	Low concentration	Larger scale transfections. Stable cell line with Ig-κ signal peptide used**	Yes	V5	WB	1500
CUB1/2		No	40	Yes	-	-	Yes	Myc	WB	40

Table 3.2 Mammalian ADAMTS13 domain constructs expressed, purified and quantitated in this study

Predicted molecular weights (MW) of mammalian expressed ADAMTS13 domains including His tag, detection tag and predicted glycans are given. Concentrations of purified protein were determined by ELISA, comparative Western blotting (WB) and confirmed if pure by Nanodrop.
[§]construct MD has no myc tag as this was removed for earlier work * construct kindly gifted by Sadler ** construct kindly gifted by Voorberg

The fragment TCS did not express in repeated small scale and larger scale transfections. The TCS vector was checked again by resequencing, but transfection attempts remained unsuccessful. TSP5-8 was expressed but not secreted and was retained intra-cellularly (Figure 3.6 - TSP5-8 is seen in the cell lysate, but is not in the conditioned media).

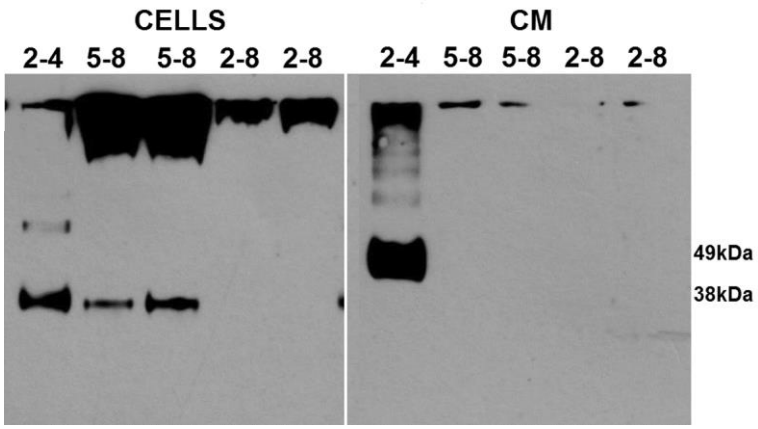


Figure 3.6 Western blot of expression of mammalian C-terminal TSP domain fragments

2-4=TSP2-4 domain fragment, 5-8=TSP5-8, 2-8=TSP2-8, Cells=cell lysate, CM=conditioned media. TSP2-4 is expressed and secreted, but is of incorrect size (see later). TSP5-8 is retained intracellularly. TSP2-8 has not expressed in this particular small scale transient transfection. Western blot probed with anti-His antibody.

Similarly, MDTC truncated at glycine^{560} was not secreted. On review of the literature, MDTC terminating at cysteine^{555} had been successfully synthesised by another group (49). I therefore cropped the vector to that point. Despite this, a further attempt to express MDTC terminating at cysteine^{555} in the pcDNA3.1/myc-His vector was also not secreted (Figure 3.7). However, MDTC^{555} in a pcDNA3.1/V5-His vector was kindly gifted by J.E Sadler and this construct was successfully expressed and secreted, albeit at a lower level than full length ADAMTS13. Possible reasons for this are considered in the discussion section of this chapter. I checked that MDTC was likely correctly folded (despite the low secretion levels) by confirming that it had activity in a FRETS assay.

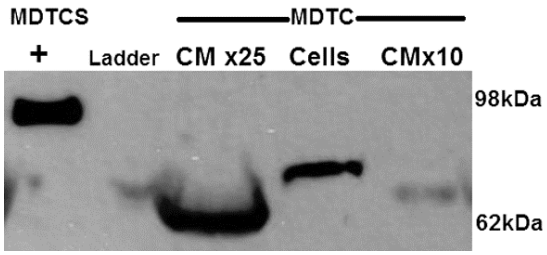


Figure 3.7 Western blot of expression of MDTC^{555} in pcDNA3.1/myc-His vector

Western blot probed with rabbit polyclonal anti-ADAMT113 antibody. A band is seen at 80kDa in the cell lysate indicating that MDTC^{555} has expressed, but there is no corresponding band in the 25-fold concentrated (CMx25) or 10-fold concentrated (CMx10) conditioned media. A non-specific band is seen at approximately 66kDa in the highly concentrated conditioned media. This is likely to represent BSA (rabbit polyclonal anti-ADAMTS13 antibody is known to contain antibodies *vs.* BSA).

The fragment TSP2-8 was secreted at an extremely low level, making use of transient transfections impractical as a source of this material. The stable cell line HEK293 TSP2-8 V5 His, which has an Ig-κ signal peptide cloned in front of the coding region of the TSP2-8 construct in order to increase the level of expression of the variant (50), was kindly gifted by J. Voorberg and I used the stable cell line to provide sufficient material for purification.

3.2.2.3 ADAMTS13 fragment purification by FPLC

I then went on to partially purify full-length ADAMTS13 and all the ADAMTS13 fragments on a Ni^{2+}-chelating HiTrap column. Figure 3.8 illustrates this purification step for the fragment CUB1/2. Fractions of the flow-through, elution and stripping peaks were analysed by SDS-PAGE to determine which fraction contained the protein of interest. The mammalian expressed ADAMTS13 fragments were present in the elution peaks for each construct.

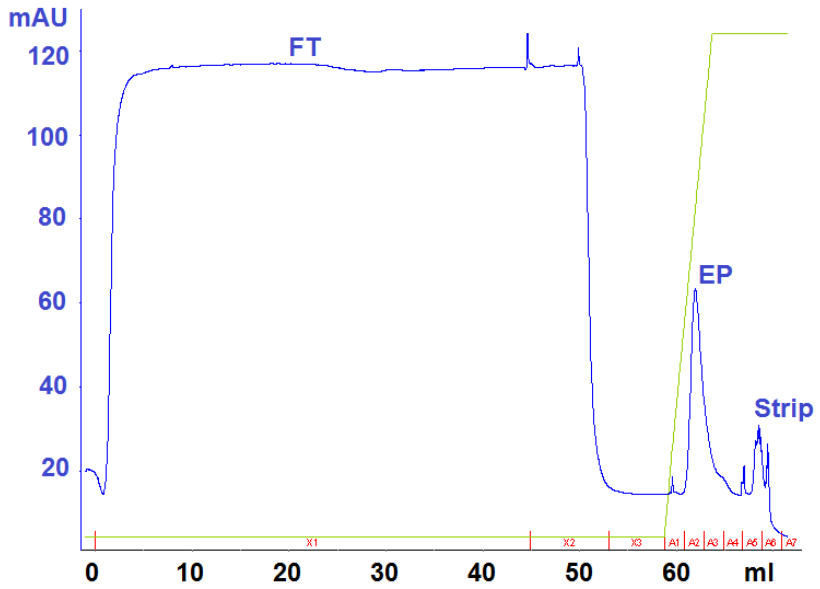


Figure 3.8 FPLC chromatogram of the purification of CUB1/2

The 280nm absorbance (blue line) is plotted against the elution volume over time.
Buffer gradients are shown in green. The unfolded protein is loaded onto the column in a denaturing buffer with low imidazole concentration to reduce non-specific protein binding. The CUB1/2 protein binds via the metal ion binding His-tag, and the other non-tagged proteins appear in the flow through (FT) as an absorbance peak containing large amount of protein (approx. 120mAU). The desired protein is eluted by a gradient over 10 minutes into elution buffer containing a higher concentration of imidazole (elution gradient shown in green). This aims to gradually increase the protein concentration in the eluted fractions and thus reduce the risk of aggregation. The smaller elution peak (EP) is seen, which should contain the desired protein (CUB1/2). The column is stripped with EDTA-containing stripping buffer (Strip= stripping peak) to remove any aggregated protein from the column matrix and remove the Ni2+ ions.

3.2.2.4 Analysis and characterisation of mammalian expressed proteins

The ADAMTS13 fragments expressed spanned the enzyme from the metalloprotease domain to CUB1/2. Predicted molecular weights of the various fragments including tag are shown in Table 3.2. The construct MD does not have a myc tag as this was removed for earlier work, and MDTC and TSP2-8 have V5 epitopes. Western blot analysis using anti-tag antibody confirmed that the purified ADAMTS13 fragments were mainly isolated as single undegraded bands of the expected molecular weight with little multimerisation.

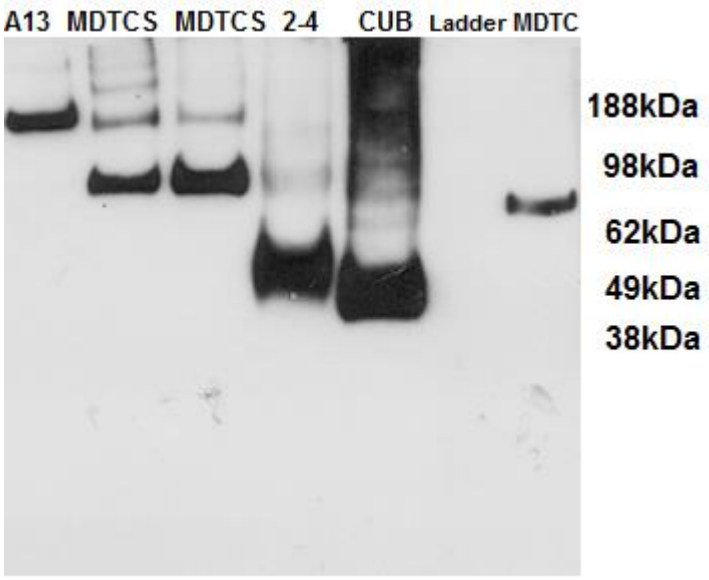


Figure 3.9 Semi-quantitative Western blot of mammalian expressed ADAMTS13 fragments with anti-tag antibodies

A13 = full length ADAMTS13, MDTCS = metalloprotease-spacer domain fragment, MDTC =metalloprotease-cys-rich domain fragment, 2-4= TSP2-4 domains, CUB = CUB1/2 domains. Blot probed with anti-myc-HRP (A13, MDTCS, 2-4, CUB) + anti-V5-HRP (MDTC)

Figure 3.9 shows one of series of semi-quantitative Western blot used to ensure approximately equal loading (in molar terms) of each domain fragment for subsequent analysis of patient antibodies. Bands are seen at 190kDa for full-length ADAMTS13, 95kDa for MDTCS, 50kDa for TSP2-4, 40kDa for CUB1/2 and 80kDa for MDTC.

The fragment TSP2-4 was not reliably detected by Western blotting with anti-myc HRP and was poorly detected by the rabbit polyclonal antibody anti-TSP2-4 in Western blots. It migrated at approximately 50kDa (Figure 3.9), although the predicted molecular weight was closer to 25kDa. The vector was again resequenced and confirmed to be correct. Interestingly, the TSP2-4 harvested from the cell lysate was the correct size, with a smaller band seen at approximately 50kDa (Figure 3.6). TSP2-4 has free cysteines, suggesting that the 50kDa band might represent dimeric material.

A reducing gel of TSP2-4 (using DTT as the reducing agent and including a reducing agent in the running buffer, ClearPAGE, C.B.S. Scientific) with anti-myc-HRP was performed to see if the protein was dimerising, but did not detect the fragment. A sample of TSP2-4 was deglycosylated with PNGase F (NEB), and the size of the glycosylated and deglycosylated forms assessed by Western blot with anti-myc HRP. However, the deglycosylated TSP2-4 still ran at greater than 40kDa – significantly bigger than the predicted molecular weight. Use of the fragment TSP2-4 was therefore abandoned, due to the difficulty in detecting it reliably in Western blots and concerns about the size of the fragment.

Quantitation of purified full-length ADAMTS13, MDTCS and MDTC by ELISAs revealed concentrations of 3850nM, 750nM and 150nM respectively. Semi-quantitative Western blotting using anti-tag antibodies was used to estimate the concentration of ADAMTS13 fragments relative to each other and to a standard (full-length ADAMTS13 of known concentration determined by ELISA), by altering the volume loaded to give bands of approximately equal size. This allowed the concentrations of the smaller fragments to be estimated (Table 3.2). Analysis of the purity of the ADAMTS13 fragments by Coomassie revealed that MDTCS and MD migrated as single bands of expected molecular weight (Figure 3.10). The concentration of these purified samples was assessed by absorbance at 280nm, which corroborated the concentrations obtained by ELISA/Western blot.

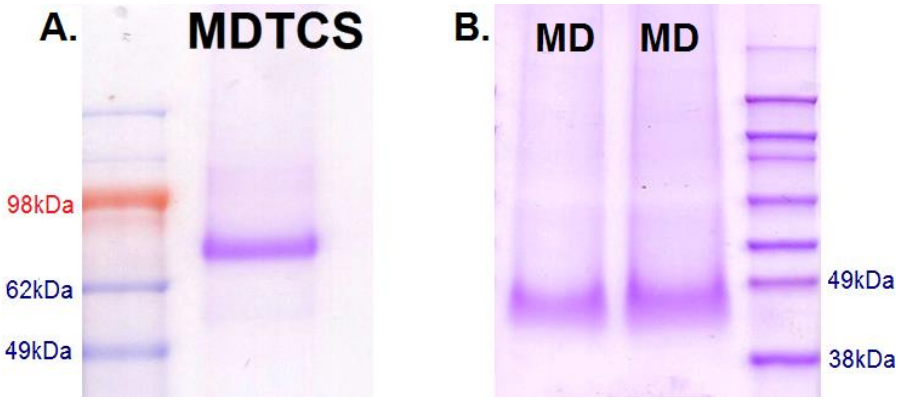


Figure 3.10 Coomassie of mammalian expressed ADAMTS13 fragments MDTCS and MD

MDTCS and MD migrated as single bands of expected molecular weight (95kDa and 40kDa respectively).

3.2.3 Determination of domain specificity of TTP patient autoantibodies

3.2.3.1 Immunoblotting using bacterially expressed material

This strategy was chosen as immunoblotting is potentially straightforward, gives a good overview of the domains affected by an autoimmune response and allows major immunogenic regions to be identified. Bacterially expressed material was used in the first instance due to the ease of expression allowing synthesis of large quantities (up to mg amounts of protein).

Repeated attempts were made to optimise this assay. Initial high background was improved by altering membrane blocking by increasing the percentage of blocking agent and incubating overnight. Background was further reduced by using purified IgG (rather than plasma or PEX) as a source of anti-ADAMTS13 antibodies.

The only specific band seen in Western blotting with TTP IgG was the positive control: mammalian expressed full-length ADAMTS13 (Figure 3.10). Non-specific bands were seen in the normal control. Many human plasma samples contain anti-*E.coli* antibodies, and there is the possibility that contaminating bacterial proteins could give rise to false positive results.

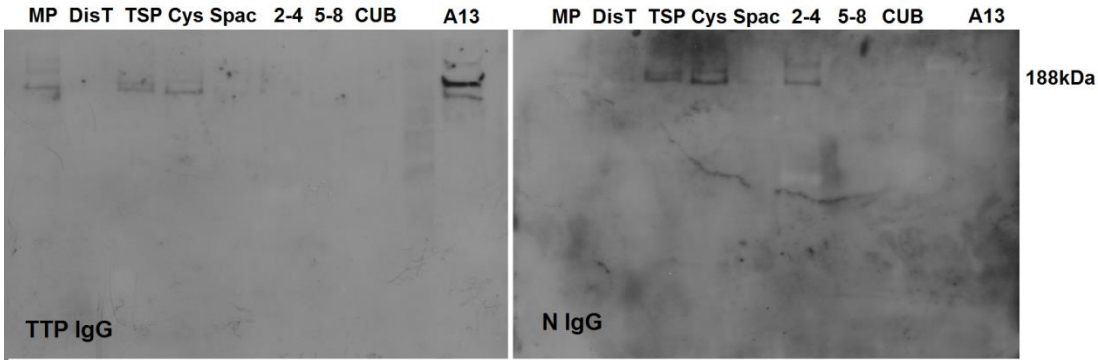


Figure 3.11 Western blot of bacterially expressed domains of ADAMTS13 probed with TTP IgG and normal IgG in non-reducing conditions

No specific bands are seen other than the positive control- mammalian expressed full-length ADAMTS13. Note high background.

To determine whether the lack of reactivity of TTP IgG with the bacterially expressed fragments might be due to non-native conformation under reducing conditions, I repeated the Western blots under non-reducing conditions but this did not alter the results. No improvement was seen using a PVDF membrane rather than nitrocellulose.

The monoclonal human-derived anti-spacer antibody recognised mammalian-expressed full length ADAMTS13 but did not recognise the bacterially expressed spacer domain (not shown) suggesting the material was not correctly folded / displaying physiological epitopes.

3.2.3.2 Immunoblotting using mammalian expressed material

The approach was changed to use mammalian expressed material when it became clear that the bacterially expressed material, in particular the critical spacer domain, was not recognised by anti-ADAMTS13 antibodies from TTP patients in the context of Western blotting. Expression of proteins in mammalian cells is more time-consuming with much lower yields, but is more likely to result in correctly folded protein.

Repeated attempts were made to optimise an immunoblotting assay using mammalian expressed material by altering the amount of protein loaded; the blocking strategy; the buffer (TBS *vs.* PBS) and using purified IgG rather than plasma. Initially the fragment MDTCS was recognised by TTP IgG (Figure 3.12), but no antibodies were detected against full-length ADAMTS13 or other fragments.

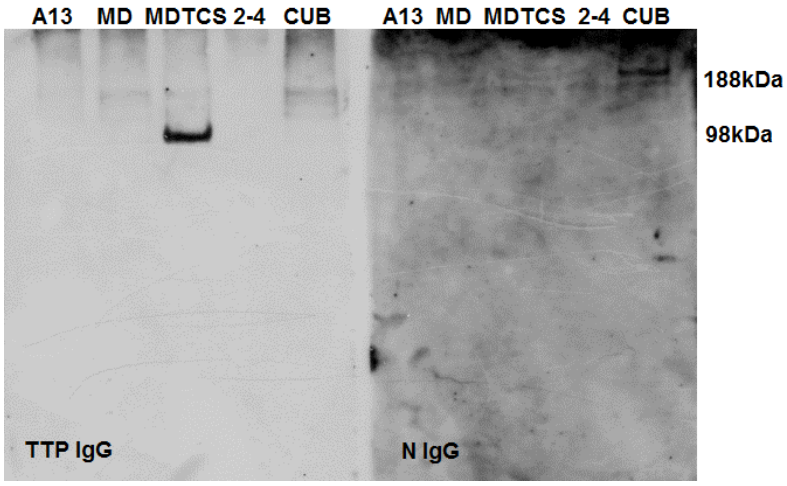


Figure 3.12 Western blot of mammalian expressed fragments of ADAMTS13 probed with TTP IgG and normal IgG in non-reducing conditions

TTP IgG extracted from PEX shows reactivity against MDTCS but not full-length ADAMTS13. Note high background with normal IgG.

Some improvement was seen using a PVDF membrane rather than a nitrocellulose membrane, in that antibodies against full-length ADAMTS13 could be demonstrated as well as antibodies against MDTCS (Figure 3.13). No antibodies were detected that recognised MD, MDTC or the C-terminal domains in three patients examined. However, problems remained with high background and non-specific binding, and the decision was made to try an alternative strategy of immunoprecipitation.

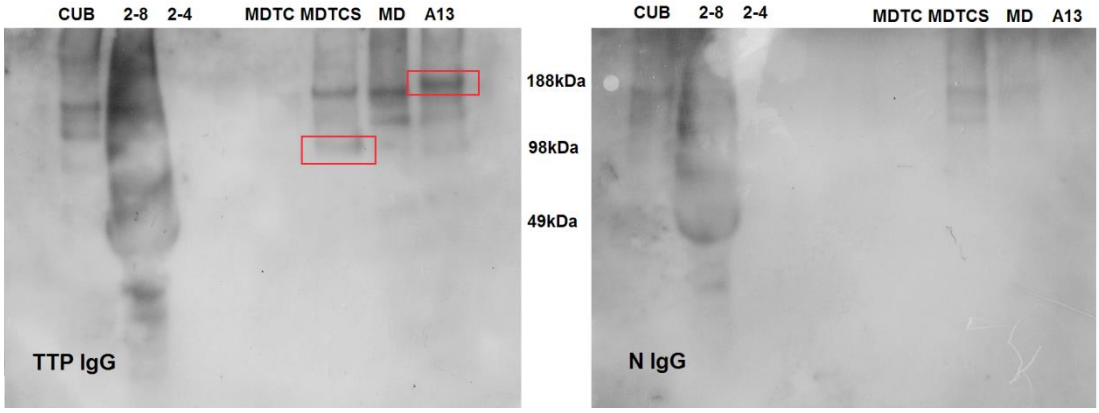


Figure 3.13 Western blot of mammalian expressed fragments of ADAMTS13 using PVDF membrane probed with TTP IgG and normal IgG in non-reducing conditions

Immunoblotting with TTP IgG showing reactivity against full length ADAMTS13 and MDTCS (boxed in red). Non-specific bands are seen with both TTP and normal IgG.

3.2.3.3 Immunoprecipitation

Plasma exchange fluid from patients with TTP was able to immunoprecipitate full length ADAMTS13 and the N-terminal fragment MDTCS (Figure 3.14 A and B).

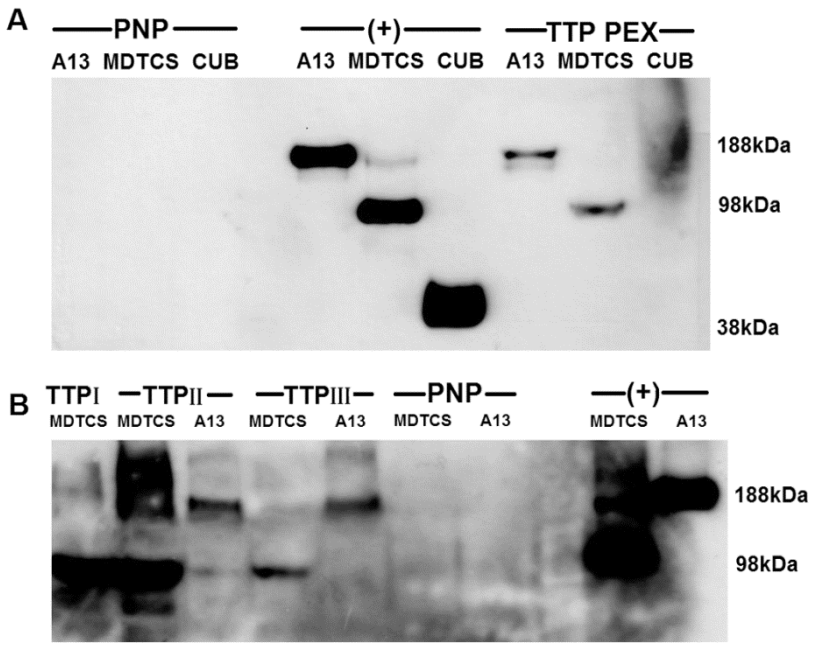


Figure 3.14 Immunoprecipitation of full length ADAMTS13 and MDTCS by TTP PEX fluid

+=positive control (20-40ng of his-tag purified recombinant A13, MDTCS or CUB). Detection with anti-myc-HRP

MDTCS was generally more readily immunoprecipitated than the full length ADAMTS13 as shown in Figure 3.14B by TTP PEX fluid from two different patients - TTPI and TTPII. (Interestingly, patients TTPI and TTPII were later demonstrated to have solely antibodies directed against the N-terminal domains of ADAMTS13, whilst TTPIII, where the preferential IP of MDTCS was not seen, was shown to also have a large proportion of antibodies against the C-terminal domains).

Increasing the volumes of protein G, antibody and antigen for a given elution volume meant that the assay could be utilised in patients with a more moderate titre of anti-ADAMTS13 IgG, thus improving sensitivity (Figure 3.15).

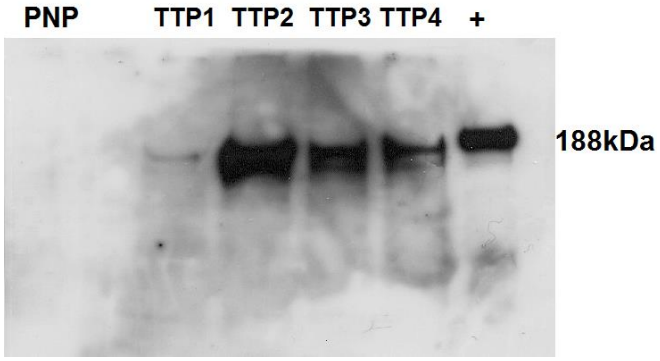


Figure 3.15 Immunoprecipitation of full-length ADAMTS13 by plasma exchange fluid from TTP patients

PNP=pooled normal plasma TTP=PEX from TTP patient
+=positive control (20ng his-tag purified recombinant A13). Detection with anti-myc-HRP
The amount of protein G, antibody (volume of PEX fluid) and ADAMTS13 were doubled for TTP2 and trebled for TTP1 for the same elution volume to try to improve the sensitivity of the assay. This allowed detection of anti-ADAMTS13 antibodies by immunoprecipitation in a sample with a moderate titre (TTP1 =40%)

PEX from four patients tested was able to immunoprecipitate MDTCS but not MDTC, suggesting that the antibodies immunoprecipitating MDTCS are directed against the spacer domain. PEX from seven patients were screened for reactivity against the fragment TSP2-8, two of whom had detectable anti-TSP2-8 antibodies (Figure 3.16)

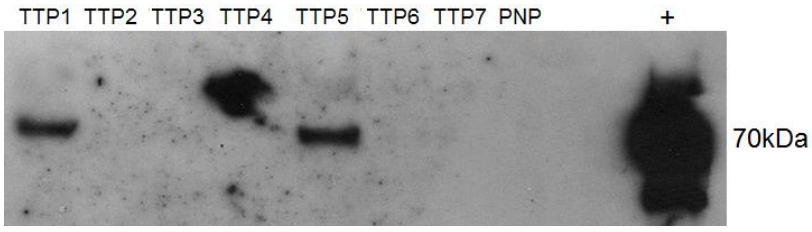


Figure 3.16 Immunoprecipitation of the fragment TSP2-8 by plasma exchange fluid from TTP patients

+ = positive control (50ng his-tag purified TSP2-8). Detection with anti-V5-HRP
TTP1 and TTP5 have anti-TSP2-8 antibodies

PEX from the same seven patients was screened for anti-CUB antibodies using this technique with no reactivity found. As material remained at the top of the lanes in the Western blots of the CUB immunoprecipitation, these were repeated in reducing conditions. However, no reactivity against the CUB domains could be detected in the samples.

The plasma requirement for immunoprecipitation (170µl) was prohibitive, meaning this assay could not be rolled out to patient samples. Thus, an alternative strategy of an ELISA-based assay was sought.

3.2.3.4 ELISA

3.2.3.4.1 Initial assay development

I decided to develop an ELISA for detection of domain-specific antibodies as such an approach could potentially allow high-throughput with screening of multiple samples against different fragments of ADAMTS13 with lower plasma requirements.

I tried various strategies including using polyclonal rabbit anti-human ADAMTS13 or anti-His tag antibodies to capture the ADAMTS13 fragments with detection of bound anti-ADAMTS13 antibodies with anti-human IgG-HRP. Coating the plate with purified IgG from TTP patients was also attempted with bound proteins detected with anti-tag antibody.

Capturing the his-tagged full length ADAMTS13 or ADAMTS13 fragments using anti-His antibody was tried first (Figure 2.7A), as this method was used in the original description of an ELISA to detect anti-ADAMTS13 IgG (81). However, this strategy did not result in good capture of my partially purified full length ADAMTS13, and did not permit detection of anti-ADAMTS13 IgG. This was not improved by using a more specific anti-human IgG HRP (Abcam *vs* Dako).

Capturing ADAMTS13 using the in-house rabbit polyclonal anti-ADAMTS13 antibody (Figure 2.7C) allowed detection of anti-ADAMTS13 antibodies in

TTP PEX. However, this strategy could not have been used for all the fragments, as the antibody has been depleted of anti-TSP2-4 activity.

Inverting the assay and using patient IgG to coat the plate and detecting captured ADAMTS13 with a polyclonal biotinylated anti-TSP2-4 antibody and streptavidin-HRP to amplify the signal (Figure 2.7B) allowed me to distinguish between TTP PEX and normal plasma, but gave only a weak signal. This is likely to be because only a very small percentage of total IgG will be anti-ADAMTS13 IgG, even in the acute setting. A different detection system, e.g. anti tag antibodies, would also be required to roll this assay out to all the fragments.

The most promising strategy was to coat the plate directly with full-length ADAMTS13 or fragments, and I developed this assay further. However, direct coating did not work for some of the ADAMTS13 fragments e.g. MDTCS and CUB1/2, presumably because the proteins bound to the plate through their antigenic surfaces. This was circumvented by using both a direct ELISA for the fragment TSP2-8, and spiking other fragments (MDTCS, MDTC, MD, CUB) into an ELISA detecting antibodies against full-length ADAMTS13.

The development and results of these novel domain specificity assays at presentation of acute TTP are discussed in chapter 4.

The results of the domain specificity assays longitudinally through therapy, remission and relapse are discussed in chapter 6.

3.2.4 Antibody extraction

Total IgG extracted from TTP PEX or plasma or pooled normal human plasma using protein G spin columns was used for initial work. It was utilised for immunoblotting with ADAMTS13 fragments synthesised in both bacterial and mammalian expression systems. I was able to demonstrate antibodies against mammalian expressed full-length ADAMTS13 and the N-terminal fragment MDTCS using protein G extracted IgG. A protein G approach was also used to extract IgG from PEX for immunoprecipitation, which allowed me to demonstrate antibodies against full-length ADAMTS13, MDTCS and TSP2-8, albeit with a large plasma requirement.

However, when work was commenced optimising the VWF115 activity assays using IgG extracted from TTP PEX, no inhibition could be detected. The ability of these TTP IgG to bind ADAMTS13 was tested using the ELISA described in section 2.3.4 for detection of anti ADAMTS13 antibodies, and no binding was detected. Quantitation of the total IgG by Nanodrop and IgG ELISA (Bethyl Laboratories) revealed a concentration of only 1mg/ml. Normal plasma IgG concentration is 10-15mg/ml. The protocol resulted in an approximately 2.6 fold dilution of the IgG, meaning that only 20-30% of the available IgG was being extracted using this method.

Melon gel IgG extraction was therefore tried instead. Work using VWF115 assays to compare the inhibitory potential of IgG extracted from TTP PEX using melon gel with protein G extracted IgG showed that melon gel extracted antibodies retained their inhibitory potential. However, the melon gel protocol results in a 10-fold dilution of the IgG, so I used a buffer exchange protocol prior to melon gel extraction to avoid this dilution step.

Total IgG purified using melon gel with buffer exchange to eliminate dilution step had concentrations of 8-15 mg/ml, commensurate with plasma IgG concentrations. There was good agreement between values obtained by IgG ELISA and by measuring absorbance at 280nm. Total IgG extracted from

patient samples or pooled normal plasma using this protocol was therefore used in subsequent VWF115/106 assays and FRETS assays (chapter 5).

3.3 Discussion

In order to generate material for use in assays investigating the domain specificity of anti-ADAMTS13 antibodies, I expressed and purified recombinant proteins spanning ADAMTS13 synthesised in both bacterial and mammalian systems. I used a novel on-column refolding strategy for bacterially expressed domains purified from inclusion bodies which yielded soluble protein for all domains, except the spacer. The spacer domain lacks disulphide bonds which normally give structural stability to a protein. The spacer domain is stabilised by multiple hydrophobic interactions both within the domain and with the neighbouring cys-rich domain, and loss of the cys-rich domain may adversely affect its structure.

The domains expressed in *E.coli* (with the exception of MP) were recognised by a rabbit polyclonal anti-human ADAMTS13 (raised against full-length ADAMTS13 expressed in mammalian cells) giving initial hope that these domains might also serve as tools for exploring TTP patients' autoantibody specificity. However it should, of course, be remembered that this polyclonal antibody was invoked by immunisation of rabbits with human ADAMTS13, a much more immunogenic scenario than an autoimmune response.

However, my preliminary work on antibody detection using immunoblotting with bacterially expressed material was unsuccessful, with none of the domains being specifically recognised by TTP patient plasma or IgG purified from patient plasma or PEX. This was not due to antibody concentration as the TTP plasma/IgG recognised the positive control: mammalian expressed full-length ADAMTS13. This is most likely to reflect a problem with correct folding of the bacterially expressed material, or possibly lack of post-translational modifications such as glycosylation. The fact that the patient-derived monoclonal anti-spacer antibody II-1 failed to recognise the bacterially expressed spacer domain confirms that the critical antigenic

surface involving $Arg^{660,}$ $Tyr^{661,}$ and Tyr^{665} (RYY- see Figure 1.12) was not available or correctly folded to enable antibody binding.

A previous study had successfully used bacterially expressed domains in a Western blotting strategy to study the domain specificity of anti-ADAMTS13 antibodies (192). The difference is likely to lie in the different expression/refolding strategies used and also the choice of fragments synthesised. Klaus *et al* used a pBAD/Thio-TOPO expression vector where the ADAMTS13 fragments were produced as thioredoxin fusion proteins with His tags. HP-thioredoxin acts as a translation leader to facilitate high-level expression and, in some cases, solubility (Invitrogen), and TSP1 was indeed obtained from the soluble fraction in the study by Klaus *et al*, although the other fragments were from the insoluble fraction (192). It may be that my on-column refolding strategy was not as effective, although it did generate soluble protein for all the ADAMTS13 domains, except the spacer domain. Importantly, Klaus *et al* also mainly expressed larger domain fragments (MDT, cys-rich/spacer, TSP2-8) rather than single domains, and did not attempt to express the spacer domain in isolation (192). These larger domain fragments may have been more likely to adopt a native fold than isolated domains. As discussed above, the spacer domain lacks disulphide bonds and is instead stabilised by multiple hydrophobic interactions both within the domain and with the neighbouring cys-rich domain.

I went on to express domain fragments spanning ADAMTS13 in a eukaryotic system. Full-length ADAMTS13 and ADAMTS13 fragments MDTCS, TSP2-8 and CUB1-2 were successfully expressed by large-scale transient transfection of HEK23T cells. I encountered some issues with expressing other domain fragments and used different strategies to investigate this further in an attempt to resolve the problems. ADAMTS13 fragments TSR5-8 and MDTC (truncated at Gly^{560}) in the pcDNA3.1/myc-His vector were not secreted and were retained intracellularly. Cropping MDTC in pcDNA3.1-myc-His vector to Cys^{555} did not improve secretion. However, the fragment $MDTC^{555}$ in a V5-tagged vector was secreted (albeit at reduced levels compared to full-length ADAMTS13). This may be because the V5-epitope is

larger and possibly plays a protective role. The cys-rich domain has a large interface with the spacer domain and lack of the spacer may cause misfolding: the V5 tag may act as a surrogate for the spacer domain and help correct folding.

The fragment TSP2-4 was expressed and His-tag purified, but there was difficulty in detecting it reliably in Western blots. It migrated at approximately 50kDa, although the predicted molecular weight was 25kDa and the vector was confirmed by resequencing. I investigated whether it might be dimerising or heavily glycosylated, but as the domains were included in the fragment TSP2-8, this material was used instead for assay development.

Detection of TTP autoantibodies by Western blotting using mammalian expressed ADAMTS13 fragments allowed detection of antibodies that specifically recognised either / both full-length ADAMTS13 or the N-terminal fragment MDTCS. However, high background and additional non-specific bands remained a problem despite repeated attempts to optimise assay conditions. No antibodies were detected that recognised MD, MDTC or the C-terminal domains in three patients examined, either because the antibodies detected in these patients were solely directed against the spacer domain, or because Western blotting is unable to pick up antibodies against other domains because conformation-specific epitopes are lost, as Western blotting solely detects antibodies directed against linear epitopes.

Immunoprecipitation offered a more physiological approach as antibody-antigen binding occurs in solution with the antigen in native conformation, not denatured as in SDS-PAGE and then immobilised on a membrane, or unfolded as on an ELISA plate. I was able to reproducibly detect antibodies against the N-terminal fragment MDTCS using this approach. Indeed, MDTCS was more readily immunoprecipitated, and thus appears more readily recognised by anti-ADAMTS13 antibodies, than the full-length ADAMTS13 of the same concentration. This is likely to reflect the availability of the critical antigenic surface in the spacer domain that may be shielded / partially shielded in ‘closed’ conformation full-length ADAMTS13 by the C-

terminal domains. Further support for this concept is provided by South *et al* who studied IP of full-length ADAMTS13 by the patient-derived anti-spacer mAb II-1. When converted to its open conformation by binding to VWF D4CK fragment, recognition of full-length ADAMTS13 increased dramatically (53).

Immunoprecipitation demonstrated anti-TSP2-8 antibodies in PEX from two out of seven TTP patients tested. However, immunoprecipitation did not allow me to detect any anti-CUB antibodies in the seven samples tested. This may be because these patients lacked antibodies directed against the CUB domains but, given the frequency of C-terminal antibodies in the literature (widely varying but around 30% in several studies (50, 196)), may represent a sensitivity issue when detecting these antibodies using immunoprecipitation. The washing conditions for the assay are fairly stringent and might be causing dissociation of the antigen from the antibody, if antibodies against CUB domains are of lower affinity.

Initial attempts at developing an ELISA to assess domain specificity demonstrated that direct coating of the fragment on the plate represents the best strategy, as opposed to antibody-mediated capture. Full-length ADAMTS13 appears to be relatively more easily recognised by autoantibodies in this ELISA than in immunoprecipitation, likely reflecting a conformational change induced by direct coating on the plate, thus 'opening' up ADAMTS13 and unmasking important antibody recognition sites that are hidden in solution phase. However, direct coating did not work for some of the ADAMTS13 fragments e.g. MDTCS and CUB1/2, presumably because the proteins bound to the plate through their antigenic surfaces.

I went on to develop a combination of direct ELISAs and competition assays (chapter 4), which allow analysis of the domain specificity of anti-ADAMTS13 antibodies. These have been used to determine the domain specificity of anti-ADAMTS13 antibodies, both at presentation of acquired TTP (chapter 4), and longitudinally through therapy, remission and relapse (chapter 6). The inhibitory potential of the anti-ADAMTS13 antibodies directed against different domains is discussed in chapter 5.

4 Domain specificity of TTP patient autoantibodies at presentation

Autoantibodies against ADAMTS13, predominantly IgG, are present in the majority of acquired TTP patients and cause profound loss of VWF-cleaving function (83-85, 138). Antibodies that bind the N-terminal domains of ADAMTS13 (MDTCS) are detected in most patients, although antibodies recognising the C-terminal domains of ADAMTS13 have also been reported (50, 192-196). Little work has been done on the clinical significance of these antibody patterns.

The aim of the work presented in this chapter was to determine the domain specificity of anti-ADAMTS13 antibodies at presentation of acquired TTP using novel ELISAs and competition assays, and also to study the clinical correlates of the patterns of domain specificity. We currently have only limited ability to identify those individuals with a more severe disease phenotype who are most at risk of death, or to determine which patients are more likely to relapse. Thorough investigation of the antibody repertoire in TTP patients may improve our ability to prognosticate in acquired TTP.

4.1 Methods

4.1.1 Development of domain specificity assays

To examine the domain specificity of anti-ADAMTS13 autoantibodies in TTP patients at presentation, combinations of ELISA-based and competition assays were developed using the full-length ADAMTS13 and ADAMTS13 fragments that I had expressed in mammalian cells and purified by Ni^{2+} chelating chromatography (section 2.2.4).

In the direct ELISA to detect all antibodies recognising full-length ADAMTS13, recombinant ADAMTS13 was coated on Maxisorb plates. Once blocked, diluted TTP plasma or PNP was added. After washing, bound anti-ADAMTS13 antibodies were detected using an HRP-conjugated secondary anti-human IgG (Dako).

All TTP patient plasmas exhibited robust immunoaffinity for full-length ADAMTS13 immobilised on a 96-well plate (Figure 4.4) and I proceeded to optimise certain variables in this assay. The amount of ADAMTS13 coated on Maxisorb plates was titrated (1, 2.5 and 5μg/ml), with 2.5μg/ml providing maximum signal and being chosen for coating as being most sensitive to detect anti-ADAMTS13 IgG (5μg/ml did not give a further increase in signal).

Plasma dilution was also titrated with 1 in 100, 1 in 50 and 1 in 20 dilutions being assayed. A 1 in 50 dilution of plasma was used routinely as it was sufficiently sensitive, but a 1 in 20 dilution of plasma was used to detect lower titre antibodies (anti-ADAMTS13 IgG<35%).

I then tried to develop direct ELISAs for the other ADAMTS13 fragments I had expressed in mammalian cells and purified. Interestingly, when MDTCS was immobilised directly to a 96-well plate, this material was comparatively poorly recognised by patient antibodies even when 1 in 20 plasma dilutions were used (data not shown), suggesting that direct coupling of MDTCS to the plate appreciably compromises its immunoreactivity (or that antibodies do not recognise these domains. However, given previous data on antibody specificity, this latter contention was unlikely).

To circumvent the apparent loss of immunoreactivity of MDTCS when directly immobilised on the plate, I developed a competition assay in which the TTP plasma dilutions were preincubated both with and without an excess of MDTCS in solution prior to incubation with wells coated with full length ADAMTS13. The concentration of MDTCS spiked was determined by titration using a patient sample with one of the highest total anti-ADAMTS13 titres (Figure 4.1).

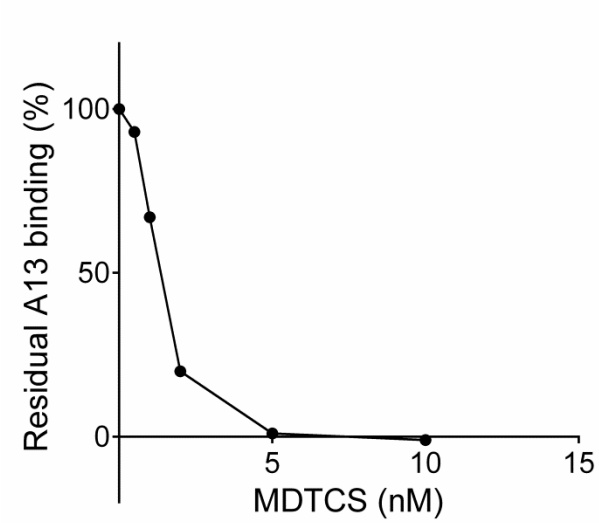


Figure 4.1 Titration of MDTCS concentration required for competition assay

5nM of MTCS was able to compete for 100% of ADAMTS13 binding in patient sample #16 (anti-ADAMTS13 IgG titre=105%). The IC_{50} was approximately 1.5nM. Based on these results, 10nM MDTCS was chosen for the competition assay.

When the assays were performed in parallel (i.e. ±MDTCS preincubation), this approach enabled estimation of the proportion of total anti-ADAMTS13 antibodies that recognised MDTCS. The residual binding detected (i.e. % non-MDTCS autoantibodies) was likely, therefore, to represent autoantibodies that recognised the C-terminal TSP2-8 and/or CUB1/2 domains (Figure 4.3).

I attempted to develop a direct ELISA for the two C-terminal domain fragments TSP2-8 and CUB1/2. When the fragment CUB1/2 was immobilised directly on the plate, it was not recognised by anti-ADAMTS13 antibodies in TTP patient plasma, despite coating up 20nM and using plasma at up to 1 in 10 dilution. Higher concentrations of CUB1/2 could not be used because of the limited amount of recombinant protein available.

However, the fragment TSP2-8 was recognised by anti-ADAMTS13 antibodies in patient plasma in a direct ELISA and I went on to optimise this assay further by determining the coating concentration required to give the most robust/sensitive detection (100nM) and the optimal plasma dilution (1 in 50 for higher titre anti-ADAMTS13 IgG, 1 in 20 for samples with anti-

ADAMTS13 IgG<35%). A cut-off of 2x the mean signal for pooled normal plasma was used to assign positivity in the direct TSP2-8 ELISA. Seven samples had previously been tested for the presence or absence of anti-TSP2-8 antibodies by immunoprecipitation (Figure 3.16), and the results of the IP were in agreement with those from the TSP2-8 ELISA.

In the final optimised assays, the direct anti-ADAMTS13 ELISA was performed in parallel with the competition assay and samples tested for the same time for their reactivity against TSP2-8 (Figure 4.2).

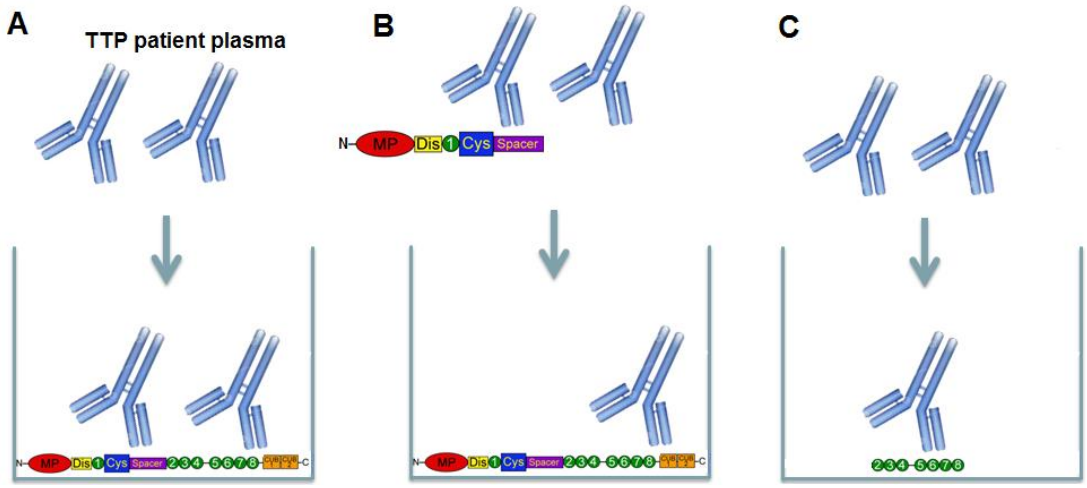


Figure 4.2 Novel assays for analysis of domain specificity of anti-ADAMTS13 IgG

Diluted TTP patient plasmas were analysed in parallel for IgG that recognised immobilised full length ADAMTS13 both without (A) and with (B) preincubation of plasmas with 10nM MDTCS. Plasma samples were also analysed for the presence of anti-TSP2-8 IgG in a separate ELISA (C).

Each assay (direct ADAMTS13 ELISA, MDTCS competition assay and TSP2-8 ELISA) was performed in duplicate at the same time. Pooled normal plasma was used on each plate and acted as an internal control. The intra-assay coefficients of variation (CV - average value calculated from the individual CVs for all of the duplicates) for each assay were 6.7% for the direct ADAMTS13 ELISA, 7.3% for the MDTCS competition assay and 8.1% for the TSP2-8 direct ELISA.

The results of the assay were left as mean absorbance, as the assay was not quantitative in absolute terms, but rather provided a description of the pattern of the antibody repertoire in a given patient. For the ADAMTS13 ELISA ±MDTCS preincubation, the mean absorbance at 492nm (optical density, OD) for the PNP on the plate was subtracted from the mean OD of each sample, before converting the result into a proportion of non-MDTCS antibodies (C-terminal antibodies) using the formula:

$$\frac{\text{(Mean sample OD– mean PNP OD) with MDTCS precincubation}}{\text{(Mean sample OD - mean PNP OD) without MDTCS preincubation}} \times 100$$

Ten samples were run on three separate dates for the MDTCS competition assay (once as part of the main assay to determine domain specificity, once for MDTC competition and once for MD + MDTC competition). The average difference in % C-terminal reactivity on different runs was 4.2% (mean difference 4.2%; range 0-8%), and no sample changed category (i.e. N-terminal only *vs*. N+C antibodies).

4.1.2 Methodology for domain specificity assays

4.1.2.1 Competition ELISA to detect antibodies against the N-terminal fragments MDTCS, MDTC and MD

Maxisorb 96-well plates (Nunc) were coated with 2.5µg/ml His tag purified ADAMTS13 in 50mM sodium carbonate buffer pH 9.6. Wells were washed with phosphate buffered saline/0.1% Tween-20 (PBST) and blocked with PBS/2.5% BSA/1% fetal calf serum (FCS) and incubated for 2 hour with shaking. TTP patient, or pooled healthy control plasmas were diluted 1 in 50 (or 1 in 20 if patient anti-ADAMTS13 IgG titre was <35%) in PBS/1% BSA/1% FCS and incubated with wells (100µl/well in duplicate) for 1 hour (Figure 4.2A). Wells were washed and bound anti-ADAMTS13 antibodies were detected with anti-human IgG-HRP (Dako). Wells were washed, and OPD (Sigma) was used for colour development, which was stopped with 2.5M H_2SO_4, and absorption at 492nm measured.

In parallel, identical experiments were set up except that diluted plasma samples were incubated at 37°c for two hours in duplicate with and without either 20nM MD, 20nM MDTC or 10nM MDTCS (Figure 4.2B), before applying to the plate.

The ability of each of these fragments to compete for the binding of the autoantibodies to immobilised full length ADAMTS13 was used to assess the proportion of total anti-ADAMTS13 antibodies that recognised these domain fragments.

4.1.2.2 TSP2-8 ELISA

A second direct ELISA to detect antibodies directed against the TSP2-8 domains was performed as above for full-length ADAMTS13, except TSP2-8 was immobilised on the plate (Figure 4.2C). Maxisorb plates were coated with 100nM His-tag purified TSP2-8 in 50mM sodium carbonate pH 9.6 and incubated overnight at 4°C. Wells were washed 4 times between each step with PBST. The plate was blocked with PBS/2.5%BSA/10%CM and incubated for at least 2 hour at RT with shaking. Plasma was diluted 1 in 50 (or 1 in 20 if anti-ADAMTS13 IgG titre<35%) in PBS/1%BSA/10%CM before applying to the plate and incubating for 1 hour. Pooled normal plasma was used as a control. Bound anti-ADAMTS13 antibodies were detected with anti-human IgG-HRP (Dako) at a dilution of 1 in 1000 in PBS/ 1% BSA/10%CM for 1 hour at RT. Finally, plates were washed and incubated with 100µl OPD for colour development, which was stopped with 50µl 2.5M H_2SO_4. Absorption at 492nm was read using a spectrophotometer. In this assay, the OD is proportional to the titre of anti-TSP2-8 antibodies

4.1.2.3 Classification of domain specificity of anti-ADAMTS13 antibodies

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.

Further spiking experiments to confirm the presence of anti-CUB antibodies were performed where diluted plasma samples were incubated in duplicate with and without 10nM MDTCS, or 10nM MDTCS plus 20nM CUB, before applying to the plate.

4.2 Results

4.2.1 Patient characteristics

Presenting samples from 92 acute episodes of TTP were included in the domain-specificity study. A flowchart of sample distribution of the 92 acute episodes is shown in Figure 4.3.

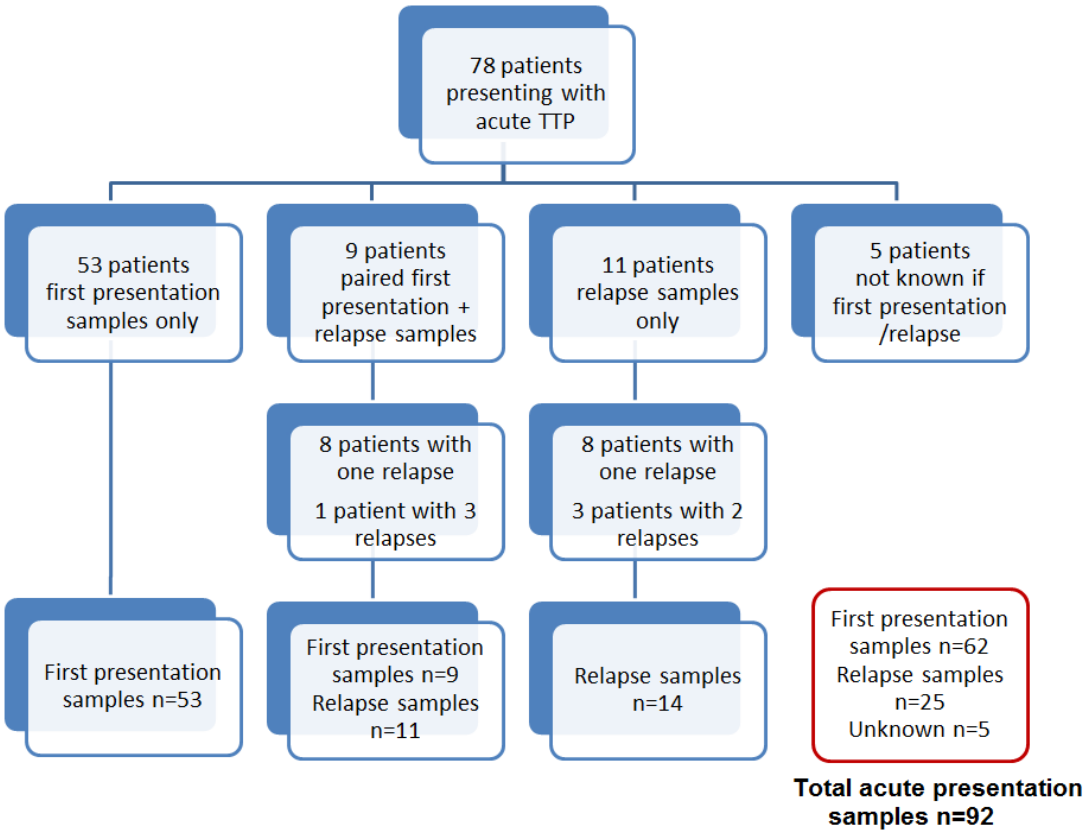


Figure 4.3 Flowchart of the 92 acute TTP episodes

The clinical and laboratory characteristics of the 92 TTP episodes in 78 patients are summarised in Table 4.1. 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 .

Patients were selected based on their medium/high anti-ADAMTS13 titre (i.e. >15%). Through exclusion of patients with low-titre anti-ADAMTS13 antibodies, this cohort of patients was consequently enriched for those patients that relapsed 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.

There were 62 *de novo*, and 25 relapse presentations. Data on whether samples were first or subsequent presentations were not available on the remaining five presentations. Initial presentations were more severe than relapses, as reflected by the increased frequency of neurological symptoms (median 81% *vs*. 60%, $p<0.05$); lower haemoglobin (Hb) [median 8.0 g/dl (range 3.6-11.8 g/dl) *vs.* 10.8 g/dl (4.8-13.8 g/dl), $p<0.0005$]; higher lactate dehydrogenase (LDH) [2046 IU/l (165-5000 IU/l) *vs.* 921 IU/l (276-3174 IU/l), $p<0.005$]; higher anti-ADAMTS13 IgG titre [65% (18-164%) *vs.* 34% (9-101%), $p<0.0001$] and the increased plasma volumes (pv) of PEX required to achieve remission (21.5 pv (4-92) *vs*. 12.25 pv (4-32.5), $p<0.005$).

Patients presenting with relapsed episodes were more likely to experience further relapse than those at first presentation [15/25 (60%) *vs.* 12/62 *(*19%), $p<0.005$]. Of the 21 deaths amongst the patients in this cohort, 16 were during first presentation and three during a relapse episode (it was not known if the remaining two deaths occurred during first presentation or a relapse). The high mortality rate amongst this cohort of patients reflects the selection of patients with higher anti-ADAMTS13 IgG titre, and hence more severe disease (139).

	TTP Episodes			
	All (n=92)	**First (n=62)**	**Relapse (n=25)**	***p***
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, $x10^9$/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, % activity (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)			

Table 4.1. Demographic and clinical features at presentation

Tabulation of the parameters for all cases, subsequently divided into first episode and relapses, in a selected cohort based on high anti-ADAMTS13 IgG antibody levels. Hb = haemoglobin, LDH = lactate dehydrogenase, NR = normal range, pv = plasma volumes. Data is presented as medians (range). Differences in clinical and laboratory parameters are statistically presented. Comparison of first presentation and relapses by § Mann-Whitney test for non-normally distributed continuous variables or * Fisher's exact test (two tailed) for categorical variables.

4.2.2 Domain specificity of TTP autoantibodies at presentation

In 89/92 (97%) of presentation samples, MDTCS competed for >5% of binding to full length ADAMTS13, suggesting that most acquired TTP patients have detectable autoantibodies against the N-terminal domains of ADAMTS13. Only in 3/92 patient samples (patients #64, #91 and #92) did MDTCS fail to detectably compete for full length ADAMTS13 binding, suggesting that the autoantibodies present in these three patients primarily recognise epitopes located in the C-terminal domains alone. Of note, these patients had lower titre antibodies.

38/92 (41%) patients (patients #1 to #38) were designated to have autoantibodies that predominantly recognised epitopes within the N-terminal domains, as MDTCS competed for >85% of full-length ADAMTS13 binding (Figure 4.4) and these patient samples exhibited no, or very low immunoreactivity for the immobilised TSP2-8 domains in a separate ELISA.

Using a cut-off of 2x the signal for pooled normal plasma in a direct TSP2-8 ELISA to assign positivity, 26/92 (28%) patients had autoantibodies that recognised the TSR2-8 domains. 25 of these 26 patients also had antibodies that recognised MDTCS.

The remaining 28/92 (31%) patients for whom MDTCS only partially competed for binding to full length ADAMTS13, but that exhibited no or little immunoreactivity against the TSP2-8 domains, were, by elimination, determined to have antibodies that recognised the CUB1/2 domains. 26 of these 28 patients also had antibodies that recognised MDTCS.

This enabled me to assign patients to different groups based on the domain specificity (i.e. 38/92 “anti-N-terminal alone”, 51/92 “anti-N- & C-terminal” and 3/92 “anti-C-terminal alone” - Figure 4.5).

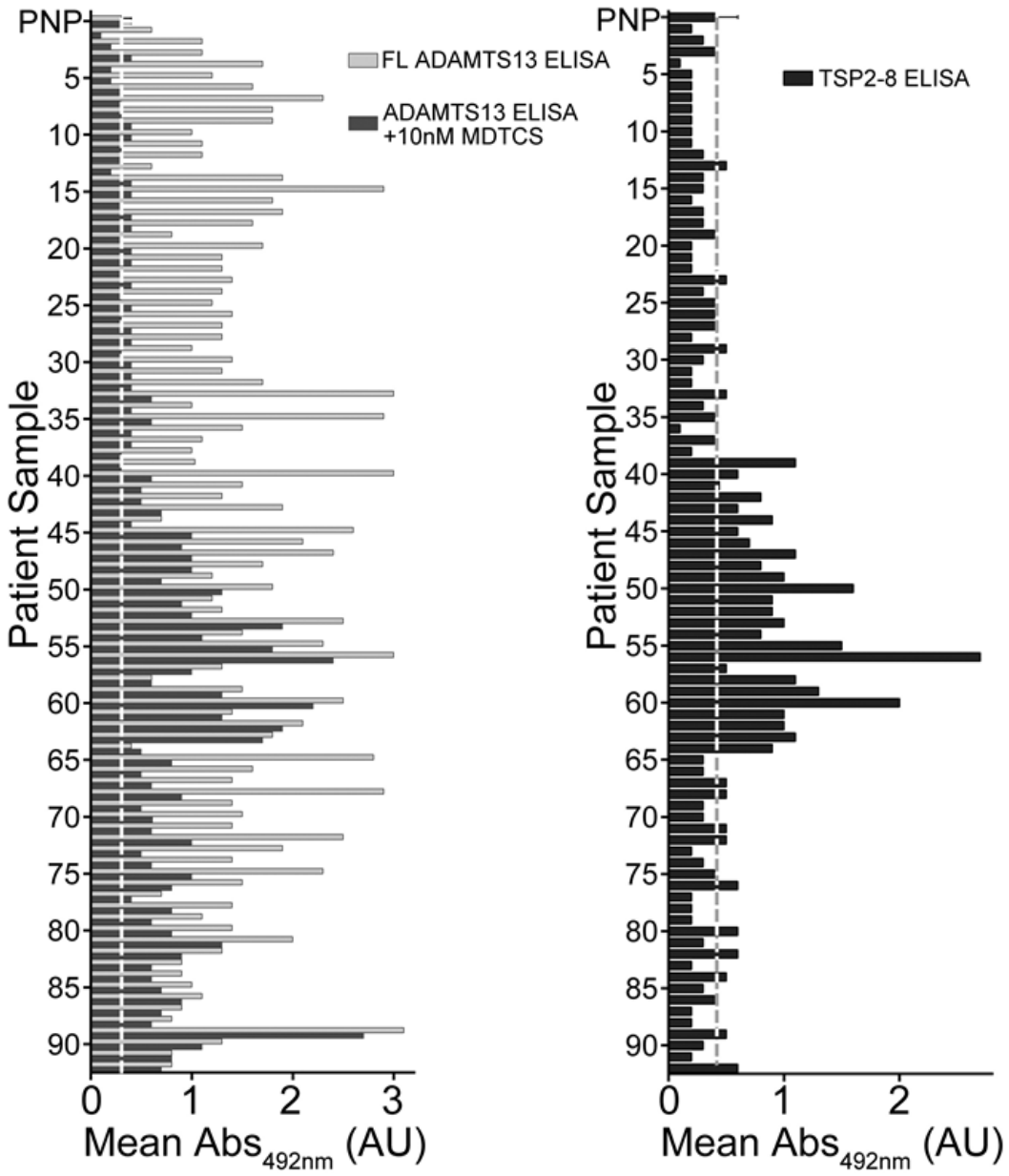


Figure 4.4 Graphs depicting anti-ADAMTS13 domain specificity ELISAs for 92 TTP patient episodes

Left graph; TTP patient plasmas (diluted 1/50) were analysed for IgG that recognised immobilised full length ADAMTS13 (grey bars) and also when preincubated with 10nM MDTCS at 37°c for two hours (dark grey bars). Mean absorbance for every sample is shown. All samples (±MDTCS) were performed in parallel on the same plate. The background associated with pooled normal plasma (PNP) is shown and marked by the dotted line. Right graph; TTP patient plasmas (diluted 1/50) were analysed for IgG that recognised immobilised TSP2-8 (black bars). Mean absorbance for every sample is shown. The background associated with pooled normal plasma (PNP) is shown and marked by the dotted line.

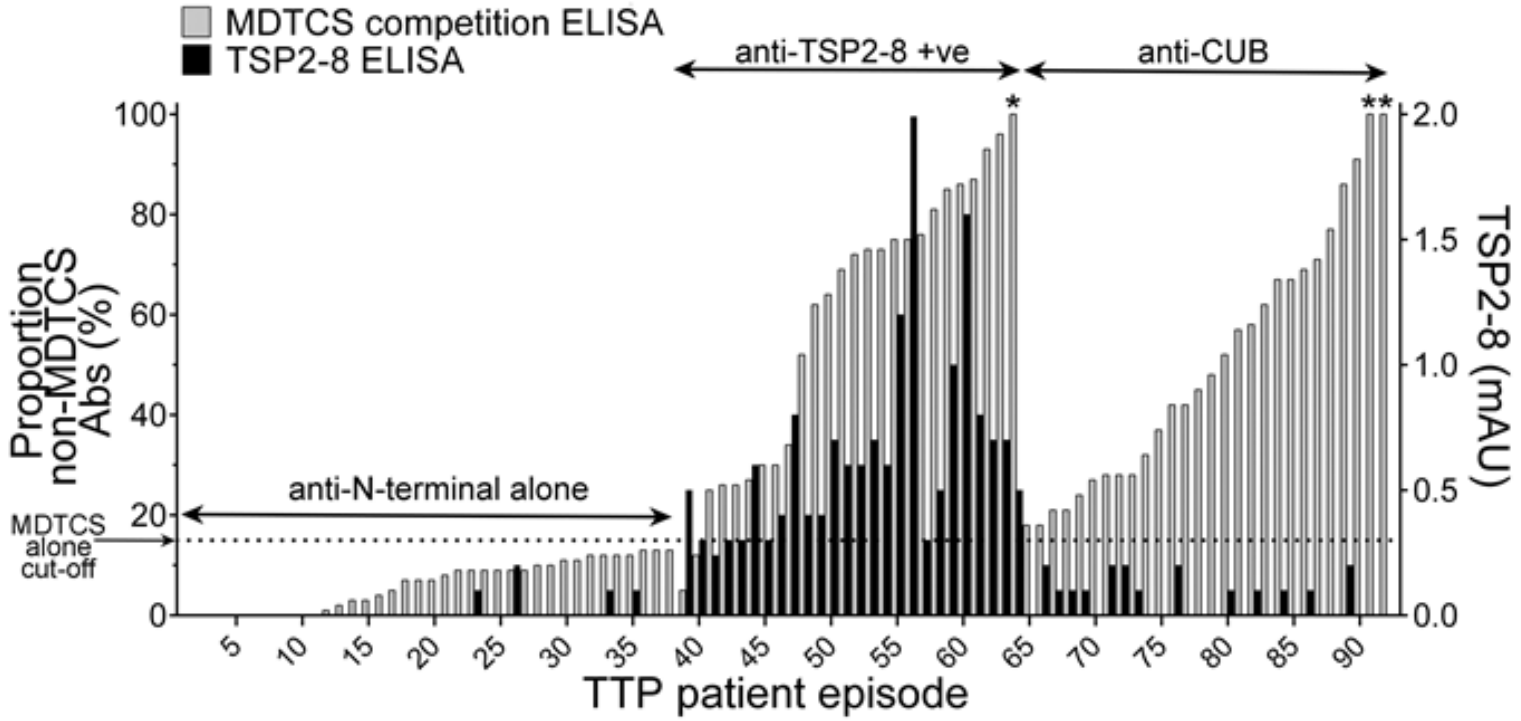


Figure 4.5 Summary of domain specificity ELISA results for 92 TTP patient episodes

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/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/very low recognition of TSP2-8.

I further analysed a subgroup of 25 patients that had a high proportion of antibodies recognising MDTCS (total anti-ADAMTS13 IgG >40%, >80% anti-MDTCS), using a competition ELISA specifically to detect antibodies against either MD or MDTC domain fragments. Interestingly, none of the patients tested exhibited any evidence of immunoreactivity against either MD or MDTC (Figure 4.6). There was no effect on the signal when TTP plasmas were pre-incubated with these fragments before applying to the plate, in contrast to the profound drop in absorbance seen with MDTCS incubation. This strongly suggests that autoantibodies recognising the N-terminal domains MDTCS primarily bind epitopes located within the ADAMTS13 spacer domain.

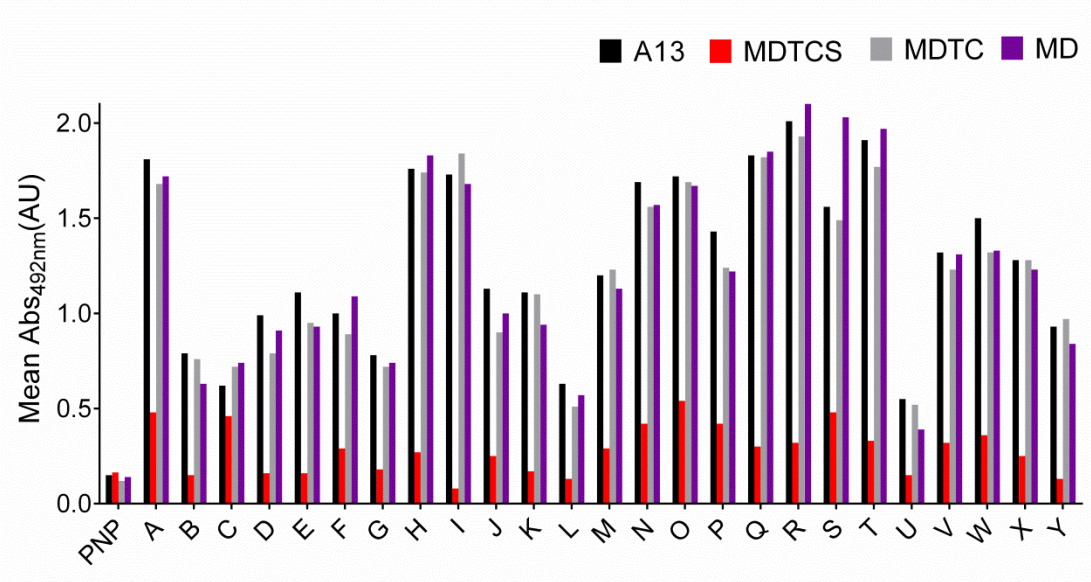


Figure 4.6 Competition ELISAs with MD, MDTC and MDTCS.

TTP patient plasmas (diluted 1/50) were analysed for IgG that recognised immobilised full length ADAMTS13 (black bars) and also after preincubation with 10nM MDTCS (red), 20nM MDTC (grey) or 20nM MD (purple). Mean absorbance for each sample is shown. All samples (±MDTCS/MDTC/MD) were performed in parallel on the same plate. The background associated with pooled normal plasma (PNP) is shown. There was no evidence of immunoreactivity against MD or MDTC.

The presence of anti-CUB antibodies was confirmed in a small number of patients by another competition ELISA (Figure 4.7), using the fragments MDTCS and CUB. Patient episodes #55 and 56, with a high proportion of anti-C terminal antibodies but known anti-TSP2-8 antibodies, show no significant reduction in absorbance after preincubation with CUB in addition to MDTCS. In contrast, episodes #77 and 90, with a high proportion of anti-C-terminal antibodies but no detectable anti-TSP2-8 antibodies, demonstrate a reduction in ADAMTS13 binding after CUB spiking, consistent with the presence of anti-CUB antibodies. However, this assay could not be used more widely as it required patients to have a high proportion of anti-C terminal antibodies in order to be sensitive to a drop in OD after CUB spiking, and utilised a large amount of recombinant CUB antigen.

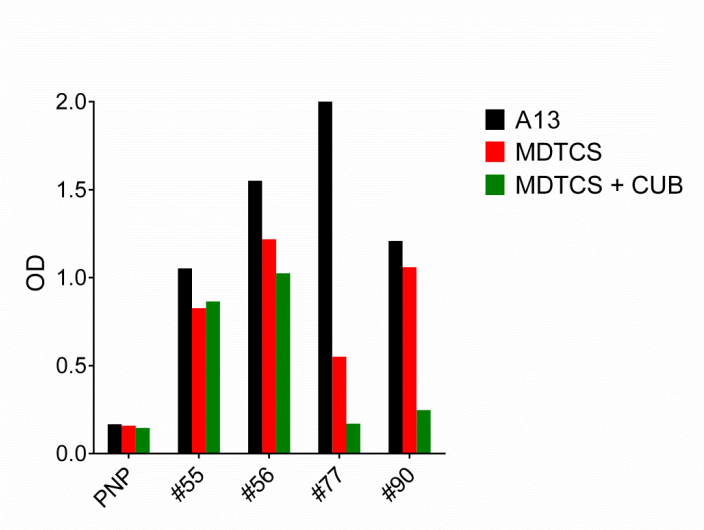


Figure 4.7 Detecting anti-CUB antibodies by competition ELISA

TTP patient plasmas (diluted 1/100) were analysed for IgG that recognised immobilised full length ADAMTS13 (black) and also when preincubated with 10nM MDTCS (red) or 10nM MDTCS plus 20nM CUB (green).

A summary of the autoantibody domain specificity results at TTP presentation is shown in Figure 4.8.

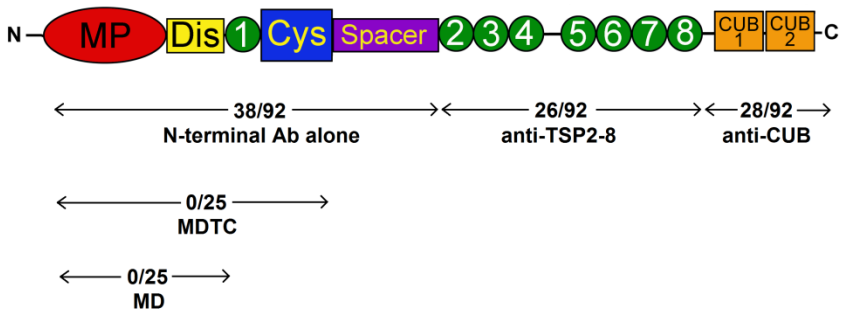


Figure 4.8 Summary of domain specificity of anti-ADAMTS13 antibodies at presentation of acquired TTP in the 92 patient episodes studied

4.2.3 Domain specificity and total anti-ADAMTS13 IgG titre

There was no difference in median total anti-ADAMTS13 IgG titre between the 38 patients assigned to the “anti-N-terminal alone” group, and the 54 patients with anti-C-terminal antibodies – i.e “anti-N- & C-terminal” + “anti-C-terminal alone” – Figure 4.9. Thus, antibodies directed against the C-terminal domains could be detected even in patients with lower titres of anti-ADAMTS13 antibodies indicating that it is not a question of assay sensitivity. Conversely, domain specificity does not appear to influence titre.

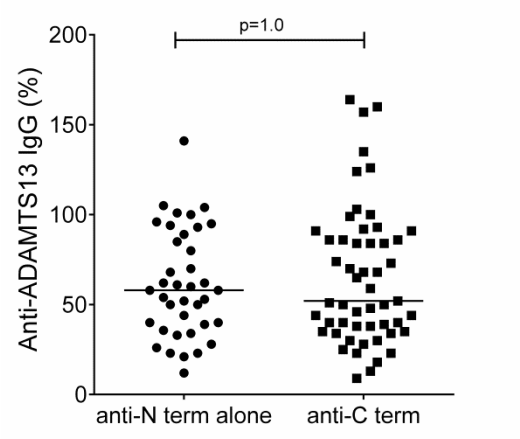


Figure 4.9 Total anti-ADAMTS13 IgG titre at presentation by domain specificity of anti-ADAMTS13 IgG

There was no difference in median total anti-ADAMTS13 IgG titre (horizontal line) between patients with anti-N-terminal antibodies alone and patients with anti-C-terminal antibodies [median 58% anti-N-terminal alone (range 12-150) vs. 52% (9-164), p=1.0, Mann-Whitney U test for non-normally distributed continuous variable].

I then went on to study whether there is a difference in how antibodies against different domains might influence the disease. Patients with anti-C-terminal antibodies at first presentation were older than those in the anti-N-terminal alone group (Figure 4.10), although there were very similar age ranges. Neither sex nor ethnicity differed between domain specificity groups.

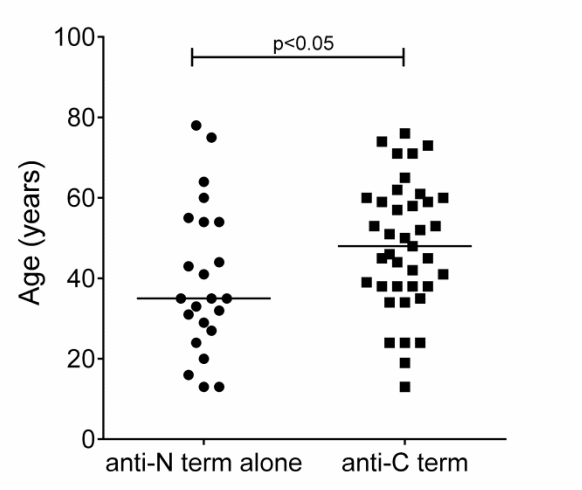


Figure 4.10 Age of patients at first presentation in patients by domain specificity of anti-ADAMTS13 IgG

Patients with anti-C-terminal antibodies at first presentation were older than those in the anti-N-terminal alone group [median 48 years (13-76) *vs*. 35 years (13-78), p<0.05, Mann-Whitney U test]. Median age is shown by horizontal line.

4.2.4 Domain specificity and disease severity

Analysis of the prognostic implications of domain specificity of anti-ADAMTS13 antibodies was performed only on the 62 first presentation samples to avoid intra-patient confounding.

4.2.4.1 Domain specificity and mortality

Sixteen patients died during their first presentation. The median Hb and LDH at presentation were not different in patients who died. The platelet count at presentation was slightly lower in patients who died [median 10x10^9/l in deaths (range 5-19 x10^9/l) *vs*. 14x10^9/l in survivors (range 3-60 x10^9/l), $p<0.05$, Figure 4.11A]. However, this was skewed by a handful of survivors who had much higher presenting platelet counts, suggesting that platelet count at presentation is not a robust prognostic indicator. Although anti-ADAMTS13 IgG titre did not correlate with disease severity as previously reported (139), this was likely due to those patients with lower titre antibodies being excluded from this study during patient selection. Domain specificity was examined in patients who died and those who survived (Figure 4.11B). There was no difference in domain specificity between those who died and survivors.

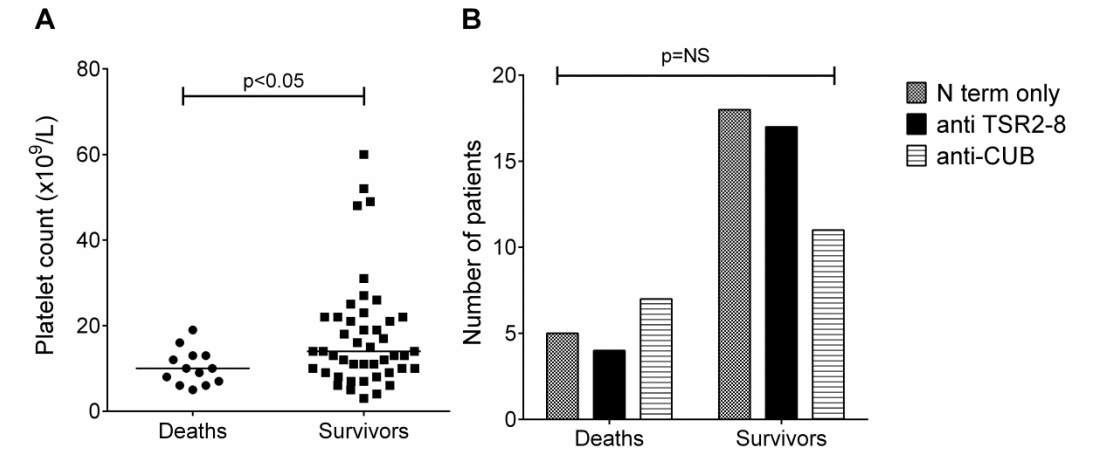


Figure 4.11 (A) Platelet count and (B) Domain specificity of anti-ADAMTS13 antibodies at presentation in patients who died or survived their first TTP episode

(A) Median platelet count (horizontal line) was slightly lower in patients who died [median 10x10^9/l in deaths (range 5-19 x10^9/l) *vs*. 14x10^9/l in survivors (range 3-60 x10^9/l), $p<0.05$, Mann-Whitney U test].
(B) There was no difference in outcome (deaths or survivorship) for patients with N terminal antibodies only, anti TSP2-8 antibodies or anti-CUB antibodies by Chi-square tests.

4.2.4.2 Domain specificity and clinical presentation

Severe neurological or cardiac involvement at presentation is associated with a worse patient outcome (57). Domain specificity was not different in patients with or without cardiac features at presentation (Figure 4.12A), suggesting that the domains recognised by the autoantibodies do not alter the clinical presentation. Neurological presentation was less common in patients with anti-TSR2-8 antibodies at presentation (14/20 patients with anti-TSR2-8 antibodies (70%) *vs*. 36/38 patients without anti-TSR2-8 (95%), p<0.05; Figure 4.12B), although the numbers here are small, which warrants caution in extrapolating these data.

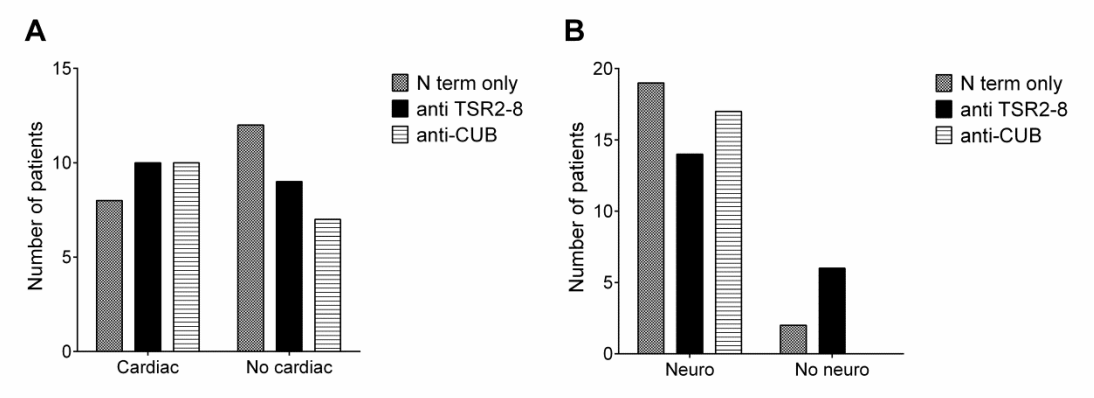


Figure 4.12 Domain specificity and clinical presentation

(A) Domain specificity at presentation in patients with and without cardiac involvement
(B) Domain specificity at presentation in patients with and without neurological involvement

There was no difference in likelihood of cardiac or neurological presentations for patients with N-terminal antibodies only or anti-CUB antibodies, by Chi-square tests. Neurological presentation was less common in patients with anti-TSR2-8 antibodies at presentation (14/20 patients with anti-TSR2-8 antibodies (70%) *vs*. 36/38 patients without anti-TSR2-8 (95%), p<0.05; Chi square test)

4.2.4.3 Domain specificity and Hb, platelets and LDH at presentation

In this cohort, there was no difference in median platelet count between patients with antibodies directed against the C-terminal domains and those with solely N-terminal antibodies (Figure 4.13), once the potential confounding factor of first *vs.* relapsed presentation was removed - as relapse is often caught earlier and thus platelet counts tend to be higher.

Haemoglobin levels at first presentation did not differ between patients with antibodies directed against the C-terminal domains (8.0g/dl (3.6-11.8g/dl) and those with N-terminal antibodies alone (7.5g/dl (5.2-11.3g/dl); p=0.54) Similarly, LDH levels did not differ with domain specificity of the autoantibodies (median 1190iu/dl N-terminal antibodies alone (165-5000iu/dl) *vs.* 1870iu/dl N+C terminal antibodies (498-4177iu/dl; p=0.42)).

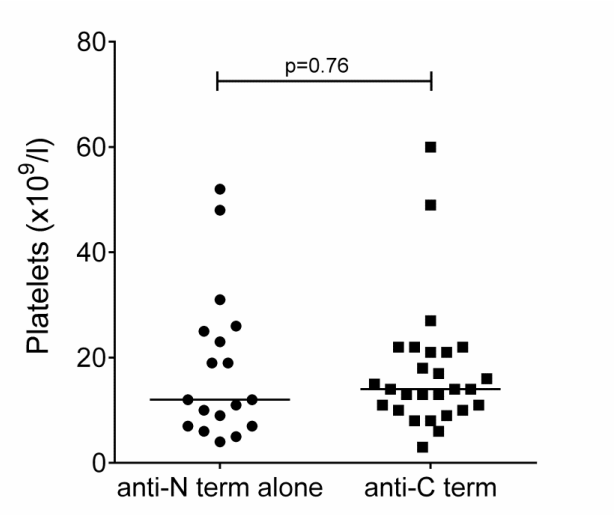


Figure 4.13 Domain specificity of anti-ADAMTS13 antibodies and platelet count at first presentation

There was no difference in median platelet count at first presentation (horizontal line) between patients with antibodies directed against the C-terminal domains (14 x10^9/l (range 3-60x10^9/l) and those with solely N-terminal antibodies (median 12 x10^9/l, (4-52 x10^9/l); p=0.76, Mann-Whitney U test).

4.2.4.4 Domain specificity and plasma exchanges to remission

The median number of plasma exchanges to remission, another marker of disease severity, did not differ with the domain specificity of the anti-ADAMTS13 autoantibodies at first presentation (Figure 4.14).

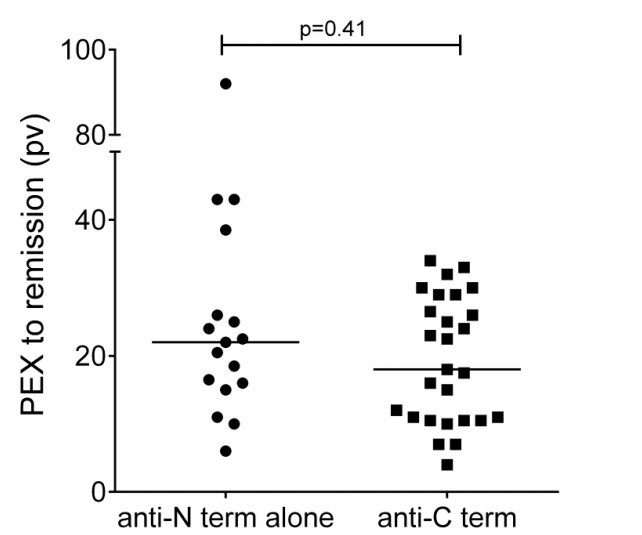


Figure 4.14 Domain specificity and number of plasma exchanges to complete remission

Median plasma volumes of PEX to remission (horizontal line) did not differ with the domain specificity of the anti-ADAMTS13 autoantibodies at first presentation (median PEX to CR N terminal antibodies alone 22pv (6-92) *vs.* 18pv (4-34) for N+C terminal antibodies; p=0.41, Mann-Whitney U test). pv=plasma volumes.

4.2.5 Domain specificity and relapse

4.2.5.1 Domain specificity at first presentation vs. relapse

The domain specificity of the 62 first presentations was compared to the 25 relapse episodes. There was no difference in the proportion of patients with anti-N-terminal alone, and those with anti-C-terminal antibodies at first presentation and at relapse (Figure 4.15). There was a trend to fewer anti-TSR2-8 antibodies in relapsed presentations, but this did not reach statistical significance [4/25 (16%) in relapses *vs*. 21/62 (34%) first presentations, p= 0.25].

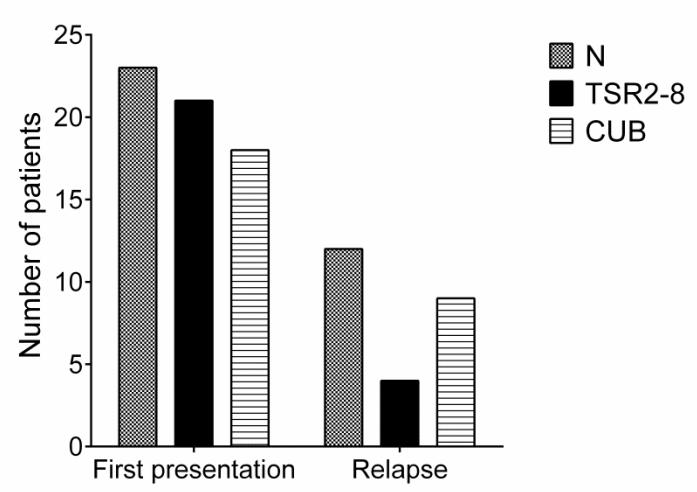


Figure 4.15 Domain specificity of anti-ADAMTS13 antibodies at first presentation and in relapsed episodes

There was no difference in the proportion of patients with anti-N-terminal alone, and those with anti-C-terminal antibodies (TSR2-8 plus CUB) at first presentation and at relapse [C terminal antibodies in 39/62 patients (63%) first presentation *vs*. 13/25 (52%) relapses, p=0.47, Fisher's exact test]. There was a trend to fewer anti-TSR2-8 antibodies in relapsed presentations, but this did not reach statistical significance [4/25 (16%) in relapses *vs*. 21/62 (34%) first presentations, p=0.25, Fisher's exact test].

4.2.5.2 Domain specificity as a predictor of relapse

Relapse rate was examined in those patients who had received rituximab during the acute episode of TTP. Rituximab is known to alter the biology of the disease and there is increasing evidence that it may reduce relapse risk (61), therefore only patients receiving anti-CD20 therapy were studied to avoid confounding. Only first presentations were analysed to prevent intra-patient confounding.

There were 44 first episodes where patients received rituximab. Three patients died leaving 41 analysable episodes. Nine patients relapsed after a median of 27 months (4-50 months). Patients were followed up for a median of 5.7 years (2.1- 7.9 years). Cumulative incidence and Gray's test were used to compare the incidence of relapse between groups. There was no association of domain specificity of ADAMTS13 autoantibodies at first presentation and subsequent relapse rate (Figure 4.16).

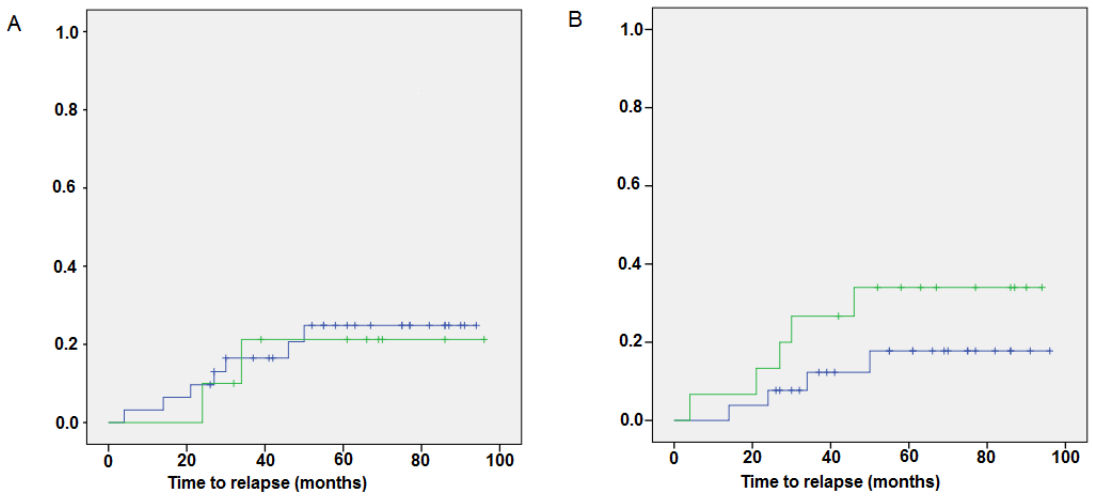


Figure 4.16 Kaplan Meier plots of relapse by domain specificity of anti-ADAMTS13 antibodies at first presentation

A) Anti-CUB antibodies and relapse. Cumulative incidence and Gray's test were used to compare the incidence of relapse between groups. Time to relapse in patients with (blue line) and without anti-CUB antibodies (green line) at first presentation of acquired TTP. There was no difference in relapse rate (p=0.84).
B) Anti TSP2-8 antibodies and relapse. Time to relapse in patients with (blue line) and without anti-TSP2-8 antibodies (green line) at first presentation of acquired TTP. There was no difference in relapse rate (p=0.22).

4.3 Discussion

I developed novel assays to determine the domain specificity of anti-ADAMTS13 antibodies using the mammalian full-length ADAMTS13 and ADAMTS13 fragments I had expressed and purified. All TTP patient plasmas exhibited robust recognition of full-length ADAMTS13 immobilised on a 96-well plate, however, direct coupling of MDTCS to the plate appreciably compromised its immunoreactivity. This was circumvented by devising the competition assay in which dilutions of plasma from acquired TTP patients were pre-incubated both with and without an excess of the N-terminal fragment of ADAMTS13 (MDTCS) in solution, prior to incubation with wells coated with full-length ADAMTS13. When the assays were performed in parallel (i.e. ±MDTCS pre-incubation), this approach enabled estimation of the proportion of total anti-ADAMTS13 antibodies that recognised MDTCS, with the residual binding representing autoantibodies recognising the C-terminal TSP2-8 and/or CUB1/2 domains. Samples were tested for the same time for their reactivity against TSP2-8 in a direct ELISA, giving an indication of the autoantibody repertoire in each patient.

I then used these domain-specificity assays to investigate a large well-characterised cohort of acquired TTP patients (92 acute episodes in 78 patients).

This patient group represents a typical TTP cohort in terms of age, sex and clinical features (neurological 71% patients, cardiac 41%), when compared to other registries (79, 85). It is the largest cohort of TTP patients studied to date in which domain specificity of anti-ADAMTS13 antibodies have been analysed (50, 196), and has the unique benefit of detailed accompanying clinical and outcome data, allowing the clinical significance of the different antibody patterns to be studied both in terms of disease severity and relapse.

Similar to a previous report, initial TTP presentations were more severe than relapses (64), with increased frequency of neurological symptoms; lower Hb;

higher LDH; higher anti-ADAMTS13 IgG titre and increased plasma volumes of PEX required to achieve remission.

At presentation of an acquired TTP episode, 89/92 (97%) patients had detectable antibodies against the N-terminal domains MDTCS. Of these, 38 patient samples had antibodies only against these N-terminal domains. 54/92 (59%) patient samples had antibodies against the C-terminal domains, with 28% having immunoreactivity against TSR2-8. There was no difference in total anti-ADAMTS13 IgG titre between patients with anti-C-terminal antibodies and those with only anti-N-terminal antibodies, meaning it was not just an issue of assay sensitivity.

In contrast to Klaus *et al*. and Zheng *et al*. who, respectively, found antibodies against MDT in 56% and 12% of patients (192, 196), I found no evidence of antibodies that recognised either MD or MDTC in 25 patients tested. The advantages of my novel assays and possible reasons for this discrepancy with previously published studies are discussed in the final chapter of this thesis.

In this TTP patient cohort, patients with anti-C-terminal antibodies at first presentation were older (median 48 years) than those with anti-N-terminal antibodies alone (median 35 years). The reason for this remains unclear, but this could perhaps be due to an increase in clonality with age. In contrast, there was no difference with sex or ethnicity.

Importantly, 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. A previous study found the presence of IgG antibodies against TSP2-8 and/or CUB was inversely correlated with patient platelet counts on admission (196). However in this 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 published studies, I found no association between autoantibody / inhibitor titre and domain specificity (192, 196).

There was no difference in the proportion of patients with anti-N-terminal alone, and those with anti-C-terminal antibodies at first presentation and at relapse. Relapse rate was examined in patients who had received rituximab during their first acute episode of TTP and there was no association of domain specificity of ADAMTS13 autoantibodies at presentation and relapse rate.

Part of my original hypothesis was that the identification and characterisation of the antibody repertoire in a number of patients with acute idiopathic TTP might provide a means to identify those most likely to achieve remission and those at higher risk of relapse. However, it is not possible to risk stratify patients, either in terms of disease severity or risk of relapse, based on their antibody pattern at presentation using these assays.

5 Pathogenic mechanisms of TTP patient autoantibodies

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* (209). Autoantibodies against different ADAMTS13 domains likely inhibit enzyme function to different extents, and may cause deficiency *in vivo* via distinct mechanisms. The aim of the work presented in this chapter was to determine the inhibitory potential of different anti-ADAMTS13 antibodies and investigate the pathogenic mechanisms of TTP patient autoantibodies.

5.1 Methods

5.1.1 VWF115 and VWF 106 activity assays

See section 2.5.1.3-4

5.1.2 FRETS VWF73 activity assay

See section 2.5.2.

5.1.3 Measurement of ADAMTS13 antigen levels

See section 2.2.4.3

5.2 Results

5.2.1 VWF 115 activity assay

To explore the inhibitory potential of TTP patient autoantibodies, functional analyses were performed using total IgG isolated from TTP patient plasmas. To assess the ability of purified total IgG from TTP patients to inhibit the proteolysis of the short VWF A2 domain substrate VWF115, full-length ADAMTS13 was incubated with VWF115 at 37°C in the presence or absence of either control or patient IgG. At various time points (0, 30 and 60 minutes) reactions were stopped with EDTA and analysed by SDS-PAGE. Proteolysis was assessed by the generation of the 10kDa and 6.9kDa cleavage products and the disappearance of the uncleaved 16.9kDa VWF115 (Figure 5.1).

2nM full-length ADAMTS13 proteolysed the VWF115 (16.9kDa) to completion, into 10kDa and 6.9kDa fragments, within 60 minutes (Figure 5.1, -IgG). Normal human IgG (17μM, ~1/4 normal plasma concentration, the highest concentration that could be achieved with the reaction conditions) did not inhibit this reaction, as is seen by the appearance of the 10kDa and 6.9kD fragments by the 30 minute timepoint. In contrast, addition of rabbit polyclonal anti-ADAMTS13 (7μM) (86) led to complete inhibition with no cleavage products being seen even at the 60 minute timepoint. (The vast excess of anti-ADAMTS13 antibody was simply used to demonstrate antibody-mediated inhibition of the enzyme and provide a positive control).

Purified total IgG (17μM) from a TTP patient at presentation with high titre anti-ADAMTS13 IgG (105%, normal range cut-off < 6.1%) directed solely against the N-terminal domains (patient episode #16) appreciably inhibited VWF115 proteolysis, shown by the persistence of the uncleaved 16.9kDa VWF115 at the 30 and 60 minute timepoints. However, trace quantities of cleavage products (10kDa and 6.9kDa bands) were detected, suggesting that inhibition of ADAMTS13 was not complete under these conditions. A contaminating band from IgG extraction is seen at approximately 12kDa.

In contrast, purified total IgG from a patient with a very similar anti-ADAMTS13 titre that had anti-C-terminal, but undetectable anti-N-terminal, antibodies (episode #81, remission sample, anti-ADAMTS13=97%)had no inhibitory effect upon VWF115 proteolysis with appearance of the 10kDa and 6.9kD fragments by the 30 minute timepoint, consistent with the C-terminal domains of ADAMTS13 not playing a role in this assay.

Patient #61 at presentation with a similar anti-ADAMTS13 IgG titre (99%) and with antibodies directed against both N-terminal (~15% of total anti-ADAMTS13 IgG) and the C-terminal domains, demonstrated less inhibition of VWF115 cleavage at this concentration. This is shown by persistence of the uncleaved 16.9kDa band out to the 60 minute timepoint, despite appearance of the 10kDa and 6.9kDa cleavage products.

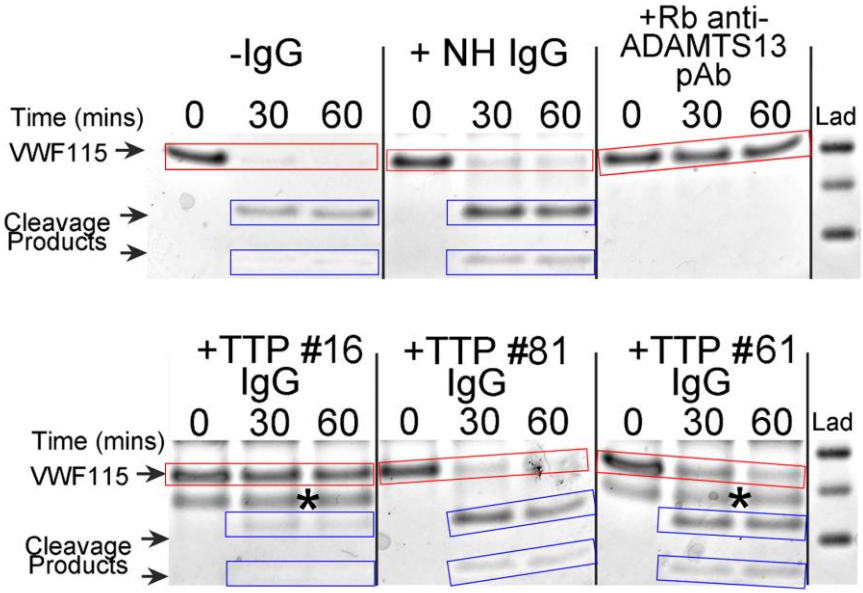


Figure 5.1 Inhibition of VWF115 proteolysis by ADAMTS13

ADAMTS13 activity assays using VWF115 in the presence and absence of isolated IgG samples from patients with high titre anti-ADAMTS13 IgG (TTP#16=105%,#81=97%, #61=99%). 2nM ADAMTS13 proteolysed VWF115 (boxed in red) into 10kDa and 6.9kDa fragments (boxed in blue) (-IgG), within 60 minutes. 17μM normal human IgG (NH IgG) did not inhibit this reaction, whereas 7μM rabbit polyclonal anti-ADAMTS13 led to complete inhibition. Identical reactions containing isolated total IgG (17μM) from TTP patient samples #16, #81 (remission sample) and #61 are shown (* denotes contaminating band from IgG extraction seen in #16 and #61).

5.2.2 VWF 106 activity assay

To further examine the domain specificity of inhibitory autoantibodies, I examined the proteolysis of VWF106, which is identical to VWF115, but lacks 9 residues from its C-terminus that are critical to ADAMTS13 spacer domain binding (Figure 2.8). As full-length ADAMTS13 proteolyses VWF106 more slowly than VWF115, a higher concentration of ADAMTS13 (3.5nM) was used over a longer reaction time. Under these conditions, VWF106 was partially proteolysed by ADAMTS13 after 120 minutes (Figure 5.2). This is shown by persistence of the uncleaved 16kDa band out to the 60 minute timepoint, despite appearance of the 10kDa and 6kDa cleavage products.

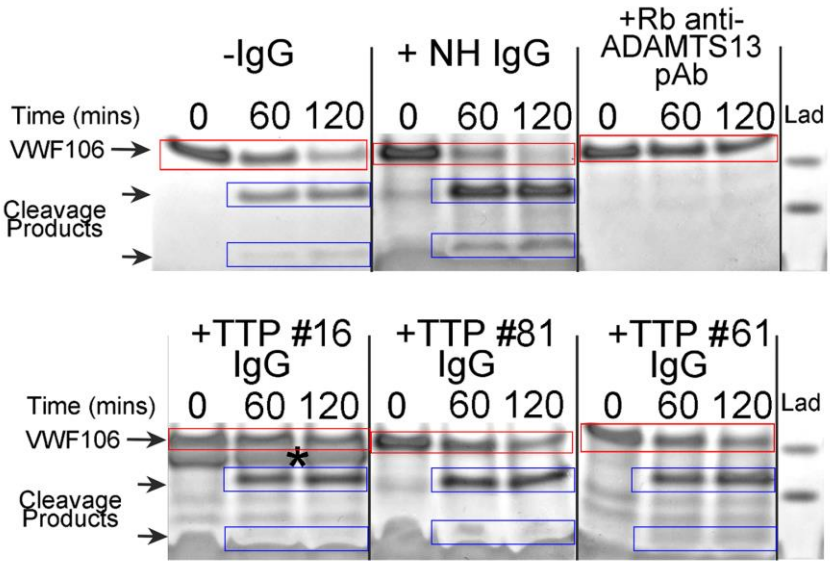


Figure 5.2 Inhibition of VWF106 proteolysis by ADAMTS13

ADAMTS13 activity assays using VWF106 in the presence and absence of isolated IgG samples. The proteolysis of VWF106, which lacks 9 residues that are critical to ADAMTS13 spacer domain binding, was investigated using 3.5nM ADAMTS13 and 2 hour reaction times. VWF106 is boxed in red with cleavage products boxed in blue. Under these conditions, VWF106 was only partially proteolysed by ADAMTS13 after 120 minutes (-IgG). Cleavage was unaffected by normal IgG (NH IgG), but completely inhibited by rabbit polyclonal anti-ADAMTS13. Reactions containing isolated total IgG (29μM) from TTP patient samples #16, #81 and #61 are shown. TTP patient IgG does not inhibit proteolysis of VWF106. (Note a contaminating band from IgG extraction is seen at approximately 12kDa is seen in the reaction with TTP#16 but not with a different preparation of IgG from TTP#61).

As before, cleavage was unaffected by normal IgG, but completely inhibited by rabbit polyclonal anti-ADAMTS13. Again, purified total IgG from a patient anti-C-terminal antibodies alone (episode #81, remission sample) had no inhibitory effect upon VWF115 proteolysis with appearance of the 10kDa and 6kD fragments by the 30 minute timepoint, consistent with the C-terminal domains of ADAMTS13 not playing a role in this assay.

When ADAMTS13 was preincubated with isolated IgG from #61 and #16, neither exhibited an inhibitory effect upon VWF106 proteolysis with appearance of the 10kDa and 6kD fragments by the 30 minute timepoint, strongly suggesting that in both cases the inhibition observed in the VWF115 assay (Figure 5.1) is mediated by antibodies that recognise the spacer domain. Moreover, it suggests that any autoantibodies in these samples that recognise the MDTC domains either do not impair ADAMTS13 function and / or are only present at very low concentrations.

Whilst these assays are not quantitative, they allowed me to generate the hypothesis that anti-spacer antibodies are the main inhibitory antibodies in acquired TTP and I proceeded to test this hypothesis with another assay.

5.2.3 FRETS VWF73 activity assay

To examine further the inhibitory potential of the autoantibodies in a larger number of samples, I 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 5.3A-G).

The first group of patients (n=10; TTP episodes #1, 2, 6, 14, 16, 21, 22, 23, 32, 34) consisted of those assigned to the anti-N-terminal alone group (termed here Group I). Total IgG from all of these patients dose-dependently inhibited 125pM MDTCS (median IC_{50} 1.0μM; range 0.2-2.8μM) (Figure 5.3A

and 5.3D, inhibition at 0.7μM total IgG shown), whereas total IgG from normal human plasma had no effect upon ADAMTS13 function.

To determine the contribution of anti-spacer domain antibodies to the inhibitory function of these antibodies, reactions were set up in parallel in which varying concentrations of total IgG were first preincubated with an 80-fold excess (10nM) of mammalian expressed purified MDTC (purified MDTC exhibited little/no FRETS-VWF73 cleaving activity in the control IgG samples – Figure 5.3A). In these experiments, MDTC would compete with MDTCS (125pM) for all antibodies that recognise the N-terminal domains except for those against the spacer domain. In all 10 patient episodes from this group, preincubation with MDTC failed to detectably alter the IC_{50} (Figure 5.3A). These results strongly suggest that anti-spacer domain antibodies are the primary inhibitory antibodies, and that any antibodies against the MDTC domains are unlikely to contribute appreciably to ADAMTS13 inhibition.

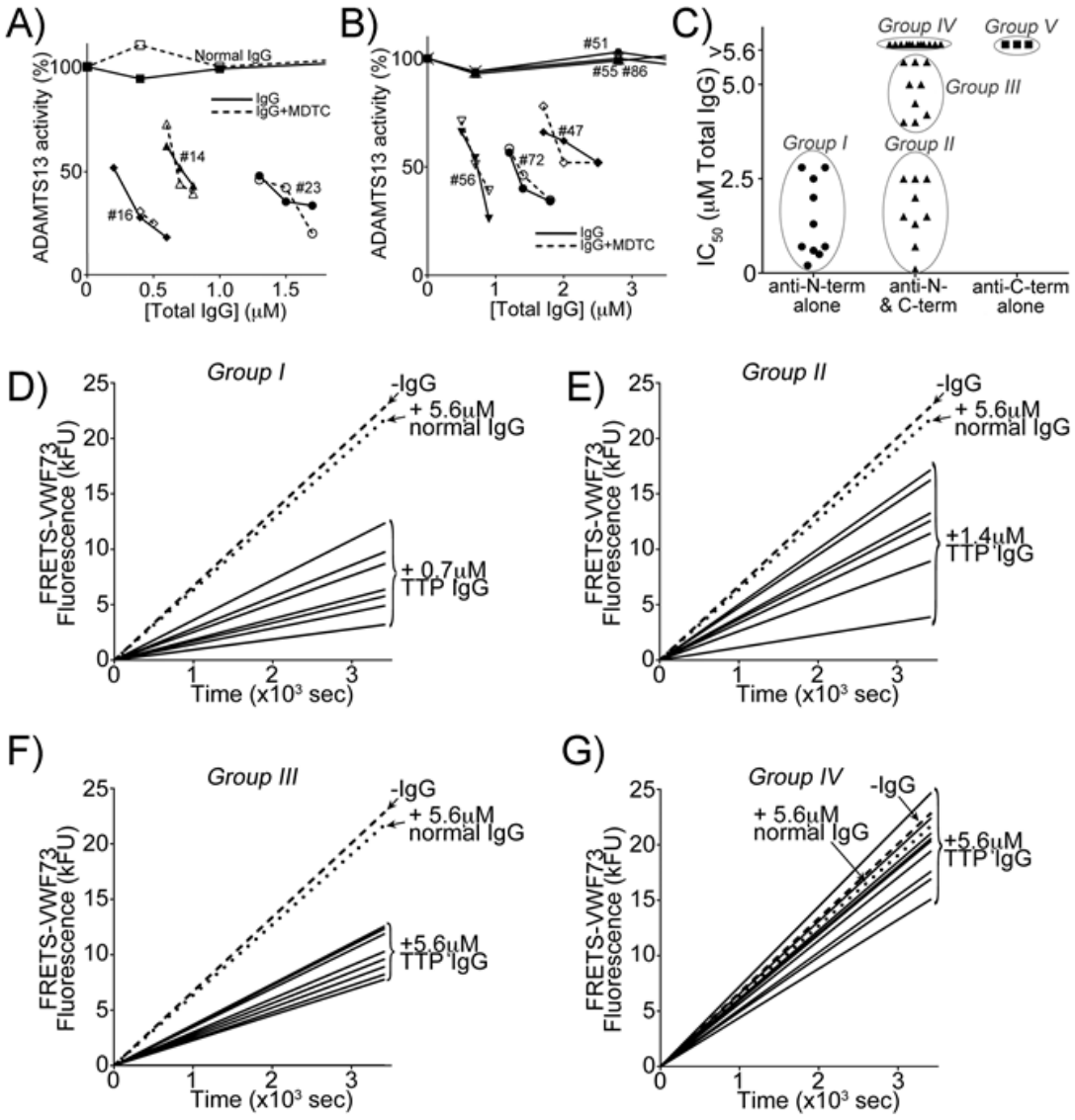


Figure 5.3 Analysis of the inhibitory potential of total IgG isolated from acquired TTP patients

A) and **B)** 125pM 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 10nM 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 preparation 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 (*Group I-V*) based on their domain specificity and the inhibitory potential of their IgG. **D)** Inhibition of 125pM MDTCS by 0.7μM total IgG isolated from *Group I* samples, all samples shown inhibit MDTCS appreciably at this concentration. **E)** Inhibition of 125pM MDTCS by 1.4μM total IgG isolated from *Group II* samples. Samples shown inhibit MDTCS variably at this concentration. **F)** Inhibition of 125pM MDTCS by 5.6μM total IgG isolated from *Group III* samples. Samples shown inhibit MDTCS by ~50% at 5.6μM total IgG. **G)** Inhibition of 125pM MDTCS by 5.6μM total IgG isolated from Group IV samples. At 5.6μM total IgG little or no inhibition of MDTCS was detected for these samples.

The inhibitory potential of total IgG isolated from the three patients with only anti-C-terminal domain antibodies (termed Group V) was examined (Figure 5.3C). As expected, these antibodies failed to inhibit the activity of MDTCS even at the highest IgG concentration tested (5.6µM). This corroborates the earlier findings that these patients have no autoantibodies that recognise MDTCS.

Finally, the inhibitory potential of IgG isolated from 30 TTP patient episode samples containing varying proportions of anti-N- and anti-C-terminal antibodies was examined. Interestingly, in 12/30 (40%) patient samples (termed Group IV), either no, or minimal inhibition of MDTCS was detected at the highest IgG concentration tested (5.6µM) (Figure 5.3C and 5.3G). These patient samples (#51-55, 57, 59, 60, 85, 86, 88, 90) all had comparatively low proportions (<33%) of total anti-ADAMTS13 antibodies that recognised the N-terminal domains (Figure 4.2). The remaining 18/30 (60%) patient samples with both anti-N- and anti-C-terminal antibodies were inhibitory. However, the median IC_{50} (3.3µM; range 0.2-5.6µM) was appreciably higher than for the 10 patient 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) (Figure 5.3C and 5.3E, inhibition at 1.4µM total IgG shown), and those with modest inhibitory potential (Group III, n=9) (Figure 5.3C and 5.3F, inhibition at 5.6µM total IgG shown).

The nine Group II patient samples were also investigated using the MDTC competition assay to test for the presence of inhibitory antibodies other than those that recognise the spacer domain. In all (9/9) samples, MDTC failed to influence the estimated IC_{50}, once again strongly suggesting that the inhibitory antibodies present in these samples are limited to those that recognise the spacer domain.

TTP episode	Group	Age /sex	Episode number	Domain specificity	Rituximab	Outcome	TTR (mo) or F/U
#51	IV	34F	1	N+2-8	Y	No relapse	86
#52	IV	51M	1	N+2-8	Y	No relapse	58
#53	IV	71M	1	N+2-8	Y	No relapse	42
#54	IV	42F	1	N+2-8	Y	No relapse	77
#55	IV	24F*	1	N+2-8	N	Relapse	34
#57	IV	52M	1	N+2-8	Y	No relapse	94
#59	IV	38F	1	N+2-8	N	Died	-
#60	IV	73F	1	N+2-8	N	Died	-
#64	V	65F	2	2-8 only	Y	Died	-
#85	IV	61F	1	N+CUB	N	Died	-
#86	IV	74M**	3	N+CUB	Y	Relapse	5
#88	IV	63M	3	N+CUB	Y	Relapse	2
#90	IV	75M**	4	N+CUB	Y	Relapse	13
#91	V	40F	2	CUB only	Y	No relapse	41
#92	V	27F*	3	CUB only	N	Relapse	19

Table 5.1 Characteristics of fifteen patient episodes with no evidence of anti N-terminal inhibitory antibodies (IC_{50}>5.6μM) at presentation of a TTP episode (groups IV and V)

TTR=time to relapse
* and **subsequent episodes in same patient – all other episodes are different individuals.

The inhibition assays suggested that the anti-ADAMTS13 autoantibodies from patients in *Groups IV* (n=12) and *V* (n=3) listed in Table 5.1 (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.

I therefore hypothesised that the loss in ADAMTS13 activity at TTP presentation in these patients may be due to antibody-mediated depletion of ADAMTS13 antigen, and went on to measure the ADAMTS13 antigen levels at presentation in these and all the presentation samples.

5.2.4 ADAMTS13 antigen levels at presentation – the role of antigen clearance

In 67 healthy normal volunteers, median plasma ADAMTS13 antigen levels were 951ng/ml (range 515-1829ng/ml; IQR 799-1143ng/ml) (Figure 5.4), very similar to previously published normal ranges (41, 42, 46, 47). In the 91 TTP presentation samples tested (one sample was lost to analysis due to insufficient sample size), ADAMTS13 concentrations were significantly lower ($p<0.0001$, Mann Whitney U test): median ADAMTS13 antigen level was 58ng/ml (6% normal) with a range of 0-450ng/ml (0%-47%). 83/91 of samples had <25% normal ADAMTS13 antigen levels, and 42/91 had ADAMTS13 antigen levels <5% normal.

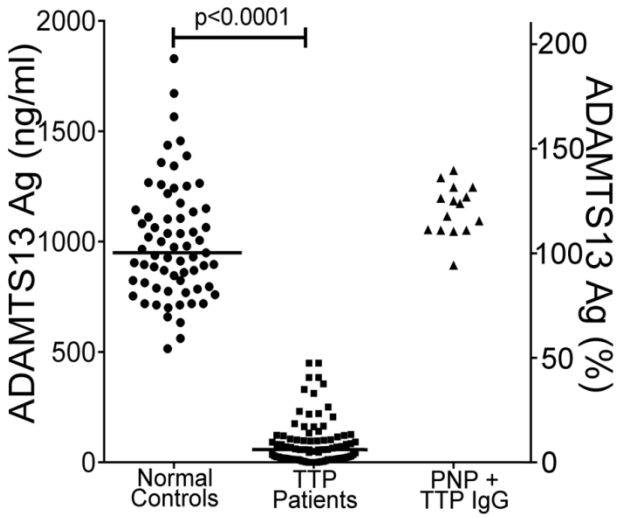


Figure 5.4 ADAMTS13 antigen levels at presentation of TTP

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. Median antigen levels are shown by the horizontal lines. 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.

To ensure that the reduced ADAMTS13 levels detected were not attributable to epitope masking by the autoantibodies preventing efficient capture / detection by the rabbit polyclonal ADAMTS13 IgG used in the antigen assay, ADAMTS13 levels were measured in pooled normal plasma in the presence of IgG isolated from 14 different TTP patient episodes. In this control experiment, the presence of TTP patient IgG only led to very minor differences in ADAMTS13 concentration measured, suggesting that the magnitude of the reduction seen in the TTP plasmas likely represents a true picture of the ADAMTS13 concentrations in these samples.

At first presentation, patients with anti-N-terminal antibodies alone (n=23) had a median ADAMTS13 concentration of 81 ng/ml (8.5%); range 13-331ng/ml (Figure 5.5A). Those patients at first presentation with no evidence of any inhibitory antibodies in the FRETS-VWF73 assays (n= 9) had a significantly lower ADAMTS13 concentration [median 2 ng/ml (0.2%) range 0-141ng/ml; p=0.005]. Indeed, only three of these samples were above the detection threshold of the ELISA. Those patients at first presentation with both anti-N- and C-terminal antibodies that exhibited evidence of inhibitory function showed a trend to lower ADAMTS13 antigen levels by comparison to those with anti-N-terminal alone [median 30 ng/ml (3.2%); range 0-356ng/ml; p=0.08] (Figure 5.5A), but that were significantly higher than those without inhibitory antibodies ($p<0.05$). The same trend / pattern was also observed when the ADAMTS13 antigen levels were plotted in patients at first presentation assigned to Groups I-IV (Figure 5.5B).

These data suggest that antigen depletion is the major cause of ADAMTS13 deficiency – however, in the presence of inhibitory antibodies, depletion does not need to be as severe/profound.

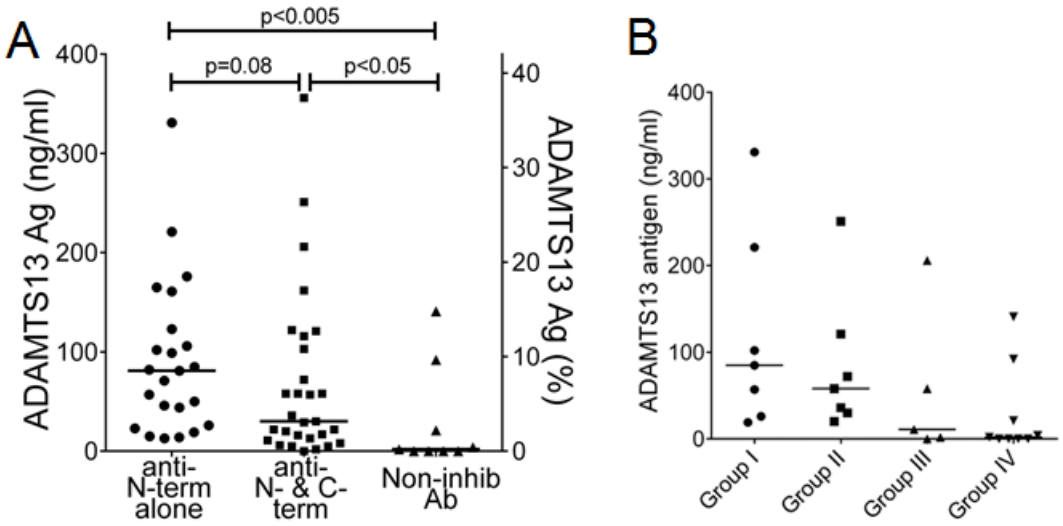


Figure 5.5 ADAMTS13 antigen levels by domain specificity

A. Plasma ADAMTS13 antigen levels at 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* and *V* patients from Figure 5.3C)
B. Plasma ADAMTS13 antigen levels in acquired TTP patients separated into patients with anti-N-terminal alone (*Group I*), and anti-N- and C-terminal antibodies that were potently inhibitory (*Group II*), mildly inhibitory (*Group III*) and apparently non-inhibitory antibodies (*Group IV*) from Figure 5.3. Individual concentrations are shown and the median represented with a horizontal line.

5.2.5 Clinical correlation of ADAMTS13 antigen levels at presentation

To examine the importance of antigen depletion as a pathogenic mechanism, I analysed the relationship between ADAMTS13 antigen levels and mortality. ADAMTS13 antigen levels were significantly lower at presentation in patients that died during their first episode [median antigen 12 ng/ml (0-165) deaths *vs.* 57 ng/ml (0-356) in survivors; p<0.05, Figure 5.6]. Moreover, severe deficiency of ADAMTS13 antigen [ADAMTS13 antigen levels in the lowest quartile (<13.5ng/ml)] was associated with increased mortality (odds ratio 5.4; 95% confidence interval 1.5 to 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% confidence interval 1.5-21.8; p=0.01).

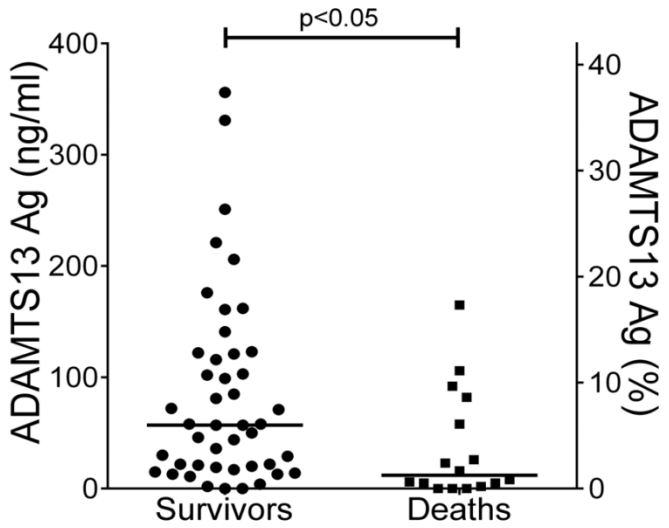


Figure 5.6 ADAMTS13 antigen levels and mortality

Plasma ADAMTS13 antigen levels in acquired TTP patients at first presentation that survived their first episode or died. Individual concentrations are shown; the median is represented by a horizontal line. Comparison of antigen levels in survivors and deaths by Mann-Whitney U test.

5.2.6 Prognostic effect of non-inhibitory anti-ADAMTS13 antibodies alone (IC_{50}>5.6μM) at presentation

The relationship between the presence of solely non-inhibitory antibodies at first TTP presentation (IC_{50}>5.6μM, *group IV*) and patient outcome was investigated. Again, analysis was performed only on the first presentation samples to avoid intra-patient confounding. IC_{50}>5.6μM was not associated with an effect on mortality, or disease severity as assessed by likelihood of a cardiac / neurological presentation, total anti-ADAMTS13 IgG levels, Hb, platelets or LDH at presentation, or number of PEX to complete remission.

However, none of the five patients with non-inhibitory antibodies at first presentation treated with rituximab went on to relapse during a 42-94 month follow up period, in contrast to 9/36 patients with inhibitory antibodies (TTR 27 months, range 4-50 months, Figure 5.7). This difference did not reach statistical significance because of the small numbers involved (p=0.22).

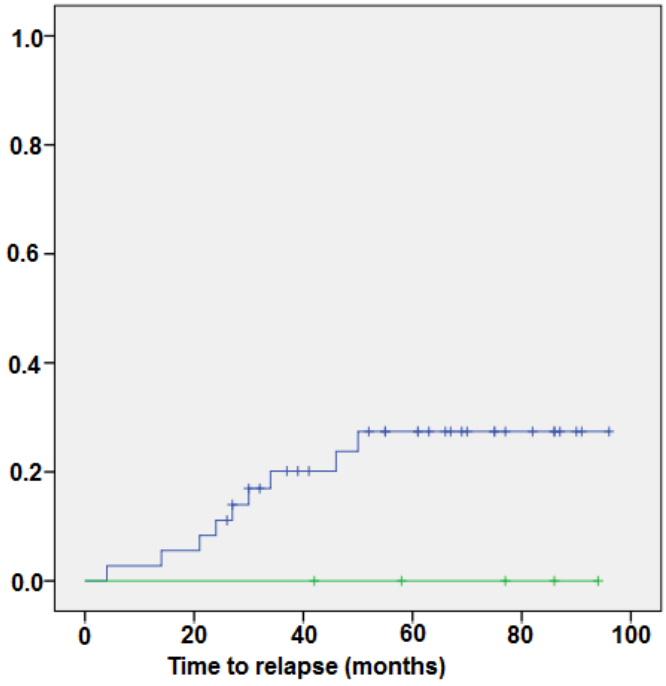


Figure 5.7 Kaplan Meier plot of relapse by inhibitory potential of anti-ADAMTS13 antibodies at first presentation

All patients received rituximab as part of initial therapy. Time to relapse in patients with (blue line) and without inhibitory antibodies (green line) at first presentation of acquired TTP. Cumulative incidence and Gray's test were used to compare the incidence of relapse between groups.

5.3 Discussion

Although the domain specificity of anti-ADAMTS13 autoantibodies in acquired TTP has been previously investigated, those studies were not combined with analysis of antibody function (192, 196). This work attempted to investigate the contribution of antibodies against different ADAMTS13 domains to the inhibitory potential in plasma and investigate the pathogenic mechanism of TTP patient autoantibodies.

My results demonstrate the critical role of anti-spacer antibodies in mediating ADAMTS13 inhibition. Indeed, no antibodies other than those directed against the spacer domain were detected that were capable of inhibiting MDTCS function. Although it cannot be excluded that inhibitory antibodies against the MDTC domains exist amongst the patients analysed, these results suggest that, even if such antibodies are present, their inhibitory contribution relative to those targeting the spacer domain is small. Given that 0/25 patients had evidence of non-spacer anti N-terminal antibodies in the competition ELISA (Figure 4.6), it is tempting to extrapolate these findings to all TTP patients.

The spacer domain has long been suspected as the primary antigenic target for inhibitory antibodies, which has been corroborated by a recent analysis of monoclonal antibodies derived from two acquired TTP patients (219). However, no other study has demonstrated in this many patients that inhibitory antibodies are limited to those that recognise this domain.

Despite the high frequency of anti-N-terminal antibodies, anti-spacer domain antibodies are not necessary 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 (<10%).

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. I 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, I used 0.125nM ADAMTS13, which is 1/40th of the plasma ADAMTS13 concentration. Based on the normal plasma IgG concentration of 80μM, this might suggest that in those patients with IC_{50} values >2μM (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. This finding prompted me to determine the ADAMTS13 antigen levels in all the acquired TTP patient samples.

ADAMTS13 antigen levels were significantly lower in all TTP patient plasma samples (median 6% normal), with over 90% samples having <25% normal ADAMTS13 antigen levels. Indeed, 46% samples had ADAMTS13 antigen levels of <5% - sufficient in itself to cause TTP, without the need to invoke inhibitory antibodies. These results support the contention that enhanced, antibody-mediated clearance of ADAMTS13 antigen from plasma is the major cause of deficiency in acquired TTP, and thus this mechanism should be considered to play a significant role in disease pathogenesis.

Previous studies on small numbers of less well characterised TTP patients have revealed reduced ADAMTS13 concentration (31, 32) (section 1.4.4.3). However, none of those studies have had ELISAs that have been demonstrated not to be sensitive to epitope masking, or been able to demonstrate the extent of the reduction in ADAMTS13 concentration. Moreover, other studies have not simultaneously looked at the inhibitory potential of the anti-ADAMTS13 antibodies.

Thus, although ADAMTS13 antigen levels in smaller groups of acquired TTP patients have been previously reported to be low, the magnitude of antigen clearance as a disease mechanism has been frequently underplayed, with far greater attention placed on the role of the 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 (87). However, this finding was significantly limited by the inclusion of patients with only moderately reduced / normal ADAMTS13 activity or no autoantibody in their cohort.

Importantly, ADAMTS13 antigen levels at presentation also appear to have prognostic significance. ADAMTS13 antigen levels were lower at presentation in patients who died (median 1.0% *vs.* 5.5%). This finding is in keeping with a smaller study of four patients by Yang *et al.* (91). Indeed, ADAMTS13 antigen levels in the lowest quartile at first presentation (<13.5ng/ml, <1.4% normal) increased the likelihood of mortality by approximately 5-fold. Whether this reflects more severe disease in these individuals, or that the patients presented later is unclear, but the association of antigen levels and patient outcome is consistent with clearance being an important pathogenic mechanism.

As the patient samples with no evidence of inhibitory anti-N-terminal antibodies had the lowest ADAMTS13 antigen levels, the primary pathogenic mechanism of anti-C-terminal antibodies is likely to be increased ADAMTS13 clearance. It remains possible that some anti-C-terminal antibodies may also inhibit ADAMTS13 function. For example, there were two patients at first presentation without evidence of inhibitory antibodies against MDTCS, but with antigen levels >10% (Figure 5.5A). These two individuals may thus harbour autoantibodies that recognise the C-terminal tail, but that may be capable of compromising the function of full length ADAMTS13 (rather than MDTCS), potentially either through blocking the binding of ADAMTS13 to globular VWF or through steric hindrance of spacer domain function.

It should also be noted that anti-spacer domain autoantibodies operate through clearance as well as inhibition, as patients with anti-N-terminal antibodies alone have reduced antigen levels. Another piece of evidence to support this contention is the fact that the spacer domain only contributes to the activity of ADAMTS13, it is not essential. Deletion of the spacer domain binding region of the VWF A2 domain leads to a modest (~5-6 fold) reduction in ADAMTS13 proteolysis (199). Thus, even complete inhibition of the spacer domain would leave residual ADAMTS13 activity, meaning that there must be an additional mechanism i.e. clearance.

However, not all anti-ADAMTS13 antibodies promote clearance as antibody 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 (see subsequent chapter, Figure 6.1E) but persistent high titre antibodies that recognised ADAMTS13.

The original hypothesis to be tested was that autoantibodies against different ADAMTS13 domains inhibit ADAMTS13 to different extents, and therefore contribute variably to the pathogenesis of TTP. This work has demonstrated that anti-spacer domain autoantibodies are the major inhibitory antibodies in acquired TTP. Importantly, it has also shown that depletion of ADAMTS13 antigen (rather than enzyme inhibition) is the prevailing pathogenic mechanism in the disease. The importance and relevance of this will be discussed further in the final chapter of the thesis.

6 Longitudinal analysis of the domain specificity of TTP patient autoantibodies

The majority of studies of TTP patient autoantibodies have analysed presentation samples, which provides only a snapshot of the disease. There has been very limited work on the longitudinal humoral immune response in TTP. The aim of the work presented in this chapter was to characterise the repertoire of antibodies in patients with acute idiopathic TTP and, for the first time, to monitor this through therapy, remission and relapse. In this way, important features of response to therapy, remission and also relapse might be identified.

6.1 Methods

6.1.1 Response criteria

In all TTP cases, treatment response was defined as a platelet count > $150x10^9$/l for two consecutive days, accompanied by normal/normalising LDH and stable / improving neurological deficits (220). Clinical remission was defined as a durable treatment response, lasting at least 30 days after discontinuation of PEX (220). Relapse was defined as recurrent disease 30 days or longer after remission (62).

Elective rituximab prophylaxis was given to patients considered at high risk of relapse in remission (111), and consisted of therapy once weekly for four weeks. In the majority of cases, patients were given rituximab if their ADAMTS13 activity levels fell to <5%. In two cases, patients with higher ADAMTS13 activity level (but still <15%) were treated, as they were deemed at high risk of relapse based on previous experience with the individual patients concerned (111).

6.1.2 Assays

The domain specificity of anti-ADAMTS13 antibodies was analysed at different timepoints through therapy, remission and, where applicable, relapse using the assays described in sections 4.1.2. ADAMTS13 activity and total anti-ADAMTS13 IgG were measured as described in sections

2.3.3-4. ADAMTS13 antigen levels were measured at specific points, as described in section 2.2.4.3.

6.1.3 Patient selection

Patients were selected on the basis of availability of serial samples in which there was detectable anti-ADAMTS13 IgG after treatment.

6.2 Results - patient groups

Four different patterns of clinical response to therapy were identified in patients surviving their index episode of TTP:

1. Patients who did not relapse within the study period and in whom the anti-ADAMTS13 autoantibody titre fell after therapy (n=6).
2. Patients with subsequent relapse in whom the anti-ADAMTS13 autoantibody titre fell after initial therapy, but then rose again with relapse (n=16).
3. Patients with persisting inhibitory anti-ADAMTS13 IgG in clinical remission (n=2).
4. Patients with non-inhibitory anti-ADAMTS13 IgG in clinical remission (n=2).

6.2.1 No relapse

Six patients were studied who did not go on to relapse during the study period. The domain specificity of anti-ADAMTS13 antibodies was analysed at presentation, at one month post presentation and in remission. All patients received standard therapy with PEX and steroids, and also rituximab as part of their acute therapy. Median follow up was 3.3 years (range 1-6.3 years). The results are summarised in Table 6.1.

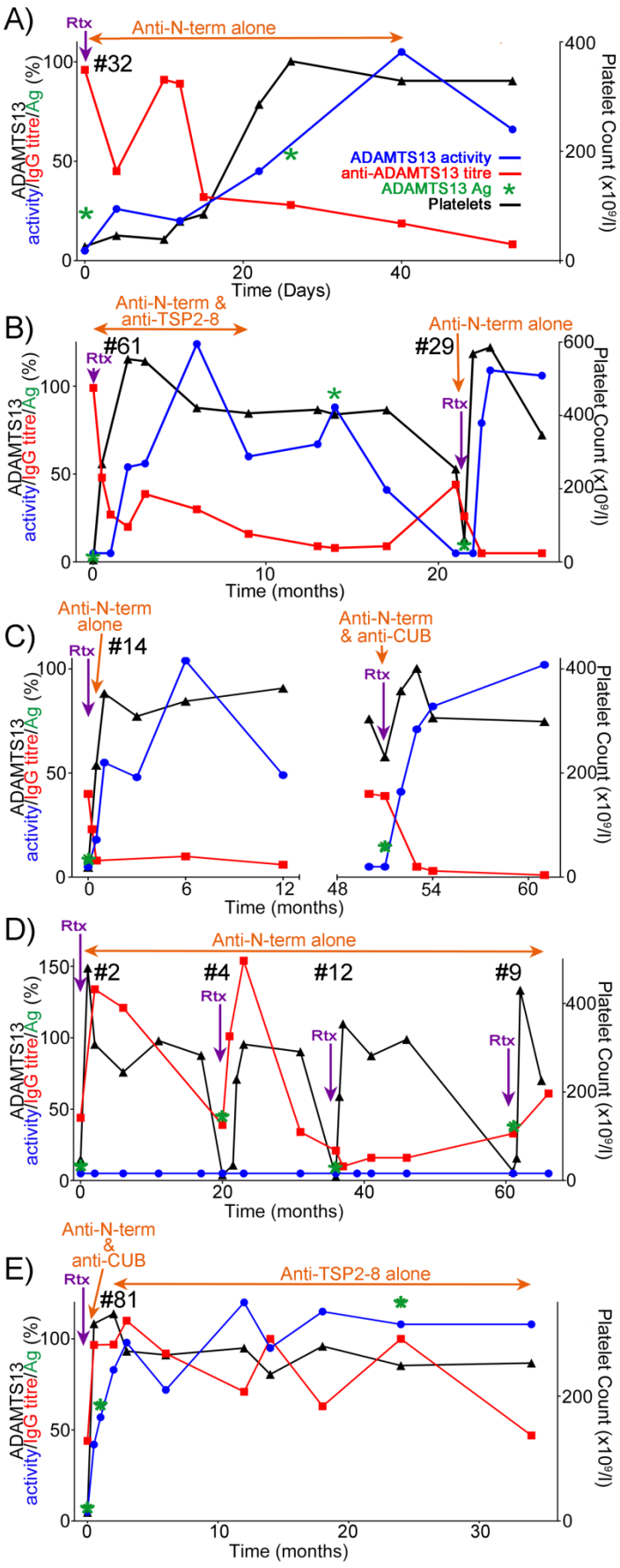
Patient	Presentation				One month				Clinical remission				
TTP episode	Domain pattern	Act (%)	αA13 IgG	Ag (%)	Domain pattern	Act (%)	αA13 IgG	Ag (%)	Domain pattern	Act (%)	αA13 IgG	Ag (%)	Timing (mo)
#32	**N**	<5	96	23	**N**	105	19	57	-	157	8	188	6
#26	**N**	<5	62	1	**N**	<5	33	31	-	106	9	147	12
#73	**N+C**	<5	23	3	**N**	<5	55	6	-	62	3	55	12
#45	**N+2-8**	<5	86	4	**N***	7	29	17	**N**	38	10	60	7
#57	**N+2-8**	<5	160	0	**N+C**	82	22	46	**N**	>66	7	91	>12
#51	**N+2-8**	<5	50	15	**N+C**	35	25	72	**-**	71	7	120	9

Table 6.1 Serial domain specificity of anti-ADAMTS13 IgG in non-relapsing patients

TTP episode number refers to Figure 4.4
Act = ADAMTS13 activity, αA13 IgG = anti-ADAMTS13 IgG titre (NR<6.1%),
Ag=ADAMTS13 antigen, - = below limit of detection of assay
*2 month time point as no detectable Ab at one month

Total anti-ADAMTS13 IgG titre fell following therapy with PEX, steroids and rituximab in 5/6 patients, as previously described (82). However, all patients studied still had detectable anti-ADAMTS13 IgG at one month that enabled analysis of ADAMTS13 domain specificity.

There were two cases with solely anti-N-terminal antibodies at presentation that disappeared with time. The results from one of the patients are shown graphically in Figure 6.1A. Four patients with both anti-N and C-terminal antibodies at presentation all cleared 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 the anti-N terminal antibodies that persisted were not pathogenic. Anti-ADAMTS13 antibodies took 3-12 months to clear, coinciding with recovery of ADAMTS13 antigen to normal levels.



A-E) Longitudinal analysis of five acquired TTP patients following an initial presentation. ADAMTS13 activity (blue), ADAMTS13 antigen (green), anti-ADAMTS13 IgG (red) shown as % normal (left axis). Platelet counts are also shown (black, right axis). TTP episode number (e.g. #32) refers to episodes denoted in Figure 4.3. Rituximab therapy (Rtx) is shown by purple arrows. Anti-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.

Figure 6.1 Longitudinal analysis of TTP patients

6.2.2 Relapse

Sixteen patients were studied who relapsed during the study period. Relapse was defined as either a clinical relapse, or acute drop in plasma ADAMTS13 activity to <5% normal during routine follow-up, with accompanying normal routine laboratory parameters, which prompted treatment with elective rituximab (111). The median time to either clinical relapse or elective rituximab was 31 months (range 4-52 months) -Table 6.2. Thirteen out of sixteen patients received rituximab as part of their acute management of the initial episode.

Nine out of sixteen 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 disease recurrence (Fig 6.1C). 3/9 patients had anti-N-terminal as well as 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. These could represent anti-CUB antibodies present at first presentation but masked by the high titre anti TSR2-8, or a fall in the anti TSR2-8 titre.

Of particular note, one patient developed novel anti-C-terminal antibodies at incipient relapse, four years after the original episode. At first presentation the patient (episode #14 from Figure 4.4) had only N terminal antibodies detectable, but when his ADAMTS13 activity fell to <5% after a prolonged period of clinical and biochemical remission, both N terminal and evolving anti-C terminal antibodies were detected (Fig 6.1C). This is of interest as these results suggest a further development of the autoimmune response to ADAMTS13, rather than just re-emergence of the pathogenic clone.

First Presentation					Relapse						
TTP episode	**AutoAb specificity**	**Activity (%)**	**αA13 IgG (%)**	**Antigen (%)**	**TTP episode**	**AutoAb specificity**	**Activity (%)**	**αA13 IgG (%)**	**Antigen (%)**	**Relapse/ Elect Rtx**	**TTR (mo)**
#72 *	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 *	N & 2-8	6	68	0	#92	N & CUB	<5	68	1	Relapse	34
#56 *	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^	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

Table 6.2 Longitudinal analysis of domain specificity of anti- ADAMTS13 IgG titre, ADAMTS13 antigen and activity in relapsing acquired TTP patients

TTP episode number refers to episodes denoted in Figure 4.1 and 4.2. N = anti-N-terminal Ab, CUB = anti-CUB, 2-8 = anti-TSP2-8 Ab.
Relapse = clinical relapse Elect Rtx = elective rituximab therapy. - = elective rituximab (patient episodes are not presented in Figures 4.4 and 4.5)
* no rituximab at initial presentation. n.d = not determined (insufficient sample). ^=post PEX sample. TTR = time to relapse (months).

Median ADAMTS13 antigen levels were not higher at relapse than at first presentation in the paired samples (6% (0-26%) first presentation *vs.* 7% (0-47%) at relapse). Antigen levels were markedly reduced in all but one of the patients who received elective rituximab for a fall in ADAMTS13 activity (median 15% (range 0-88%)). The fact that antigen levels fall when anti-ADAMTS13 IgG titres rise, and before any drop in platelet count or clinical symptoms arise, suggests that antigen clearance is a primary antibody-mediated phenomenon, rather than a secondary consumptive one seen with active thrombotic microangiopathy.

6.2.3 Inhibitory anti-ADAMTS13 IgG in clinical remission

The typical serological response to rituximab therapy has been well documented (82). Treated patients tend to clear anti-ADAMTS13 IgG over a period of several weeks to months. There is a concomitant recovery in ADAMTS13 activity and patients enter clinical remission. There is a rare group of patients who do not respond typically to rituximab. These patients respond clinically, recover their platelet counts and go into remission. However, despite no evidence of ongoing TTP symptoms, these patients have persistent low ADAMTS13 activity in conjunction with persistent anti-ADAMTS13 antibodies. Such patients generally relapse on multiple occasions, but appear to require a 'second hit' such as intercurrent infection or other physiological stressor to precipitate relapse. This pattern appears to be more common in AfroCaribbean patients (personal comm, M. Scully).

The domain specificity of the anti-ADAMTS13 antibodies in two such patients was analysed both during remission and relapse. Both patients failed to recover their ADAMTS13 activity to normal levels despite going into clinical remission and had medium to high titre anti-ADAMTS13 IgG (50-100%**,** NR<6.1%) detectable. The results from one of the patients are shown graphically in Figure 6.1D. The domain specificity of these anti-ADAMTS13 antibodies was analysed both during remission and relapse, and it could be demonstrated that the persisting inhibitory IgG in clinical remission was

directed against the N terminal domains of ADAMTS13. In further analyses using the competition ELISA described in section 4.1.2 and inhibition assay described in section 2.5.2, no antibodies other than those directed against the spacer domain were detected.

The ADAMTS13 antigen levels in these patients varied between 20-60% in clinical remission samples, although plasma ADAMTS13 activity was persistently <5%, indicating the inhibitory nature of the persisting anti-ADAMTS13 antibodies.

6.2.4 Non-inhibitory IgG in remission

Rarely, patients may have detectable anti-ADAMTS13 IgG in clinical remission and yet have normal ADAMTS13 activity. Two such patients were identified and investigated. Both patients achieved sustained remission (follow up 5·1 and 6·3 years) after standard therapy and rituximab. ADAMTS13 activity returned to, and remained at, normal levels within 1-2 months after initial treatment, 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 TSR2-8 domains (Figure 6.1E).

6.3 Discussion

A major strength of this study is the availability of serial plasma samples from each TTP patient through immunosuppressive treatment. These samples have been taken at different time points during treatment, remission, and also during relapse of TTP, allowing me to examine how the autoantibody repertoire changes during immunosuppression and remission, and also during cases of relapse. I have endeavoured to determine whether immunosuppression / remission involves reduction in antibodies with particular specificities, and whether relapse involves the reappearance of antibodies against specific domains, or whether new antibodies with alternative specificities arise.

My results show that (at least in some patients) there is continual development of the autoimmune response during treatment that can result in altered domain specificity at relapse. 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. 5/9 of the patients with altered domain specificity at relapse had loss of anti-C-terminal reactivity, leaving only N-terminal antibodies at disease recurrence, suggesting that the clones directed against the C-terminal domains had been eliminated but not those directed against the N-terminal domains. Similarly, 3/9 patients had anti-N-terminal as well as 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. These could represent anti-CUB antibodies present at first presentation but masked by the high titre anti-TSR2-8, or a fall in the anti-TSR2-8 titre below the cut-off for positivity of the assay.

However, one patient developed a novel antibody specificity at relapse, with the development of anti-CUB antibodies in addition to the anti-N-terminal antibodies detected at first presentation. This epitope spreading suggests that there may be further development of the autoimmune response, rather

than just simple re-emergence of the clones of autoimmune B cells 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, as has been described for tetanus/measles/pneumococcal specific B cells (221) (222). Indeed, a recent study has shown that the spleen harbours ADAMTS13 specific memory B cells following acute acquired TTP (219). This may explain part of the mechanism of action for splenectomy as a (historic) therapy in TTP.

There may be a difference in the domain specificity pattern of anti-ADAMTS13 antibodies when assessed at the point where patients first drop their ADAMTS13 activity and receive elective rituximab, as opposed to at full clinical relapse (which is likely to be later). Patients are treated earlier, so the period for further autoantibody development is shorter. This is likely to underestimate the degree of epitope spreading in the cohort.

It is clear from this work that not all persistent anti-ADAMTS13 antibodies in remission behave in a comparable manner. Patients may have persistent inhibitory anti-N-terminal antibodies (likely anti-spacer) causing severe deficiency of ADAMTS13 activity in remission (although only mildly reduced antigen levels). This renders them very susceptible to further relapses.

In contrast, persistent anti-ADAMTS antibodies may be non-inhibitory, non-pathogenic antibodies directed against the TSR2-8 domains, which do not affect ADAMTS13 proteolytic ability or antigen levels, at least not enough to cause quantitative or qualitative deficiency, and do not appear to affect the risk of relapse. These may be similar to the low affinity non-inhibitory anti-ADAMTS13 antibodies occurring in some healthy individuals (210), and need to be distinguished from persistent pathogenic antibodies.

7 DISCUSSION AND CONCLUDING REMARKS

The main aim of this study was to identify a better way to risk stratify patients with acquired TTP in order to identify individuals who might die from the disease, as opposed to those who would respond well to treatment, and to understand more about the pathogenesis of the disease. TTP remains a life-threatening disease with a 10-20% mortality rate, which has not improved significantly since the introduction of PEX therapy. Relapses have historically been reported in up to 50% cases (78, 130-132). We currently have only limited ability to identify those individuals with a more severe disease phenotype who are most at risk of death, or to determine which patients are more likely to relapse.

It is clear from existing work that the autoimmune response in acquired TTP is polyclonal and there have been previous studies investigating the domain specificity of anti-ADAMTS13 antibodies in acquired TTP (50, 192, 193, 195, 198, 199). However, it was not known whether or not antibodies directed against different domains of ADAMTS13 might be variably pathogenic. It was also previously unclear how the domain specificity of the antibodies might change in response to treatment, and whether re-emergence of antibodies against different domains may be indicative of/predictive of relapse.

I hypothesised that autoantibodies against different ADAMTS13 domains inhibit ADAMTS13 to different extents and therefore contribute variably to the pathogenesis of TTP. I also hypothesised that the identification and characterisation of the repertoire of antibodies in longitudinally collected samples in patients with acute idiopathic TTP might provide a means to identify those patients most likely to achieve remission and those at higher risk of relapse. This in turn, might allow a means of monitoring TTP patients during treatment and possibly tailoring therapy accordingly.

The study therefore aimed to characterise acquired TTP patient autoantibodies, both in terms of their domain specificity but also their pathogenic role.

To that end, I expressed recombinant ADAMTS13 domain fragments in both bacterial and mammalian expression systems and then used the domain fragments to develop novel assays to determine the domain specificity of anti-ADAMTS13 antibodies. I developed a competition assay in which dilutions of plasma from acquired TTP patients were pre-incubated both with and without an excess of the N-terminal fragment of ADAMTS13 (MDTCS) in solution, prior to incubation with wells coated with full-length ADAMTS13. When the assays were performed in parallel (i.e. ±MDTCS pre-incubation), this approach enabled estimation of the proportion of total anti-ADAMTS13 antibodies that recognised MDTCS, with the residual binding representing autoantibodies recognising the C-terminal TSP2-8 and/or CUB1/2 domains. Samples were tested for the same time for their reactivity against TSP2-8 in a direct ELISA, giving an indication of the autoantibody repertoire in each patient.

The domain specificity results broadly support the findings from other studies (50, 192, 196) with 89/92 (97%) patients having detectable antibodies against the N-terminal domains MDTCS. Of these, 38 patient samples had antibodies only against these N-terminal domains. 54/92 (59%) patient samples had antibodies against the C-terminal domains, with 28% having immunoreactivity against TSR2-8. In contrast to Klaus *et al*. and Zheng *et al*. who, respectively, found antibodies against MDT in 56% and 12% of patients (192, 196), I found no evidence of antibodies that recognised either MD or MDTC in 25 patients tested.

However, from the work in this thesis and elsewhere (53, 54), it is clear that the conformation of the protein is vital. Western blotting needs to be interpreted with caution, as it has the potential to pick up antibodies not recognising the native conformation of the protein. This questions the validity of the domain-specificity studies using Western blotting, as positivity of these

assays could be due to recognition of non-exposed regions which may not be relevant. Western blotting-based methods do not tell us if antibodies detected are binding to the exposed surface of the enzyme and are therefore potentially pathogenic. The same problem may apply to methods using phage display and hence investigating only linear epitopes (194, 197, 210). In addition, Klaus *et al* used bacterially expressed domains and, potentially, this methodology may have detected non–specific antibodies against residual bacterial proteins (192).

In terms of other existing assays, the GPI-anchored assay developed by Zheng's group potentially allows the antigen fragments to adopt a native conformation on the cell surface, but cannot be used to compare samples (207). It simply gives a presence or absence of antibody against the two ADAMTS13 fragments studied. Quantitation of the amount of antibody or the proportion directed against a particular fragment is not possible, as one cannot determine how much of the antigen is expressed on the surface of the cells.

Immunoprecipitation as used by some groups to investigate the domain specificity of anti-ADAMTS13 antibodies does allow antibody-antigen binding in solution and hence in native conformation, but is very laborious and difficult to do on a larger scale (50, 193, 196). Zheng *et al* and Pos *et al* used mammalian material which is more likely to be correctly folded (50, 196). However, immunoprecipitation only detects the presence or absence of antibodies against a domain fragment and there may be problems with sensitivity.

There are several advantages to the domain specificity assays I developed in this work over the existing assays looking at the domain-specificity of anti-ADAMTS13 antibodies. These novel assays use mammalian expressed material (which is, as previously discussed, more likely to be in native physiological conformation), but require only a low quantity of recombinant antigen and are easy to perform for large numbers of samples.

Another important advantage of the competition ELISA using MDTCS is that it enables the estimation of the proportion of total anti-ADAMTS13 antibodies that recognise the N-terminal domains, rather than the assessment of whether a domain fragment-specific antibody is merely present or absent, which is how all other studies investigating domain specificity of anti-ADAMTS13 antibodies have been performed. This, in combination with the anti-TSR2-8 ELISA, gives an indication of the autoantibody repertoire in each patient.

There are, of course, some limitations to the domain specificity assays developed in this thesis. The cut-off for categorising patients as having solely anti-N terminal antibodies based on MDTCS competing for >85% full length ADAMTS13 binding is arbitrary (but is certainly indicative of the primary autoimmune response being towards the N-terminal domains). In addition, whilst these assays give a broad overview of the repertoire of anti-ADAMTS13 antibodies in each patient in terms of domains targeted, and allow patterns to be determined, they do not allow for detailed epitope mapping and remain qualitative.

Thus, these novel assays allow multiple samples to be run in parallel to determine the proportion of C-terminal reactivity in each sample, as well as the presence/absence of TSP2-8 antibodies. This, in combination, with the large number of samples tested and the accompanying detailed patient data is the strength of this study.

The accompanying clinical and outcome data enabled the clinical significance of different antibody patterns to be explored. I went on to study the clinical correlates of the patterns of domain specificity of anti-ADAMTS13 antibodies at presentation of acquired TTP. The original hypothesis to be tested was that characterisation of the repertoire of antibodies in patients with acute idiopathic TTP might provide a means to identify those most likely to achieve remission, and those at higher risk of relapse, with a view potentially to tailoring therapy accordingly.

I therefore investigated whether the domain specificity of anti-ADAMTS13 antibodies at presentation might be a prognostic factor in acquired TTP. However, domain specificity of autoantibodies was not predictive of either disease severity or relapse risk. A previous study found the presence of IgG antibodies against TSP2-8 and/or CUB was inversely correlated with patient platelet counts on admission (196). However in this 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, I found no association between autoantibody/inhibitor titre and domain specificity (192, 196). The lack of association of domain specificity with disease severity suggests that antibodies against different domains may to some extent behave similarly in causing TTP, rather than antibodies against functionally important domains being more pathogenic.

In the longitudinal analysis, I demonstrated that remission involves reduction in anti-ADAMTS13 IgG with antibodies against the C-terminal domains often being cleared first. More than 50% patients studied exhibited altered domain specificity profile at relapse, but this was usually the loss of anti-C-terminal reactivity, with the reappearance of anti-N-terminal antibodies at disease recurrence. Only one patient developed novel antibodies with alternative domain specificity (anti-CUB) at relapse.

Although the domain specificity of anti-ADAMTS13 autoantibodies in acquired TTP at presentation has been previously investigated, those studies were not combined with analysis of antibody function (192, 196). In order to explore the pathogenicity of antibodies against different domains of ADAMTS13, I performed functional assays (VWF115/106 activity assays and FRETS activity assays) and identified the spacer domain of ADAMTS13 as the target of inhibitory antibodies.

I 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 (223). 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.

Importantly, this work has shed further light on the pathophysiology of acquired TTP. Over 70% of the TTP patients' samples that I analysed had antibodies with inhibitory function insufficient alone to account for the severe deficiency state, but there was severe antigen depletion (median 6% normal levels) in all acquired TTP presentation samples, supporting the contention that enhanced, antibody-mediated clearance of ADAMTS13 antigen from plasma is a major cause of ADAMTS13 deficiency in the disease.

Given the apparent importance of antibody-mediated clearance of ADAMTS13 as a significant pathogenic mechanism underlying ADAMTS13 deficiency in acquired TTP, characterisation of the mechanism(s) underlying antibody-mediated clearance of ADAMTS13 and analysis of the kinetics of clearance are necessary. ADAMTS13 antigen / antibody immune complexes (IC) have been described in acute TTP and during remission (92, 190, 191), 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. Moreover, the persisting ICs may not be the pathogenic or important ones.

The clearance of IgG-containing immune complexes (IC) is known to occur primarily in the liver (224-228). Both Kupffer cells and sinusoidal epithelial cells in the liver are believed to be involved in the clearance of ICs from the

circulation via Fc-receptor dependent uptake (227). Although Fc receptors are widely expressed, the largest relative uptake of ICs occurs in the liver, implying there may be an Fc-receptor independent uptake of ICs in this organ (224).

Complement can also play an important role in the elimination of immune complexes. When antigen / antibody ICs first form in the circulation, complement inhibits their aggregation through binding of C3b to the IC keeping them soluble (225). Erythrocytes bind these opsonised immune complexes in the circulation via C3b receptors (complement receptor 1), and deliver them to tissue macrophages (such as Kupffer cells in the liver) for elimination (226).

The spleen has also been implicated in the clearance of ICs in some studies (227), and the size and type of immune complexes may influence the relative contribution of different clearance mechanisms (224). Under some circumstances, ICs are not transported to the liver and cleared effectively, but are instead deposited in the tissues. There is, however, currently no evidence for the deposition of complement-fixing IC in organs such as the kidney in TTP, making this unlikely.

Identifying the mode of clearance may enable intervention in the clearance pathway to limit this pathogenic mechanism, and thus provide a novel therapeutic opportunity. Blockade of macrophage Fc receptors has been considered one of the mechanisms of action of intravenous immunoglobulin (IVIG) in patients with immune thrombocytopenia and other autoantibody-mediated cytopenias (229), but IVIG has no clinical benefit in TTP (230). If complement is shown to play a role in elimination of ADAMTS13 immune complexes, it is interesting to speculate on whether complement blockade at a specific point may be beneficial in reducing clearance of the enzyme.

Understanding how antibodies may alter the kinetics of clearance of ADAMTS13 will perhaps have more profound and immediate implications. ADAMTS13 has a relatively long active plasma half-life of 2-3 days (231),

suggesting its baseline rate of clearance is normally relatively slow. As up to 70% of the TTP patients' samples that I analysed had no or low inhibitory potential, this could suggest that provision of recombinant ADAMTS13 (rADAMTS13) 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.

Recombinant ADAMTS13 is already undergoing a phase I trial in the congenital form of the disease. If rADAMTS13 is well-tolerated in the early phase trials in congenital TTP, further studies of its role as an adjunct to PEX in acquired TTP will be important to determine if there is a consequent reduction in the number of PEX required to achieve remission. Given that PEX is not without complications (232), and is a lengthy procedure requiring specialised staff, this could have significant advantages. However, there is a possibility that giving rADAMTS13 might increase the antibody titre, and patients will still require immunosuppression to treat the underlying immune pathology. Further studies to explore the kinetics of ADAMTS13 antigen clearance in antibody-mediated TTP are now necessary and are in development (see later).

Some important questions remain to be answered. The mechanism of ADAMTS13 in treating TTP needs to be established in more detail: for example, does replacing ADAMTS13 merely stop the formation of new microthrombi, or does it play a role in actually clearing existing thrombi? ADAMTS13 has previously been shown to have thrombolytic effect in a ferric chloride injury model of venous thrombosis in mice (36), but whether this is the case in TTP still remains unclear.

Other recently developed potential therapeutic strategies for TTP are less likely to be effective, given the work in this thesis highlighting the importance of clearance as a mechanism. Jian *et al* have described a spacer domain mutant of ADAMTS13 which does not bind TTP autoantibodies (223). This

spacer domain mutant also has increased activity, which has recently been shown to be due to disruption of the CUB-spacer interaction, leading to conformational activation of ADAMTS13 (53). Whilst this gain-of-function mutant ADAMTS13 could potentially be effective in patients with anti-N terminal antibodies alone (in whom I found only anti-spacer antibodies), 60% patients have antibodies directed towards the C-terminal domains. In these patients the mutant is unlikely to be any more efficacious than WT ADAMTS13, as it will still be prone to clearance.

In this study, I demonstrated that the domain specificity of anti-ADAMTS13 autoantibodies at presentation was not predictive of disease severity or relapse risk. In contrast, ADAMTS13 antigen levels at presentation appear to represent a novel prognostic factor, with antigen levels in the lowest quartile amongst acquired TTP patients being associated with a five-fold increase in mortality during the first presentation. Initial interest in measuring ADAMTS13 antigen levels diminished when it was recognised to be of limited use in diagnosis, as not all patients had very low levels. In my opinion, there should now be a return to measuring antigen levels at presentation of acquired TTP, to help risk stratify patients and potentially guide intensification of therapy.

Acquired TTP is an unusual autoimmune disease in as much as it has just a single autoantigen – ADAMTS13. Unlike many other autoimmune conditions, there is no known spread to other antigens, and indeed the longitudinal results of this study show only minimal evidence of intramolecular epitope spreading with only one patient developing novel antibodies with alternative domain specificity at relapse. Although more than half of the patients studied exhibited altered domain specificity profile at relapse, this was usually the loss of anti-C-terminal reactivity, with the reappearance of anti-N-terminal antibodies at disease recurrence.

There are potential applications of the longitudinal data that could improve current and future clinical practice. For example, if inhibitory N-terminal antibodies are identified in patients in clinical remission (using a combination

of anti-ADAMTS13 IgG titre, then activity and ADAMTS13 antigen assays), then it may be appropriate to treat the patients further with immunosuppressive therapy (e.g. rituximab) to protect against relapse. However, this work has demonstrated that not all persistent anti-ADAMTS13 antibodies are pathogenic, with anti-TSP2-8 antibodies in two patients having no effect on ADAMTS13 antigen or activity levels and therefore not requiring any treatment.

In the future, use of newer monoclonal antibodies targeting B cells may be more effective at reducing anti-ADAMTS13 autoantibody production. The newer anti-CD20 agents such as ofatumumab and ocrelizumab, which target epitopes on CD20 that are distinct from the rituximab binding site, are already in use in B cell malignancies and autoimmune conditions such as multiple sclerosis, and have more complement-dependent cytotoxic (CDC) activity or antibody-dependent cellular cytotoxicity (ADCC) than rituximab (233). Unlike CD20, expression of CD19 is maintained on plasmablasts and subsets of plasma cells (234, 235). CD19 is important for regulating the threshold for B cell activation and studies of anti-CD19 therapy in murine models of autoimmunity almost completely inhibited the generation of IgG autoantibodies, suggesting targeting CD19 for B cell depletion may have a more pronounced effect on autoantibody production than CD20 (233, 236). Phase 2 trials of anti-CD19 agents in both B cell malignancies and autoimmune conditions are ongoing (NCT02200770, EudraCT: 2011-002565-38).

ADAMTS13 may be a target of an autoimmune response because it naturally can adopt different molecular conformations. Such a phenomenon has previously been described in other autoimmune diseases:
1) Anti-phospholipid syndrome, where plasma-derived β2GPI has a closed, circular conformation (237). However, after exposure to anionic structures such as negatively charged phospholipids, β2GPI binds and undergoes a conformational change exposing the epitope of the autoantibodies which can then in turn bind (238). Thus, these antibodies only recognise phospholipid bound β2GPI.

2) Heparin-induced thrombocytopenia, where the autoantibodies against platelet factor 4 (PF4) only recognise the conformation induced by binding to heparin (239).
3) Wegener's granulomatosis, where autoantibodies are directed against the neutrophil azurophil granule constituent, proteinase 3. Proteinase 3 is a serine protease zymogen which undergoes a conformational change when the activation peptide is removed. Intriguingly, the autoantibodies target this activated form, rather than the zymogen (240).
It is interesting to consider whether these autoantigens are targeted due to the existence of different conformations.

As in previous studies, I found that the majority of patients with TTP have autoantibodies against ADAMTS13 spacer domain. Recent work by South *et al* and Muia *et al* suggests that this may be because the spacer domain is naturally cryptic, shielded by the CUB domains (53, 54). They propose that ADAMTS13 circulates in 'closed' conformation, maintained by a CUB-spacer domain binding interaction (53, 54). ADAMTS13 becomes conformationally activated on demand through interaction of the TSR or CUB domains with VWF, 'opening' the enzyme up (Figure 1.7). During this process of ADAMTS13 activation, cryptic epitopes are revealed and these might be recognised as foreign during immune activation e.g. in response to infection (53).

Support for this hypothesis is provided by the increased recognition of the spacer domain by a patient-derived anti-spacer monoclonal antibody, when ADAMTS13 is in its 'open' conformation, but comparatively poorly recognised in its 'closed' form (53). The results on the conformational activation of ADAMTS13 provide insight into the potential mechanism of autoantibody formation in TTP. It remains to be seen whether the anti-spacer antibodies detected in my work are indeed conformationally sensitive, and whether the other autoantibodies targeting the TSP2-8 domains and the CUB domains are also directed against the cryptic antigenic surface or the exposed surface. This may well have an impact on the likelihood of the autoantibodies forming ICs in the circulation. This is important as it, along

with the IgG subclass of the anti-ADAMTS13 antibody, may be a major determinant of antibody-mediated clearance in acquired TTP.

As a co-applicant, I have recently been awarded a project grant from the British Heart Foundation to examine the ability of TTP patient IgG to bind to the circulating ‘closed’ and ‘open’ forms of ADAMTS13. We will determine the prevalence of different IgG subclasses (IgG1-4) that recognise ‘open’/’closed’ ADAMTS13, and explore the relative contribution of these subclasses to ADAMTS13 clearance and inhibition. We shall also ascertain the kinetics of ADAMTS13 immune complex formation, antibody-mediated ADAMTS13 clearance and mechanisms of clearance in acquired TTP patients following plasma exchange therapy.

A pilot genome-wide association study (GWAS) of acquired antibody-mediated TTP in 44 Caucasian TTP patients found multiple SNPs in the HLA-II region which were significantly associated with TTP (160), thereby validating the approach (as HLA associations with TTP have previously been characterised). Interestingly, further associations were found with genes important in B cell development and function, and a large-scale GWAS study is underway. It will be critical to pull the information gleaned from the GWAS study together with the detailed clinical and laboratory results already available to ‘deep phenotype’ acquired TTP patients. This bioinformatics approach is likely to generate important information on the different clinical subtypes seen in TTP, which may lead to more tailored therapy.

In conclusion, this study has improved our understanding of the immunological basis of acquired TTP, which accounts for the 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. The results have also implicated ADAMTS13 depletion as a dominant pathogenic mechanism underlying severe loss of enzyme activity in acquired TTP. The findings have considerable relevance to patient management and have the potential to improve outcomes. Measuring

ADAMTS13 antigen levels at presentation could help identify patients who need early intensive treatment. The appreciable proportion of acquired TTP patients with non-inhibitory / weakly inhibitory anti-ADAMTS13 IgG suggests that provision of recombinant ADAMTS13 may not result in rapid enzyme inhibition in many patients and imply that it could have therapeutic potential, potentially transforming the face of TTP treatment in the future.

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