Complement and cytokine response in acute Thrombotic Thrombocytopenic Purpura

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Summary

Complement dysregulation is key in the pathogenesis of atypical Haemolytic Uraemic Syndrome (aHUS), but no clear role for complement has been identified in Thrombotic Thrombocytopenic Purpura (TTP). We aimed to assess complement activation and cytokine response in acute antibody-mediated TTP. Complement C3a and C5a and cytokines (interleukin (IL)-2, IL-4, IL-6, IL-10, tumour necrosis factor, interferon-γ and IL-17a) were measured in 20 acute TTP patients and 49 remission cases. Anti-ADAMTS13 immunoglobulin G (IgG) subtypes were measured in acute patients in order to study the association with complement activation. In acute TTP, median C3a and C5a were significantly elevated compared to remission, C3a 63·9 ng/ml vs. 38·2 ng/ml ($P < 0·001$) and C5a 16·4 ng/ml vs. 9·29 ng/ml ($P < 0·001$), respectively. Median IL-6 and IL-10 levels were significantly higher in the acute vs. remission groups, IL-6: 8 pg/ml vs. 2 pg/ml ($P = 0·003$), IL-10: 6 pg/ml vs. 2 pg/ml ($P < 0·001$). C3a levels correlated with both anti-ADAMTS13 IgG ($r_s = 0·604$, $P = 0·017$) and IL-10 ($r_s = 0·692$, $P = 0·006$). No anti-ADAMTS13 IgG subtype was associated with higher complement activation, but patients with the highest C3a levels had 3 or 4 IgG subtypes present. These results suggest complement anaphylatoxin levels are higher in acute TTP cases than in remission, and the complement response seen acutely may relate to anti-ADAMTS13 IgG antibody and IL-10 levels.

Keywords: complement, cytokines, Thrombotic Thrombocytopenic Purpura, ADAMTS13, T cells.

Thrombotic Thrombocytopenic Purpura (TTP) and atypical Haemolytic Uraemic Syndrome (aHUS) are thrombotic microangiopathies (TMAs) that share common clinical features (microangiopathic haemolytic anaemia, thrombocytopenia and microvascular thrombosis), but typically have a different pathophysiology. This is reflected by the different target organs: typically, the central nervous system and heart in TTP, while in aHUS the kidney is primarily affected.

Key to the pathophysiology of TTP is deficiency of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) and the consequent accumulation of Ultra Large von Willebrand Factor multimers (ULVWF) in the circulation, leading to platelet aggregation and formation of microthrombi. The majority of cases are idiopathic and mediated by immunoglobulin G (IgG) antibodies to ADAMTS13 (Furlan *et al*, 1998; Tsai & Lian, 1998). aHUS however, has been shown to occur as a result

of dysregulation of the complement system, a family of serine proteases playing an important role in innate and adaptive immunity. Mutations in genes encoding tissue-bound and fluid phase complement regulator proteins (including CD46, Factor H, Factor I) can be found in approximately 50–60% of patients with aHUS (Kavanagh & Goodship, 2010a,b, 2011); dysregulation of complement leads to subsequent over-activity of complement effector proteins, including the anaphylatoxins (complement factors C3a and C5a). These are potent inflammatory mediators, which target and activate the renovascular endothelium, leading to local platelet aggregation and microvascular thrombosis.

Although complement dysregulation has a well-established role in aHUS, it is uncertain whether this mechanism plays a part in the pathophysiology of TTP. Some studies have raised the possibility of complement over-activation in TTP. Reduced levels of serum complement C3 have been found in

patients with TMAs having low ADAMTS13 levels(Ruiz-Torres et al, 2005). Another study has found increased levels of complement anaphylatoxin C3a and terminal complement pathway components SC5b-9 in patients with acute TTP; the same study found the presence of anti-ADAMTS13 inhibitors was linked to increased complement activation(Reti et al, 2012). More recently, higher complement activity has been reported in patients dying during an acute TTP episode compared to patients responding to treatment, although this study used banked citrated plasma for analysis (Wu et al, 2013).

In this study, we aimed to investigate the possible role of complement activation in antibody-mediated TTP, by measuring levels of activated components C3a and C5a in patients with acute TTP, patients with previous acute TTP now in remission, and comparing these with normal controls. We also determined whether complement activation was associated with total anti-ADAMTS13 IgG level. Given IgG antibody subtypes are known to activate different complement pathways with variable efficacy, we also assessed whether there was any association between complement activation and anti-ADAMTS13 IgG subtype. Finally, given that antibody-mediated TTP is an autoimmune disease, we also aimed to assess the association with T helper cells types 1, 2 and 17 (Th1, Th2 and Th17), which typically have an important role in autoimmunity, and to relate these to complement activation.

Methods

Patients

This was a prospective study, which included 20 patients admitted to our centre with acute TTP from June 2011 to October 2012, in whom samples were taken preceding any therapy. Remission samples were available for 15 of these cases and also for a cohort of 34 patients attending follow up clinic who had previously had an acute TTP episode. The work was approved by national ethics committees for consent of both patients and controls in this study. (MREC: 08/H0810/54, MREC: 11/LO/1153).

Thrombotic Thrombocytopenic Purpura was diagnosed based on the presence of thrombocytopenia, microangiopathic haemolytic anaemia, a normal clotting screen and an increase in lactate dehydrogenase (LDH) to at least one and a half times the upper limit of normal(Scully et al, 2012). Remission was defined as a sustained platelet count of >150 × 10^9/l for two consecutive days.

All acute patients had blood taken for complement and cytokine assays, during their initial presentation, before any therapy such as plasma exchange (PEX) or steroids was given and, where possible, during subsequent remission. The remission-only group had blood taken for complement and cytokines at a single timepoint during an outpatient hospital visit, in conjunction with routine laboratory parameters and

samples for ADAMTS13 assays. Blood was taken from normal controls to obtain local normal ranges for complement and cytokine assays.

For all acute TTP cases, ADAMTS13 activity, anti-ADAMTS13 total IgG, and anti-ADAMTS13 IgG subtypes 1–4 were measured on admission (pre-treatment). The remission group had ADAMTS13 activity measured only. Anti-ADAMTS13 IgG was only measured in remission if there was a reduction in ADAMTS 13 activity.

Sample handling

Measurement of activated complement components (including the anaphylatoxins C3a/C5a) requires special attention to sample handling, given the high risk of in vitro complement activation resulting in falsely elevated levels(Mollnes et al, 1988). Although it is known that tubes containing either EDTA or citrate both inhibit complement activation by binding Ca^{2+} and Mg^{2+} ions (and thus are vastly superior than serum tubes), previous data suggests EDTA is superior to citrate for this purpose (Mollnes et al, 1988). In order to confirm this, we measured complement C3a and C5a in a cohort of normal controls, having taken blood into EDTA, citrate and serum tubes for comparison.

Blood for complement analysis was taken into EDTA tubes, separated via centrifugation (2000 g) within 4 h of collection, and stored at −70°C prior to use. Blood for cytokine assays was drawn into serum tubes; samples were then allowed to stand for 30 mins to allow clot retraction, blood was separated via centrifugation (2000 g), and serum aspirated off and stored at −70°C prior to use.

Measurement of complement anaphylatoxins and cytokines

Measurement of complement anaphylatoxins (C3a, C5a) and cytokines [interleukin (IL)-2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF), interferon-γ (IFN-γ), IL-17a] was performed by a Cytometric Bead Array (CBA) method. This method has several advantages over conventional enzyme-linked immunosorbent assay (ELISA), including enabling the measurement of multiple analytes simultaneously (thus avoiding the need to thaw samples repeatedly), a broader dynamic range, and the use of hundreds of capture beads per analyte in each sample well (a minimum of 200 beads for each analyte per well) result in each sample having a large number of replicates, thus improving accuracy. To measure anaphylatoxins, capture beads of known fluorescence conjugated with antibodies to C3a and C5a were initially incubated with plasma, washed and then incubated with phycoerythrin (PE)-conjugated antibodies (BD Human Anaphylatoxin Kit; BD Biosciences, San Jose, CA, USA). The sandwich complexes formed between the capture beads, analyte and detection reagent were then measured using a flow cytometer (BD FACSarray™; BD Biosciences). Cytokines pertaining to Th1 (IFN-γ, IL-2, TNF), Th2

(IL-4, IL-6, IL-10) and Th17 (IL-17a) responses were measured using CBA methodology (BD Human Th1/Th2/Th17 kit; BD Biosciences). This employed the same method as for anaphylatoxins (above), but only one incubation: capture beads and PE-conjugated antibodies were incubated with plasma in one step, prior to detection using the flow cytometer. Flow cytometry data was analysed using FCAP ARRAY™ software (Soft Flow Hungary Ltd, Pécs, Hungary), to generate results for each analyte being measured. Local normal ranges were obtained for both complement anaphylatoxin and cytokine levels by taking blood samples from 17 healthy volunteers (controls).

ADAMTS13, anti-ADAMTS13 total IgG and subtypes 1–4

Plasma ADAMTS13 activity was measured using a fluorescence resonance energy transfer (FRET) assay (Kokame *et al*, 2005). ADAMTS13 activity was expressed as a percentage relative to the activity in pooled normal plasma (normal range 60–123%; lower limit of detection <5%). Anti-ADAMTS13 IgG was measured using an ELISA technique, as previously described (Chow *et al*, 2007; Scully *et al*, 2007) (normal range <6·1%). Anti-ADAMTS13 IgG subtypes 1–4 were also measured using an ELISA-based method. Microtitre plates were coated with recombinant ADAMTS13 (Baxter Innovations GmbH, Vienna, Austria) via overnight incubation at 4°C. Plates were then blocked with 2% Bovine Serum Albumin for 1 h. Diluted patient and standard/control samples were then added to wells and incubated for 2 h. Following washing, monoclonal biotinylated antibodies to either anti human IgG1, 2, 3 or 4 (Sigma-Aldrich Chemical Company Ltd, Dorset, UK) were added to wells and incubated for 90 min. Following a further wash, Streptavidin (Merck Chemicals, Nottingham UK) was added to each well and incubated for 30 min. A substrate buffer [Phosphate-citrate buffer with urea hydrogen peroxide tablets (P-9305)] (Sigma-Aldrich Chemical Company Ltd) was added and the reaction was then stopped with 2 mol/l sulphuric acid after a colour reaction was achieved. Plates were then read at 450 nm, and the amount of IgG subtype present was calculated by relating the optical density of the patient sample to the optical density of a standard (expressed as a percentage).

Statistical analysis

Complement, cytokine and IgG data was not normally distributed, and therefore non-parametric methods were used for analysis. The Mann–Whitney U test was used to compare groups, and paired data was compared using the Wilcoxon signed-rank test. Statistical dependence between variables was assessed using the Spearman's rank correlation coefficient. P-values <0·05 were regarded as statistically significant. Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA), Analyse-it® version 2.30 (Analyse-it Software Ltd, Leeds, UK), and GRAPHPAD PRISM® version 6.0 (GraphPad software Inc., La Jolla, CA, USA).

Results

Patients

Over the study period, samples were obtained from a total of 20 patients (female (F) = 11, male (M) = 9) with acute TTP, and 49 patients (F = 31, M = 18) who were in complete remission following a prior acute TTP episode. For 15/20 acute TTP patients (F = 8, M = 7) paired samples (acute and remission) were available. Detailed admission parameters for acute patients, including markers of disease severity (primarily LDH, Troponin T, and presence of neurological symptoms (fall in Glasgow Coma Score below normal or any focal neurology), are shown in Table I. Also shown are number of PEX episodes, number of rituximab infusions given and time taken to attain remission.

All acute patients ($n = 20$, median age 43 years, range 17–79 years) had ADAMTS13 < 5% and the presence of anti-ADAMTS13 IgG antibodies, with a median total IgG level of 52% (range 5–117%). Median Hb 86·5 g/l (range 48–136 g/l), platelet count 11×10^9/l (range 4–130×10^9/l) and LDH 1185 iu/l (range 346–2517 iu/l) were all in keeping with a diagnosis of acute TTP. 18/20 patients had acute *de novo* TTP, with the remaining two patients having an acute relapse. Median Troponin T was 0·025 µg/l (range 0·003–0·277 µg/l). 14/20 (70%) acute patients had neurological symptoms at presentation, and 10/20 (50%) required intensive therapy unit (ITU) admission, of which one patient was intubated. The median number of PEX episodes required to attain remission was 17·5 (range 3–57) and median number of rituximab infusions was 4 (range 1–9). The median time to remission was 15 d (range 3–43 d).

The remission group ($n = 49$, median age 45 years, range 18–81 years) had a median ADAMTS13 of 82% (range 29–130%). All patients had had at least one prior acute TTP episode, a median of 15·5 months (range 1–125 months) prior to the remission sample being taken.

Complement C3a/C5a

Effect of sample type on in vitro *complement activation.* Complement C3a and C5a levels were measured in seven control subjects, for whom blood was taken into EDTA, citrate and serum tubes for comparison. For C3a, levels obtained from serum samples were significantly higher than EDTA (median C3a 192·8 ng/ml (range 123·9–379·7) vs. 42·73 ng/ml (range 35·71–56·10) respectively, $P = 0·02$); citrate samples resulted in higher but non-significant levels of C3a compared to EDTA (median C3a 57·28 ng/ml (range 31·13–104) vs. 42·73 ng/ml (range 35·71–56·10) respectively, $P = 0·109$), Fig 1A. For C5a, serum levels were significantly

Table I. Admission parameters for the acute Thrombotic Thrombocytopenic Purpura group, $n = 20$. All patients had ADAMTS13 < 5% at presentation.

Episode	Age (years), Sex*	Ethnic group†	Previous episodes (Y/N)	Anti-ADAMTS13 IgG (%)‡	Hb (g/l)	Platelets (×10⁹/l)	LDH (iu)	Troponin, T (µg/l)§	Creatinine (µmol/l)¶	Neuro symptoms (Y/N)	ITU admission (Y/N)**	Plasma exchange episodes to remission	Number of rituximab doses	Time to complete remission (d)	Outcome
1	45, M	C	No	>100	67	17	667	0·019	101	N	N	49	8	43	rem
2	32, F	O	No	>100	96	7	382	0·003	79	N	N	18	4	16	rem
3	56, F	C	No	66	117	7	1133	0·022	113	Y	Y	11	4	12	rem
4	65, M	C	No	74	100	21	1254	0·277	83	Y	N	18	8	34	rem
5	51, F	C	No	59	51	6	NR	0·081	106	Y	Y(I)	7	1	N/A	died
6	79, F	C	No	68	87	9	1616	0·017	74	Y	Y	43	4	35	rem
7	24, M	C	No	107	48	12	1264	0·027	104	N	N	6	4	6	rem
8	23, F	C	No	52	117	10	956	0·017	80	Y	Y	9	4	8	rem
9	49, M	A/C	No	44	71	16	1258	0·118	131	Y	N	32	8	29	rem
10	53, F	C	No	117	114	6	2517	0·169	149	Y	Y	28	6	23	rem
11	45, M	C	No	96	88	20	1820	0·219	167	Y	Y	57	9	31	rem
12	42, M	A/C	Yes	5	117	8	903	0·011	90	Y	Y	7	4	7	rem
13	34, F	C	No	38	66	19	891	0·037	58	Y	N	8	4	5	rem
14	27, M	C	No	20	76	18	346	0·201	93	N	N	4	4	3	rem
15	52, M	C	No	50	128	9	1691	0·025	105	Y	N	22	8	18	rem
16	36, F	C	No	54	85	15	1185	0·018	80	Y	Y	23	6	25	rem
17	17, F	A/C	No	74	136	7	1704	0·085	63	Y	Y	17	4	12	rem
18	40, M	C	No	46	86	4	1231	0·015	120	N	N	19	4	15	rem
19	43, F	A/C	Yes	19	81	130	385	NR	90	N	N	3	4	7	rem
20	34, F	A/C	No	27	72	11	539	0·015	95	Y	Y	5	4	5	rem

*F, female; M, male.
†C, Caucasian; A/C, Afro-Caribbean; O, Other.
‡Anti-ADAMTS13 IgG normal <6·1%.
§Troponin T normal range 0–0·014 µg/l.
¶Creatinine normal range 49–92 µmol/l.
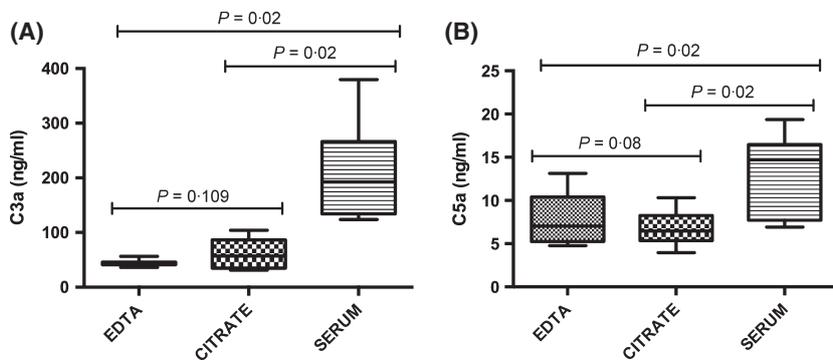**ITU, intensive therapy unit; I, Intubated; NR, not recorded; rem, remission; Y, yes; N, no.

Fig 1. Comparison of (A) complement C3a and (B) C5a levels obtained from 7 normal controls, for samples taken into EDTA, serum and citrate tubes.
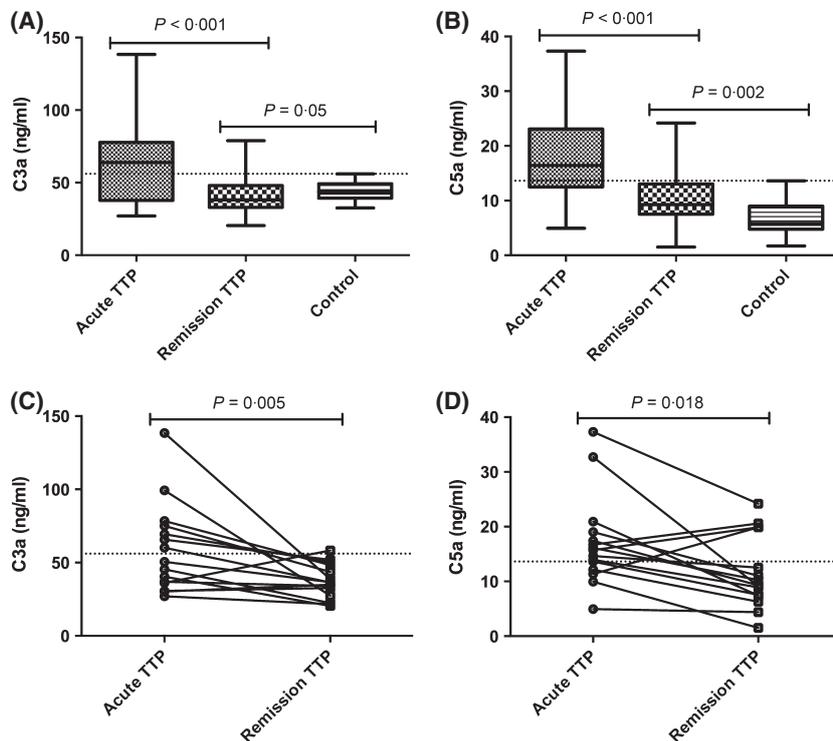

Fig 2. Complement C3a (A) and C5a (B) levels for three groups: acute Thrombotic Thrombocytopenic Purpura (TTP, $n = 20$), remission TTP ($n = 49$) and controls ($n = 17$). C3a and C5a levels were significantly higher in acute TTP vs. remission TTP ($P < 0.001$ for both). C3a (C) and C5a (D) levels for the 15 patients who had paired acute and remission samples are also shown. Levels were higher in acute TTP vs. remission TTP; C3a $P = 0.005$, C5a $P = 0.018$. The dotted line indicates the upper limit of the normal range.

higher than EDTA (median C5a 14·72 ng/ml (range 6·93–19·36) vs. 7·038 ng/ml (range 4·78–13·14) respectively, $P = 0.02$; there was no significant difference was seen between EDTA and citrate samples for C5a, $P = 0.08$, Fig 1B.

Normal controls. Complement C3a and C5a levels were measured in 17 normal healthy controls. Median control C3a levels were 43·7 ng/ml (range 32·54–56·10) and C5a levels were 5·81 ng/ml (range 1·71–13·6).

Complement activation in acute and remission patients. Complement anaphylatoxin C3a levels in the acute TTP group were significantly elevated compared to normal controls, median C3a 63·9 ng/ml (range 27·1 to 138·5) vs. 43·7 ng/ml (range 32·54 to 56·10) respectively, $P = 0.04$. C5a levels were also significantly higher than controls, median C5a 16·4 ng/ml (range 4·94–37·3) vs. 5·81 ng/ml (range 1·71–13·6)

respectively, $P < 0.001$. However not all patients had levels above the upper limit of the normal range: for C3a, 12/20 (60%) were elevated above normal, with 14/20 (70%) for C5a. Comparing the acute and remission TTP groups as a whole, both C3a and C5a were significantly higher in the acute TTP vs. remission group, median C3a 63·9 ng/ml vs. 38·2 ng/ml ($P < 0.001$), and median C5a 16·4 ng/ml vs. 9·29 ng/ml ($P < 0.001$), respectively (shown in Fig 2A, B). A significant difference in acute and remission C3a and C5a levels was also seen for the 15 patients with paired samples: median C3a 50·6 ng/ml vs. 36·5 ng/ml $P = 0.0054$ and median C5a 15·7 ng/ml vs. 9·29 ng/ml $P = 0.018$ (see Fig 2C, D respectively). For all patients as a whole, remission C3a levels were not significantly higher than normal controls, median C3a 36·5 ng/ml vs. 43·7 ng/ml respectively; conversely C5a levels in remission remained elevated, median C5a 9·29 ng/ml vs. 5·81 ng/ml, $P = 0.002$.

Three patients had higher C5a levels at remission than during the acute episode (Fig 2D); none of these were remarkable in terms of time taken to attain remission or treatment required (number of PEX episodes or rituximab infusions). Furthermore, none of these patients had either a milder clinical course at presentation or any increased risk of relapse following treatment. The two highest C3a and C5a levels seen during acute episodes were seen in four individual patients (Fig 2C, D respectively). Although the two patients having the highest C3a levels required more rituximab doses than the standard number of four infusions (six and eight infusions), there was no significant association demonstrated between C3a or C5a levels and number of PEX required, number of rituximab required or time taken to attain remission.

Cytokines

Of the cytokines assayed, only IL-6 and IL-10 were significantly elevated in the acute TTP group compared to the remission group: median IL-6: 9·785 pg/ml vs. 2·405 pg/ml ($P = 0·0013$), IL-10: 6·375 pg/ml vs. 1·8 pg/ml ($P < 0·001$) respectively (Fig 3A, B). Despite levels of these two cytokines in acute TTP being higher than remission, only seven acute cases had IL-6 levels above the upper limit of normal (>9·92 pg/ml) and five cases had IL-10 levels above normal (>7·16 pg/ml). There was no significant difference in acute vs. remission cytokine levels within the group having paired acute and remission samples. A positive correlation was present between IL-10 level and C3a level, $r_s = 0·692$ ($P = 0·006$), and IL-10 level and LDH, $r_s = 0·703$ ($P = 0·007$).

Complement, anti-ADAMTS13 IgG and anti-ADAMTS13 IgG subtype

For all acute patients a positive correlation was seen between C3a level and anti-ADAMTS13 IgG antibody level, $r_s=0·604$ ($P = 0·017$) (Fig 4A). Anti-ADAMTS13 IgG was also positively correlated with LDH $r = 0·502$, ($P = 0·028$). The anti-ADAMTS13 IgG subtype levels for acute patients are shown in Fig 4B. The highest median subtype level (as a % relative to a standard) was seen with IgG2. There was no correlation

between either C3a or C5a levels and any particular IgG subtype. However, when patients were categorized according to number of IgG subtypes (1–4) present, the highest C3a levels were seen with those patients having three or more IgG subtypes.

Complement and markers of disease severity

C3a and C5a levels were compared in patients with acute TTP having presence or absence of the following markers of disease severity: ITU admission, neurological symptoms and elevated Troponin T (>0·04 μg/ml). No significant difference was shown for either C3a or C5a for any of the three markers.

Discussion

We have presented the largest prospective study to date that describes changes in complement activation markers and cytokines in acute and remission TTP cases. Complement activation occurring acutely was demonstrated by a between-group comparison of 20 acute and 49 remission TTP cases, and a within-group comparison of 15 patients with paired acute and remission samples. Elevation in C3a and C5a was seen in acute cases, for both the between-group and within-group comparison, and acute levels were significantly higher than in remission. However, there was a wide variation in levels of C3a and C5a acutely, and not all cases had levels above the upper limit of normal. We also quantified anti-ADAMTS13 IgG levels for all acute cases, and were able to demonstrate a positive correlation between anti-ADAMTS13 IgG level and C3a level. Furthermore we looked at the relationship between anti-ADAMTS13 IgG subtype and complement activation: although no individual subtype was correlated with C3a/C5a, higher C3a levels were seen in patients having three or more subtypes present. Finally, we were also able to assess the Th1/Th2/Th17 cytokine response in acute TTP, demonstrating a higher level of IL-6 and IL-10 in acute vs. remission cases.

We confirmed the importance of using EDTA plasma for complement assays, given reduction in *in vitro* activation compared with serum; results between EDTA and citrate were comparable, but EDTA appeared to have lower C3a lev-
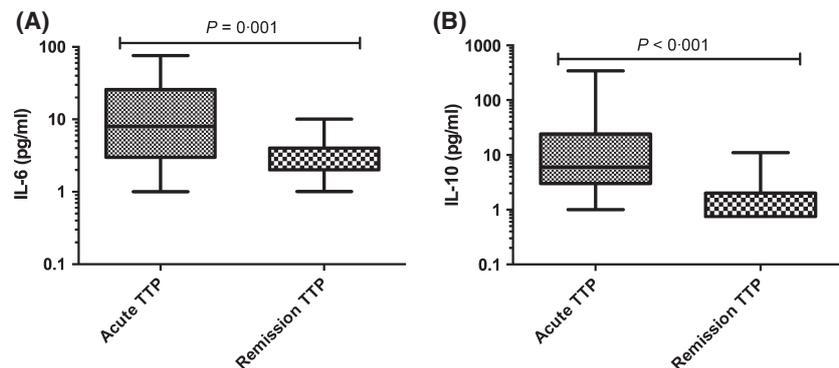
Fig 3. Levels of (A) interleukin-6 (IL-6) and (B) interleukin-10 (IL-10) for acute Thrombotic Thrombocytopenic Purpura (TTP) and remission TTP groups. For both IL-6 and IL-10, levels in acute TTP were higher than in remission, $P = 0·001$ & $P < 0·001$ respectively.
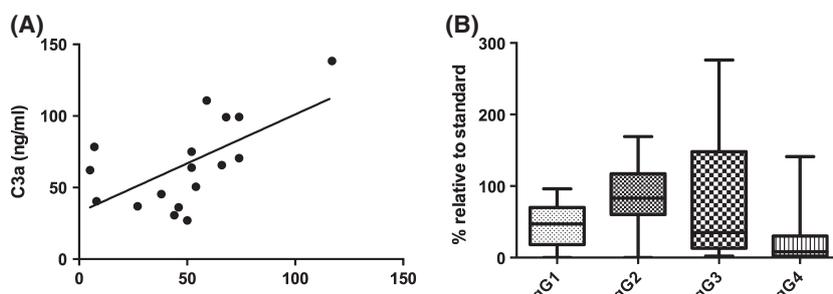
Fig 4. (A) A positive correlation was found between anti-ADAMTS13 IgG level and C3a. (B) Analysis of anti-ADAMTS13 IgG subtypes–IgG2 was found to be the subtype with the highest median level when measured as a % relative to a standard.

els and a narrower range for controls than citrate. This finding, combined with data indicating that EDTA shows better complement inhibition over time than citrate (Mollnes *et al*, 1988), leads us to conclude that EDTA plasma is superior to citrate and serum for this purpose.

The finding of complement activation in acute TTP is interesting and raises questions regarding its role in this setting. Complement can be activated directly in several ways, including via immune complexes, or infected or damaged cells. These activate different complement pathways, with immune complexes typically activating the classical pathway (Ricklin & Lambris, 2013). Therefore one potential mechanism for complement activation in TTP relates to anti-ADAMTS13 antibodies. Antigen-antibody complexes formed between ADAMTS13 and anti-ADAMTS13 IgG antibodies may directly activate the classical complement pathway, leading to downstream activation of C3 and formation of C3a. Our finding of a positive correlation between anti-ADAMTS13 IgG antibody level and C3a level would support this as a potential mechanism. Our results are consistent with those from a previous study that investigated complement activation markers in TTP (Reti *et al*, 2012), which found higher C3a levels in 13 patients with acute TTP vs. 11 remission cases. Our study comprised higher numbers of individual acute and remission patients, with the vast majority of acute cases (18/20) being *de novo*, compared with 8/13 reported by Reti *et al* (2012).

Immune complexes are also able to activate the other complement pathways (lectin and alternative), with particular antibody types and subtypes being more effective than others at activating a particular pathway. IgG subtypes 1 and 3 are particularly effective at activating the classical pathway, with IgG2 being better at activating the alternative pathway(Lucisano Valim & Lachmann, 1991). Our finding of IgG2 having the highest median level of all the four subtypes, may suggest that immune complexes in TTP could be activating the alternative as well as the classical complement pathway. Indeed markers of classical (C4d) and alternative (C3bBbP) pathway have both been shown to be elevated in acute TTP cases; both correlated with downstream effector components C3a and SC5b-9(Reti *et al*, 2012).

Aside from immune complex-mediated complement activation, there are other potential ways in which complement

might be activated in TTP. Part of the role of complement relates to recognizing and clearing damaged cells; given the organ damage occurring in TTP secondary to microvascular thrombosis, this alone may be sufficient to activate the alternative complement pathway. Over-activation of the alternative pathway has also been found to be implicated in a variety of autoimmune diseases (Klos *et al*, 2009), including systemic lupus erythematosus (Alegretti *et al*, 2012; Elkon & Santer, 2012). Indeed it is the alternative pathway that has been found to be overactive as a result of defective complement regulation in aHUS(Kavanagh & Goodship, 2010a,b, 2011). The finding of significant elevation of IL-6 and IL-10 in acute cases compared to remission, may indicate a possible mechanism driving the immune response in antibody-mediated TTP. Although IL-10 is an anti-inflammatory cytokine, it has an important role in enhancing B-cell survival, proliferation and antibody production (Iyer & Cheng, 2012); as such it is possible that IL-10 may stimulate anti-ADAMTS13 IgG production, and lead to antibody-mediated complement activation. The finding of a positive correlation between IL-10 and anti-ADAMTS13 IgG may support this hypothesis, as well as the fact that both IgG and LDH are positively correlated with LDH, a marker of disease severity in TTP. High IL-6 levels may relate to the inflammatory response associated with microvascular thrombosis in TTP (Gabay, 2006; Mihara *et al*, 2012).

The variations in degree of complement activation in acute TTP are notable, and there are various possibilities for this: given the positive correlation between IgG level and C3a, variations in complement activation may relate to antibody burden or potency of antibodies. Although we were not able to demonstrate an association between a particular IgG subtype and complement activation, the finding that patients with highest C3a levels generally have three or four IgG subtypes present, is consistent with IgG antibody burden playing a role in the degree of complement activation occurring. Furthermore, it is known that some IgG subtypes (in the form of immune complexes) are able to activate complement better than others. There is also evidence suggesting that the proportion of antibody to antigen, as well as epitope density can all affect how strongly immune complexes activate complement(Lucisano Valim & Lachmann, 1991). Variations in complement regulation between patients may also affect the degree of activation

resulting from an initial trigger. This effect may be propagated by cytokines, such as IL-10. Serum from patients with TMAs has been shown to trigger complement C3 and membrane attack complex binding, when incubated with endothelial cells; this indicates the potential for complement to activate endothelial cells, a key process in the development of TMAs (Ruiz-Torres et al, 2005). There is at present no direct evidence that complement activation in TTP is associated with a worse prognosis. We found no association between degree of complement activation (C3a and C5a levels) and markers of TTP disease severity including Troponin T, presence of neurological symptoms and ITU admission.

Elucidating the role of complement in TTP is important, given the potential role for complement inhibition, which has been used very successfully in disorders where defective complement regulation is key, including Paroxysmal Nocturnal Haemoglobinuria (Kelly et al, 2011; Hillmen et al, 2013) and aHUS (Taylor et al, 2010; Wong et al, 2013). In this study we have shown that complement activation is seen in many patients with acute TTP, but further work is needed to establish whether complement has an important role in the pathophysiology of TTP, or if complement activation occurs purely as a bystander mechanism in this disease.

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Authorship and disclosures

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