## ORIGINAL ARTICLE

# The role of ADAMTS-13 activity and complement mutational analysis in differentiating acute thrombotic microangiopathies

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## Essentials

- Molecular diagnostics has improved the differentiation of acute thrombotic microangiopathys (TMAs).
- Atypical hemolytic uremic syndrome may have features mimicking thrombotic thrombocytopenic purpura.
- We identified novel complement mutations and a high incidence of *CD46*, with favorable long term outcomes.
- Complement mutation analysis in TMA where the diagnosis is unclear and ADAMTS-13 activity is > 10%.

**Summary.** *Background:* Differentiation of acute thrombotic microangiopathy (TMA) at presentation has historically been dependent on clinical parameters. Confirmation of thrombotic thrombocytopenic purpura (TTP) is increasingly reliant on demonstrating deficient ADAMTS-13 activity. The identification of alternative complement pathway abnormalities in atypical hemolytic uremic syndrome (aHUS), along with the proven efficacy of terminal complement inhibitors in treatment, has increased the need for rapid differentiation of TTP from aHUS. *Objectives:* We describe the clinical phenotype and nature of complement mutations in a cohort of aHUS patients referred as acute TMAs. *Patients/methods:* Four-teen consecutive aHUS patients were screened for mutations in *C3*, *CD46*, *CFH*, *CFI*, and *CFB*, as well as factor H (FH) antibodies. All aHUS patients had ADAMTS-13 activity > 10%. *Results:* Of 14 aHUS patients, 11 (79%) had platelet counts $< 30 \times 10^9$/L during the acute phase. Median presenting creatinine level was 295 μmol $L^{-1}$, while five (36%) of 14 presented with a serum creatinine level $< 200$ μmol $L^{-1}$. Alternative complement pathway mutations were detected in 9 (64%) of 14 patients, including *CD46* mutations in five (36%) of 14 patients. Patients were identified with novel mutations in *CFB* and *C3* that have not been previously reported. *Conclusions:* We demonstrate that diagnostic differentiation based on platelet count and renal function is insufficient to predict an underlying complement mutation in some aHUS cases. Specifically, we demonstrate a high frequency of functionally significant *CD46* mutations which may mimic TTP. ADAMTS-13 activity > 10% in a patient with a TMA should necessitate genetic screening for complement abnormalities.

**Keywords:** atypical hemolytic uremic syndrome; CD46; complement; diagnosis; thrombotic thrombocytopenic purpura.

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The copyright line for this article was changed on 7 April 2017 after original online publication.

## Introduction

Atypical hemolytic uremic syndrome (aHUS) and thrombotic thrombocytopenic purpura (TTP) are thrombotic microangiopathies (TMA) that manifest with organ dysfunction or life threatening features requiring urgent recognition and treatment. Both present with microangiopathic hemolytic anemia and thrombocytopenia, although the clinical course of the two diseases can be markedly different. Historically, ~60% of aHUS patients

progressed to end-stage renal failure (ESRF) [1,2], whereas in TTP, neurological and cardiac features are often more prominent and severe renal dysfunction is rare.

Although it is known that aHUS and TTP are histologically distinct entities [3], diagnosis is often reliant on clinical features, such as the pattern of organ dysfunction and degree of thrombocytopenia. It has been suggested that a serum creatinine level of > 150 to 200 μmol $L^{-1}$ or a platelet count of > $30 \times 10^9$ $L^{-1}$ 'almost eliminates' a diagnosis of TTP [4]. Because the two diseases have overlapping clinical features, however, there is inherent opportunity for misdiagnosis, and studies on TTP and aHUS have been open to diagnostic bias. The term 'TTP-HUS' has been used historically to reflect the degree of diagnostic uncertainty.

The past decade has seen major advances in our knowledge of the molecular pathophysiology underlying both aHUS and TTP, accompanied by shifts in diagnostic testing and divergence of treatment algorithms for the two diseases. In aHUS, dysregulation of the alternative complement pathway leads to excessive activation of the terminal complement pathway, complement-mediated endothelial cell damage, and subsequent glomerular microthrombi [2]. Detectable complement abnormalities have been described in ~50% of patients, including loss of function mutations within membrane co-factor protein (*CD46*), complement factor H (*CFH*) and factor I (*CFI*), and autoantibodies to the factor H (FH) and factor I (FI) proteins. Gain-of-function mutations have also been identified within complement factor B (*CFB*) and *C3* [5–7].

Thrombotic thrombocytopenic purpura, caused by an acquired or inherited deficiency of the von Willebrand factor–cleaving protease ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13), results in spontaneous aggregation of platelets and ultralarge von Willebrand multimers to form platelet-rich thrombi throughout the microvasculature [8]. Assessment of ADAMTS-13 activity is now routinely performed in patients presenting with acute TMA and, when < 10%, aids in the differentiation of TTP from other causes of TMA [9,10].

Early initiation of either immunomodulatory therapy in TTP [11] or complement blockade in aHUS [12] can improve outcomes, increasing the need for prompt and accurate differentiation between the TMAs.

We describe a retrospective cohort of aHUS patients referred with acute TMA and clinically suspected TTP. We highlight their presenting clinical and laboratory features, and subsequent complement abnormalities. This study emphasizes the clinical overlap and pivotal role of ADAMTS-13 assessment in the differentiation of TMAs. We also outline response to therapy and long-term outcomes of this aHUS subgroup, before complement inhibitors were available.

## Patients and methods

Data were retrospectively analyzed for all patients with a final diagnosis of aHUS managed at University College Hospital London between January 2006 and July 2013. Twelve patients were transferred during an acute presentation with a presumptive diagnosis of TTP based on clinical parameters. Two patients were referred to TTP clinic following prior episodes of TMA to exclude a diagnosis of congenital TTP (patients 1 and 6, Tables 1 and 2). All patients had evidence of TMA, defined as the presence of thrombocytopenia with microangiopathic hemolytic anemia (anemia, schistocytes on blood film, reticulocytosis, hyperbilirubinemia, elevated lactate dehydrogenase, and negative direct antiglobulin test) [13], during their initial presentation. Citrated blood for ADAMTS-13 levels was taken prior to therapeutic plasma exchange (TPE). Patients were also tested for human immunodeficiency virus, antinuclear antibodies (ANA), lupus anticoagulant, human chorionic gonadotropin, and fecal O157 enterotoxin, if reporting diarrheal symptoms, on presentation. C3 and C4 levels were measured prior to TPE. Patients with secondary TMA, including transplant-associated microangiopathy, active rheumatologic disease, malignancy, and drug-induced TMA, were excluded.

Atypical hemolytic uremic syndrome was diagnosed according to criteria published by the UK aHUS Rare Diseases Group [14] and European guidelines [15], including the presence of both TMA and acute kidney injury [16] without ADAMTS-13 deficiency or inhibitors. One patient underwent renal biopsy for diagnostic confirmation. Two patients did not fit diagnostic criteria due to normal renal function but were investigated due to recurrent TMA with normal ADAMTS-13 activity; the identification of a functionally significant mutation in one of the aforementioned complement genes was supportive of a diagnosis of aHUS in both patients. All analyses, including mutation screening, were undertaken as part of routine patient care.

We assessed whether coexisting complement mutations were present in TTP cases and could account for increased disease severity or renal impairment. Mutation screening was undertaken in 14 TTP patients (ADAMTS-13 activity < 10% and detectable anti-ADAMTS-13 IgG auto antibodies) with either renal impairment or a severe phenotypic presentation. Sample use was approved by the local research ethics committee (reference 08/H0716/72).

### ADAMTS-13 assays

ADAMTS-13 activity was measured by fluorescence resonance energy transfer–von Willebrand factor 73 [17]. An ADAMTS-13 level < 10% (normal range: 60–123%) was confirmatory of a diagnosis of TTP in all selected patients. Patients were screened for acquired IgG inhibitors as previously described [18], with a normal range of

**Table 1** Clinical features and presenting laboratory features of aHUS patients

| Patient ID | Gender | Age (years) | Familial? | Triggering events* | ADAMTS-13 activity (%) | Platelet count (×10⁹/L) | Creatinine (μmol L⁻¹) | Hb (g L⁻¹) | LDH (IU L⁻¹) | Total bilirubin (μmol L⁻¹) | Objective neurological involvement | Dialysis | Outcome | Recurrences |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 0.92 | No | 1, 2, 3 | 107 | 11 | 290 | 88 | H | 54 | No | Yes | CR | 4 |
| 2 | M | 21 | No | 1 | 86 | 20 | 443 | 114 | 2083 | 26 | No | Yes | CR | 0 |
| 3 | M | 15 | Yes | 1 | 93 | 15 | 94 | 120 | 1986 | 40 | No | No | CR | 1 |
| 4 | M | 3 | Yes | 1 | 96 | 18 | 79 | 102 | 1066 | 38 | No | No | CR | 1 |
| 5 | M | 44 | No | 1 | 78 | 10 | 357 | 122 | 771 | 16 | No | No | CR | 0 |
| 6 | F | 28 | No | 0 | 88 | 115 | 1812 | 85 | 2162 | 32 | No | Yes | Stage 3 CKD | 0 |
| 7 | F | 35 | No | 4 | 77 | 26 | 212 | 58 | 3604 | 20 | No | No | CR | 0 |
| 8 | F | 23 | No | 1, 3 | 86 | 28 | 260 | 93 | 4621 | 51 | TIA | No | CR | 1 |
| 9 | M | 72 | No | 0 | 84 | 56 | 194 | 95 | 541 | 23 | TIA | No | CR | 1 |
| 10 | F | 36 | No | 4 | 50 | 16 | 672 | 66 | 2047 | 32 | No | No | CR | 0 |
| 11 | M | 36 | No | 0 | 72 | 34 | 137 | 89 | 2114 | 67 | No | No | CR | 0 |
| 12 | F | 23 | No | 2 | 48 | 31 | 608 | 105 | 342 | 63 | No | No | CR | 0 |
| 13 | F | 20 | No | 2 | 47 | 28 | 300 | 94 | 581 | 77 | No | No | CR | 0 |
| 14 | F | 51 | No | 0 | 120 | 58 | 198 | 72 | H | 81 | No | No | Death | 0 |

*Triggering events at initial presentation ± relapse: 0 = none identified, 1 = upper respiratory tract infection, 2 = abdominal sepsis, 3 = vaccination, 4 = pregnancy. CKD, chronic kidney disease; TIA, transient ischemic attack. Outcome: CR, complete remission, i.e., normalisation of creatinine and platelet count. LDH: H, hemolyzed.

< 6.1% calculated as the 95th percentile of 49 normal healthy controls.

### Complement assays

C3 and C4 levels were measured by rate nephelometry (Beckman Coulter Array 360, Ramsey, MN, USA). FH and FI levels were measured by radioimmunodiffusion (Binding Site, Birmingham, UK), Screening for FH autoantibodies was undertaken using ELISA as described previously [19]. FACS analysis of granulocytes from the patients was performed as described previously [20].

### Genetic analysis

Mutation screening of *CFH* [21], *CFI* [22], *CFB* [23], *CD46* [24], and *C3* [25] was undertaken using Sanger sequencing as previously described. Genotyping of the following SNPs *CFH* −331C>T (rs3753394), *CFH* c.184G>A; p.Val62Ile (rs800292), *CFH* c.1204T>C; p.Tyr402His (rs1061170), *CFH* c.2016A>G; p.Gln672Gln (rs3753396), *CFH* IVS15 −543G>A intron 15 (rs1410996), *CFH* c.2808G>T; p.Glu936Asp (rs1065489), *CD46* −652A>G (rs2796267), *CD46* −366A>G (rs2796268), *CD46* IVS9 −78G>A (rs1962149), *CD46* IVS12 +638G>A (rs859705), and *CD46* c.4070T>C (rs7144) was used to determine *CFH* and *CD46* haplotypes [26].

### Multiplex ligation–dependent probe amplification

Screening for genomic disorders affecting *CFH*, *CFHR1*, *CFHR2*, *CFHR3*, and *CFHR5* was undertaken using multiplex ligation–dependent probe amplification [26].

### Analysis of CFH c.3134-5T>C variant

RNA was extracted from peripheral blood using RNAeasy Mini kit (Qiagen, Manchester, UK). cDNA was synthesized with SuperScript III First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Paisley, UK) using random hexamers and the extracted RNA as a template. cDNA was used as a template in a polymerase chain reaction with specific primers targeting the potential splice-site mutation (*CFH* exon 20 [F-TATAA GGCGGGTGAGCAAGT] and *CFH* exon 23[AACT-GATTCACCTGTTCTCG]). Polymerase chain reaction products were separated on a 2% TBE agarose gel and sequenced using ABI Big Dye Terminator v3.1 on an ABI 3500 Genetic Analyzer (Life Technologies, Thermo Fisher Scientific).

### Western blotting

Detection of potential abnormal protein products in serum arising as a consequence of the *CFH* c.3134-5T>C variant was undertaken by Western blotting. Sera was diluted

**Table 2** Summary of complement analysis of aHUS patients with ADAMTS-13 activity > 10%

| Patient ID | Gene | Mutation identified | C3 (0.65–1.65 g $L^{-1}$) | C4 (0.16–0.54 g $L^{-1}$) | FH (0.35–0.59 g $L^{-1}$) | FI (38–58 mg $L^{-1}$) | Copies CFHR1/3 | CD46GGAAC haplotype copies | CFH H3 haplotype copies | Mutation reported previously |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CD46 | c.286+2T>C c.286+2T>G* | 0.65† | 0.14 | 0.5 | 39 | 2 | 1 | 0 | [7,20,28,45] |
| 2 | CD46 | c.175C>T; p.Arg59X | 0.56 | 0.04 | 0.5 | 49 | 1 | 2 | 0 | [27,28,46] |
| 3 | CD46 | c.470G>A; p.Cys157Tyr | 0.92† | 0.26 | 0.51 | 44 | 2 | 1 | 2 | |
| 4 | CD46 | c.470G>A; p.Cys157Tyr | 1.31 | 0.27 | 0.58 | 62 | 2 | 1 | 2 | |
| 5 | CD46 | c.191G>T; p.Cys64Phe | 1.17 | 0.17 | 0.78 | 84 | 2 | 1 | 0 | [29] |
| 6 | CFH | c.3134-5T>C | 0.87 | 0.23 | 0.62 | 53 | 2 | 0 | 2 | |
| 7 | CFH | c.3643C>T p.Arg1215X | 0.85 | 0.31 | 0.42 | 73 | 2 | 2 | 1 | [1] |
| 8 | CFB | c.1112A>G p.Asp371Gly | 0.91 | 0.3 | 0.67 | 77 | 1 | 1 | 0 | |
| 9 | C3 | c.3023C>T; p.Ser1008Leu | 1.15† | 0.34 | 0.62 | 60 | 2 | 1 | 1 | |
| 10 | Nil | – | 1.46 | 0.31 | 0.52 | 66 | 0 | 1 | 0 | |
| 11 | Nil | – | 0.56 | 0.03 | 0.55 | 52 | 2 | 0 | 2 | |
| 12 | Nil | – | 0.4 | 0.09 | – | – | 2 | 2 | 1 | |
| 13 | Nil | – | 0.94 | 0.24 | 0.62 | 50 | 1 | 2 | 0 | |
| 14 | Nil | – | 1.3 | 0.32 | 0.74 | 77 | 2 | 1 | 1 | |

*Compound heterozygote. †Complement levels measured on convalescent samples. The number of risk alleles for the CFH-H3 haplotype block that increases the risk of aHUS two- to four-fold [1,47] and the CD46 GGAAC haplotype block that has been associated with a two- to three-fold increased risk of aHUS [1,48] are shown. Complete deficiency of CFHR1 and CFHR3 has been strongly associated with factor H autoantibodies and aHUS [49–52]. Only patient 10 carried this deletion in homozygosity; however, no factor H autoantibodies were detected in this study.

1:1500, and 10 µL was electrophoresed on 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto nitrocellulose. A polyclonal antibody against FH (Calbiochem, Beeston, Nottingham, UK) was used with rabbit anti-goat horseradish peroxidase (Abcam, Cambridge, UK). Following washes in Tris-buffered saline with Tween 20, the blot was developed using Pierce ECL Western blotting substrate (Thermo Scientific).

## Results

### aHUS cases

Clinical features and laboratory parameters at presentation are detailed in Table 1. Median age at first episode was 25.5 years (9 months to 72 years), and 43% were male. The median presenting platelet count was $27 \times 10^9$ $L^{-1}$ (range 10–115), serum creatinine level was 295 µmol $L^{-1}$ (79–1812), and ADAMTS-13 activity was 80.8% (47–120%). Eleven (79%) of 14 patients had platelet counts < $30 \times 10^9$ $L^{-1}$ during the acute phase (nine of 14 at presentation; Fig. 1). Although renal involvement was more common and more severe in the group subsequently diagnosed to have aHUS, 5 (36%) these 14 patients had a serum creatinine level < 200 µmol $L^{-1}$ on transfer to our center; in two patients, serum creatinine was entirely within normal limits (Table 1, patients 3 and 4). Three (21%) of 14 maintained a creatinine
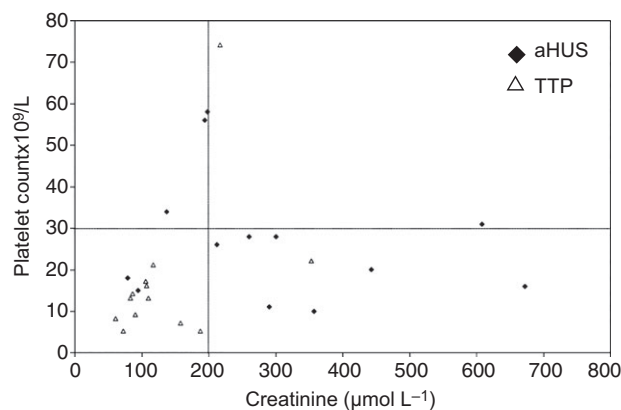


Fig. 1. Serum creatinine and platelet values at presentation in thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS) patients. Previously suggested cut-off levels for platelet count ($30 \times 10^9$ $L^{-1}$) and creatinine of 200 µmol $L^{-1}$ in aHUS [4] are highlighted. One aHUS patient, who presented with a platelet count of $115 \times 10^9$ $^1/L$ and creatinine of 1812 µmol $L^{-1}$, is censored. Two TTP patients presented with identical platelet counts and serum creatinine levels.

< 200 µmol $L^{-1}$ throughout the acute episode. Only one patient had a non-specific weakly positive ANA at a titer of 1:80 (patient 6). All aHUS patients had ADAMTS-13 activity levels > 30% (median 85%, range 47–120%) and no detectable ADAMTS-13 inhibitors.

Two patients (14%) had transient ischemic attacks: a 72-year-old man with a *C3* variant (patient 9) and a 23-year-old woman with a *CFB* variant and no preexisting cardiovascular risk factors (patient 8). Further neurological symptoms were reported in the aHUS group: five (36%) of 14 patients reported a combination of headache ($n = 4$), visual disturbance ($n = 1$) and confusion ($n = 1$). Only one aHUS patient required intubation and ventilation at presentation due to cardiorespiratory failure.

Atypical hemolytic uremic syndrome precipitants at presentation or relapse included pregnancy ($n = 2$), upper respiratory tract infection ($n = 6$), vaccination ($n = 2$), and acute abdominal pathology ($n = 3$), with a case of appendicitis, pancreatitis, and acute non-infective colitis. In three cases, there was no identifiable trigger (Table 1). Three patients had diarrhea at presentation; all were enterotoxin negative. One patient (patient 1) had a subsequent episode of enterotoxin-positive *Escherichia coli* diarrhea as a precipitant for relapse. The pregnancy-associated aHUS cases presented 3 days post cesarean section due to an abnormal cardiotocogram (patient 10) and 5 days after cesarean section at 31 weeks for presumed preeclampsia with nephrotic range proteinuria and a concurrent *Pseudomonas* bacteremia (patient 7). Subsequent identification of a pathogenic *CFH* mutation confirmed the diagnosis of aHUS in the latter case.

*Complement analysis*

C3 and C4 levels were both reduced in three (27%) of 11 patients tested during an acute presentation. In 64% (nine of 14 patients), rare genetic variants in the alternative complement pathway were identified: five *CD46* (one compound heterozygote, four heterozygotes [two *CFH*, one *CFB*, and one *C3*]. The variants identified and complement antigenic levels are detailed in Table 2.

Mutation screening in one individual (patient 1) showed a compound heterozygote *CD46* mutation (c.286+2T>C, c.286+2T>G), which resulted in complete CD46 deficiency. The c.286+2T>C variant was of maternal origin, while the c.286+2T>G was paternal. Flow cytometry analysis confirmed absent CD46 expressed on peripheral blood mononuclear cells. Both parents had 50% CD46 expression with normal serum creatinine. The CD46 variants Arg59X [27,28] and Cys64Phe [29] have been reported previously and result in impaired expression. CD46 expression data on the individuals carrying the Cys157Tyr genetic variant are not available; however, the loss of a structurally critical cysteine is highly likely to result in a non-secreted protein.

The *CFH* variant p.Arg1215X (patient 7) has been reported [1] and results in impaired protein secretion. Analysis of cDNA from the patient with the *CFH* c.3134-5T>C genetic variant (patient 6) did not detect any splice products that differed from the expected transcript sequence seen in normal control samples. The FH serum level was normal. A Western blot using a polyclonal antibody against FH did not detect any aberrant FH species (Fig. S1). Thus, from analysis undertaken to date, we cannot ascribe a functional consequence to this variant.

The *CFB* variant p.Asp371Gly (patient 8) has not previously been described and is not reported to be present in the general population from public mutation databases (NHLBI GO ESP [National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project]) [30]. Polymorphism phenotyping v2 (PolyPhen-2) [31] predicted that this variant is benign; however, previous *in silico* analysis of *CFB* mutants has demonstrated poor correlation with functional studies [32]. Structural analysis using an available crystal structure of complement C3b in complex with FB demonstrates that this variant resides in the von Willebrand factor domain of FB [32] in close proximity to four other aHUS variants where functional analysis has demonstrated increased activity (Fig. 2A).

The C3 variant p. Ser1008Lys (patient 9) has not previously been described and is not reported in the NHLBI GO ESP [30]. PolyPhen-2 predicted that this variant is probably damaging, although bioinformatic evaluation should be taken with caution. A co-crystal structure of CCPs1–4 of FH and C3b demonstrates that the variant resides in the thioester-containing domain adjacent to three other functionally significant aHUS-associated mutations Ala1094Val, Asp1115Asn, and Gln1161Lys [25] (Fig. 2B).

No patients with FH autoantibody were identified, although most were screened after TPE or while in clinical remission. At-risk *CFH-H3* or *CD46*GGAAC haplotypes were identified in all 14 patients tested (Table 2).

*Treatment and outcomes*

Thirteen of 14 aHUS patients were treated initially with TPE; one patient (patient 13) had resolving TMA at the time of transfer and did not require treatment. One patient (patient 14) developed multiorgan failure and died within 24 h of presentation after receiving corticosteroids and one TPE. A post mortem study was not performed. Three patients required renal replacement therapy (RRT) during episodes of TMA with either peritoneal or hemodialysis on a total of five occasions (two at relapse) for a duration of between 4 days and 18 months. Median time from presentation to recovery of platelet count $\geq 150 \times 10^9$ L$^{-1}$ was 9 days (range 4–29 days). Only one patient, with a *CFB* variant (patient 8), had thrombocytopenia refractory to TPE, defined by lack of platelet response to 1 week of TPE, and received eculizumab 13 days after presentation at a dosage of 900 mg weekly for 3 weeks, with complete response in both thrombocytopenia and renal function. Eculizumab was not available for any other patient in this study at presentation or relapse.

Median follow-up for the aHUS cohort was 2 years (range 1–28 years). Five of 13 patients had recurrent
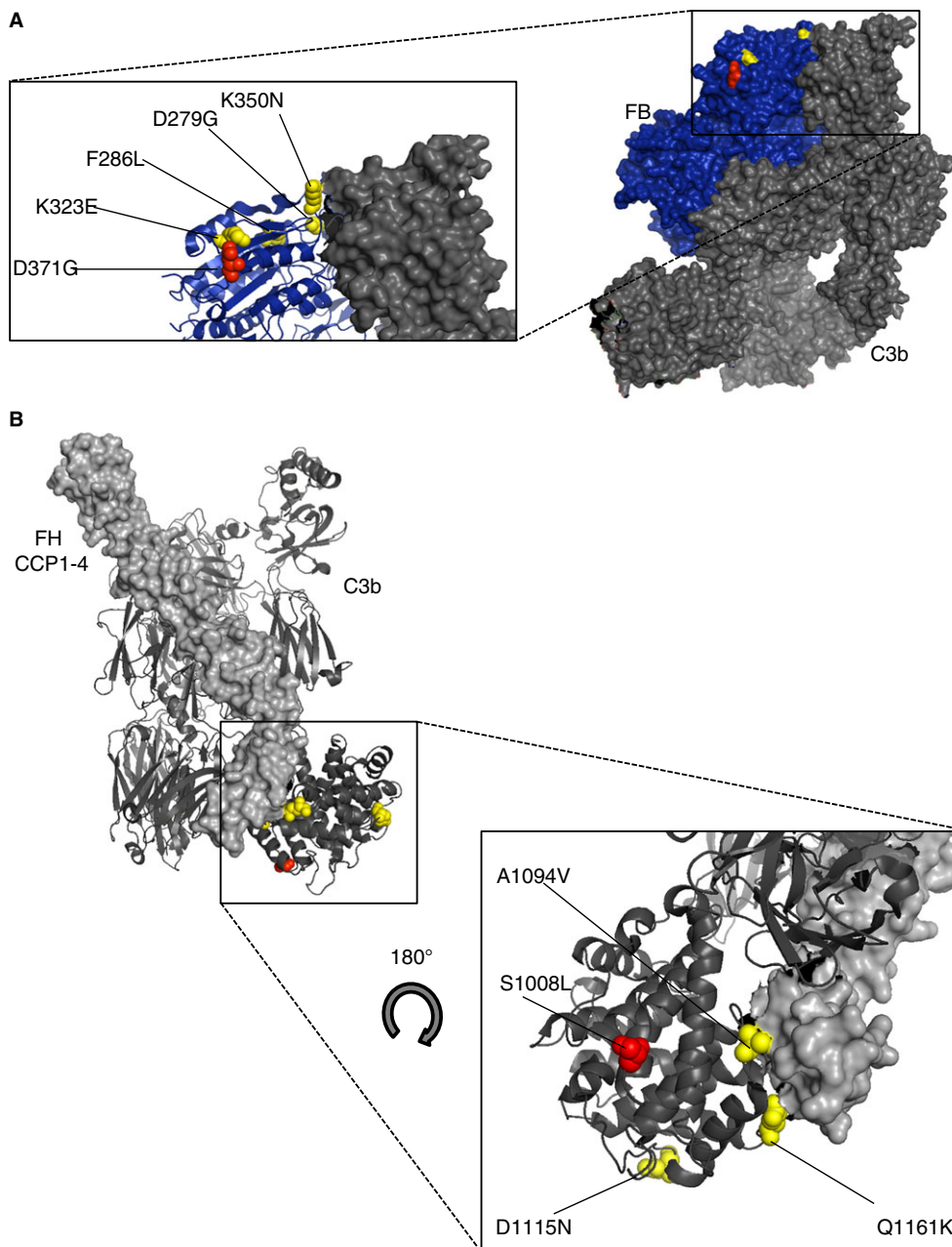
**Fig. 2.** Location of novel *CFB* and *C3* variants described in this study. (A) The *CFB* genetic variant Asp371Gly displayed (red sphere) on the C3b (dark gray): FB (blue) co-crystal structure (Protein Data Base ID code 2XWJ) [53]. Previously reported functionally significant atypical hemolytic uremic syndrome (aHUS)-associated *CFB* genetic variants Asp279Gly, Phe286Leu, Lys323Glu, and Lys350Asn (yellow spheres) are shown residing in the von Willebrand type A domain, inset [54]. (B) An X-ray–derived co-crystal structure of FH/C3b was also used to model the genetic variant in *C3*. The location of the Ser1008Leu C3 variant (red sphere) is shown within the co-crystal structure of an FH CCPs1–4 (light gray):C3b (dark gray) complex (Protein Data Base ID code 2WII) [55]. Previously reported functionally significant aHUS-associated *C3* genetic variants Ala1094Val, Asp1115Asn, and Gln1161Lys (yellow spheres) are shown inset [54].

episodes; all had confirmed complement pathway genetic abnormalities (three *CD46*, one *CFB*, one *C3*). Two of the relapses occurred within 1 year of the first episode (range, 1 month to 8 years after index presentation) and primarily triggered by viral/bacterial infection or vaccinations (Table 1). The precipitant was unclear in one case (patient 9), who relapsed just over 1 month after discharge with his initial presentation. The patient with a *CFB* mutation (patient 8) relapsed > 1 year after achieving remission, precipitated by both vaccination and viral infection, and required an additional two doses of eculizumab. Of interest, she had an uncomplicated pregnancy between presentations, for which she did not receive eculizumab.

At most recent clinical review, all aHUS patients had platelet counts above $150 \times 10^9$ $L^{-1}$, and 12 of 13 had normal serum creatinine levels; one patient (patient 6) had stage 3 chronic kidney disease with a serum creatinine level of 122 µmol $L^{-1}$. Two of 12 patients had persistent proteinuria (one with chronic renal impairment and one with disease recurrence), and four of 12 patients required treatment for persistent hypertension. None required long-term RRT or progressed to ESRF.

### Complement analysis in TTP patients

Fourteen TTP patients underwent complement mutation analysis. The median platelet count was $13 \times 10^9$ $L^{-1}$ (range 5–74), and serum creatinine level was 106.5 µmol $L^{-1}$ (61–353). Selection according to severe phenotypic presentation was based on a combination of clinical features. Four patients (29%) required intubation and ventilation at presentation, and two patients had a stroke. Four patients (29%) had marked renal impairment on presentation with creatinine > 150 µmol $L^{-1}$. Five patients (36%) had thrombocytopenia refractory to 7 days of TPE. Six (43%) had experienced recurrent relapses (median of three acute presentations, range 2–6). All had ADAMTS-13 activity below the lowest level of quantification (< 5%) with detectable ADAMTS-13 inhibitors. No rare genetic variants were identified in *C3, CFB, CFI, CFH*, or *CD46* in this cohort.

### Discussion

Many studies have shown that TMA patients without ADAMTS-13 deficiency have significantly higher median creatinine levels and platelet counts at presentation than those with confirmed acquired TTP [9,33,34]. Although our findings were consistent with this, neither of these laboratory parameters demonstrated adequate specificity for aHUS, in a cohort of patients of whom most did not present with oliguric/anuric renal failure. The majority (13 of 14) of our aHUS patients would be misdiagnosed based on clinical criteria for differentiation of TTP from other TMAs suggested in previous publications [4,33]. Coppo *et al.* reported that either a platelet count $\leq 30 \times 10^9$ $L^{-1}$, creatinine level of $\leq 200$ µmol $L^{-1}$, or positive ANA has a 85% positive predictive value and 93.3% negative predictive value for severe ADAMTS-13 deficiency in a large TTP/TMA cohort. Using only platelet count and serum creatinine, however, six of 54 patients (11%) with ADAMTS-13 activity > 10% were incorrectly predicted to have ADAMTS-13 deficiency based on these clinical variables alone. The intention of this study, which focused on diagnosis of TTP rather than aHUS, was not to define TMA classification according to clinical presentation, and the authors emphasize the need for pretreatment assessment of ADAMTS-13 activity [33]. Although the aHUS cohort presented here represents a selected group of patients, referred with suspected TTP, 13 of our 14 aHUS patients would be predicted to have severe ADAMTS-13 deficiency. It is evident that patients with both TTP and aHUS present with laboratory values outside expected limits; therefore, ADAMTS-13 testing is important for diagnosis and subsequent therapy.

Screening for complement abnormalities in TMA patients with renal impairment provides valuable diagnostic and prognostic information, although ~ 50% of patients have no detectable abnormalities [6]. Diagnosis in these cases remains reliant on clinical features. Published studies on aHUS in the acute TMA setting often define this group by parameters such as serum creatinine [35]. There are no established assays to measure global alternative complement pathway activity. In a search for potential biomarkers, C5a levels were recently shown to be significantly higher in aHUS than TTP, but there was significant overlap and no clear cut-off could be suggested as a diagnostic tool [35]. C3 or C4 levels are not reliable predictors, confirmed in our aHUS cohort.

In the absence of a rapidly available diagnostic test or biomarker for aHUS, the presence of ADAMTS-13 deficiency has an important differentiating role in acute TMAs. In the majority of TTP studies, patients with ADAMTS-13 activity > 10% had a significantly higher incidence and severity of renal impairment, a proportion of whom, in the absence of an identified secondary cause, may have had aHUS [36]. Many predated knowledge of alternative complement pathway abnormalities in aHUS, and none included routine screening for complement mutations in those without severe ADAMTS-13 deficiency. Cataland *et al.* reported that ADAMTS-13 levels < 10% were 100% sensitive and 100% specific for TTP in a cohort of 57 patients compared with 57 TMA patients with alternative diagnoses [10]. It has also been demonstrated that using an ADAMTS-13 activity cut-off of 10% can be safely used to guide TTP therapy [9].

Sixty-four percent of our patients diagnosed with aHUS had variants in complement genes, consistent with a prevalence of 44–51% in larger cohorts [5–7]. Given that complement analysis was performed largely on convalescent or post-TPE samples, the presence of FH autoantibodies may potentially have been missed. Analysis for thrombomodulin [5,37] and diacylglycerol kinase, epsilon (*DGKE*) [38] mutations was not undertaken. *DGKE* mutations are primarily detected in childhood, however, and all those presenting at a young age had mutations identified.

We identified a much higher frequency of isolated *CD46* variants (36% vs. 5–13%) and lower incidence of *CFH* variants (14% vs. 20–30%) than were reported in other studies [5–7,27]. Patients with isolated *CD46* mutations are known to have a milder clinical phenotype with reduced incidence of progression to ESRF (6–19%) [5,6,27]. The higher proportion of *CD46* variants may account for the better outcomes in our aHUS cohort with

reduced severity of renal impairment and favorable long-term outcomes, compared with a 60% incidence of ESRF or death at 3 years in the largest aHUS cohort [6]. CD46 is a membrane-bound protein where the role of TPE in treatment is questionable and the majority of patients with isolated *CD46* variants have complete resolution of disease with or without TPE [5,27].

We identified two *CD46*-mutated patients (patients 3 and 4), presenting with a relapsing/remitting TMA clinically indistinguishable from TTP, with normal renal function and ADAMTS-13 levels. Preceding the availability of ADAMTS-13 assays, both were diagnosed with congenital TTP, based on their young age of presentation and relapsing episodes. In similar cases, confirmation of normal ADAMTS-13 activity should trigger a thorough assessment for alternative causes of TMA, including complement mutation analysis. Future studies should redefine the normal ADAMTS-13 'TTP' group and incorporate molecular diagnostics to a greater extent.

Of particular interest is patient 1 with compound heterozygous deficiency of CD46. Currently, < 10 patients have been shown to have complete CD46 deficiency. It is reported that 50% of those patients have common variable immunodeficiency and recurrent chest infections. It has been suggested that impaired *CD46-Jagged1* crosstalk is responsible for the recurrent infections in subpopulations of these patients [39]. Our patient had recurrent upper respiratory tract infections and three episodes of reported chickenpox in childhood but has not experienced recurrent infections throughout adulthood.

All except one patient in this cohort received TPE as the primary treatment with positive clinical outcomes overall, before eculizumab was available nationally. With the confirmation of a pathogenic mutation, however, there is now a precedence to initiate eculizumab at relapse of an aHUS episode [4,12]. In our cohort, where only one individual received short intermittent courses of eculizumab, the median time to normalization of platelet count was 9 days and all except one patient (stage 3 chronic kidney disease) had complete recovery of renal function. A brief reintroduction of TPE or eculizumab (in one case) controlled relapsed disease in all patients. The duration of terminal complement blockade needs further clarification with reports suggesting eculizumab may be safely discontinued in selected patients with appropriate clinical monitoring [40,41], a finding mirrored by our own experience.

Activation of the classical and alternative complement pathways in TTP has been demonstrated in several studies [42–44], although the relative contribution of complement to disease pathogenesis has not been fully elucidated. The TTP cohort presented here represented an exploratory group selected due to a severe clinical phenotype but was not intended for direct comparison with aHUS patients based on clinical parameters. We did not identify any congenital or acquired aberrations of the alternative complement pathway in this select group, suggesting the presence of dual pathologies is exceptional and that routine screening for complement mutations in TTP patients with ADAMTS-13 levels < 10% and transient severe renal impairment is not necessary. It should be noted, however, that only four TTP patients in this study presented with creatinine levels > 150 μmol L$^{-1}$. Larger-scale studies would be required to further validate these findings.

## Conclusion

This aHUS cohort demonstrates the difficulty in clinically differentiating TTP from complement mediated TMAs. We highlight that diagnostic differentiation based on platelet count and renal function was insufficient to predict an underlying complement mutation. This distinction is increasingly important with the proven efficacy of complement inhibitor therapy in targeting complement activation in aHUS. In any patient with acute presentation of idiopathic TMA, identification of an ADAMTS-13 activity > 10% without detectable anti–ADAMTS-13 autoantibodies necessitates consideration of aHUS. Specifically, we demonstrate a higher frequency of functionally significant *CD46* mutations, closely mimicking relapsing/remitting TTP. We have illustrated that remissions can be associated with TPE in less severe phenotypic presentations, although with the availability of eculizumab, patient management is evolving. In a subgroup of patients with aHUS, continuous therapy is not required to maintain disease remission.

## Disclosure of Conflict of Interests

M. A. Scully, D. Kavanagh, T. H. J. Goodship, and E. K. S. Wong report personal fees and/or non-financial support from Alexion Pharmaceuticals, outside the submitted work. The other authors state that they have no conflict of interest.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** FH analysis from the patient with a *CFH* c.3134-5T>C genetic variant. Western blot using a polyclonal antibody against FH demonstrates the absence of aberrant FH species. The FH concentration from the patient with the *CFH* c.3134-5T>C is > 2× the control sample in an attempt to elucidate aberrant species.

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