Eculizumab in an anephric patient with atypical haemolytic uraemic syndrome and advanced vascular lesions

Zivile D. Békássy1, Ann-Charlotte Kristoffersson1, Mats Cronqvist2, Lubka T. Roumenina3,4,5, Tania Rybkine3,4,5, Laura Vergoz3,5, Christophe Hue3,4,5, Veronique Fremeaux-Bacchi3,6 and Diana Karpman1

1Department of Pediatrics, Clinical Sciences Lund, Lund University, Lund, Sweden, 2Department of Radiology, Skåne University Hospital, Lund, Sweden, 3INSERM UMRS 872, Cordeliers Research Center, Paris, France, 4Université Paris Descartes Sorbonne Paris-Cité, Paris, France, 5Université Pierre et Marie Curie (UPMC-Paris-6), Paris, France and 6Assistance Publique-Hopitaux de Paris, Hopital Européen Georges-Pompidou, Service d’Immunologie Biologique, Paris, France

Correspondence and offprint requests to: Diana Karpman; E-mail: diana.karpman@med.lu.se

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ABSTRACT

Background. Atypical haemolytic uraemic syndrome (aHUS) is associated with dysfunction of the alternative pathway of complement. Disease activity subsides as renal failure progresses but recurs upon renal transplantation, indicating that viable renal tissue contributes to disease activity. We present evidence of cerebrovascular occlusive disease indicating that vascular injury may occur in the absence of kidneys.

Methods. A currently 12-year-old girl developed renal failure at the age of 20 months. She underwent bilateral nephrectomy and renal transplantation but lost the transplant due to recurrences. She was on haemodialysis for 7 years. At 10 years of age she developed a transient ischaemic attack. Imaging, genetic investigation and mutation characterization were performed.

Results. Imaging demonstrated occlusion and stenosis of the carotid arteries. Two complement mutations, a novel mutation in factor B and a previously described mutation in factor I, and the H3-factor H haplotype, were identified. The factor B mutation, L433S, did not induce excessive complement activation in vitro. Measurement of C3 degradation products indicated ongoing complement activation. In spite of the patient being anephric, treatment was initiated with eculizumab, a humanized anti-C5 antibody that blocks terminal complement activation. She underwent a successful kidney transplant 9 months later and has not developed a recurrence or progression of vascular stenosis 1 year later.

Conclusions. The course of disease in this patient with aHUS suggests that complement-mediated vascular injury may occur in the total absence of renal tissue and overt recurrences. To our knowledge, this is the first description of eculizumab treatment in an anephric aHUS patient.

INTRODUCTION

Patients with atypical haemolytic uraemic syndrome (aHUS) develop symptomatic disease and recurrences (defined as acute episodes of non-immune haemolytic anaemia, thrombocytopenia and acute renal failure) as long as there is residual renal function and after renal transplantation, indicating that
viable renal tissue triggers disease activity. aHUS is, in most cases, associated with mutations or complex rearrangements in complement regulators and factors such as factor H (CFH), factor I (CFI), membrane-cofactor protein (MCP), C3, factor B (CFB) and thrombomodulin, as reviewed in Refs [1, 2]. Certain patients have circulating anti-factor H antibodies [3] often associated with deletions in factor H-related proteins (CFHRS) 3/1 [4]. Treatment with the inhibitor of the terminal pathway of complement eculizumab blocks disease progression and enables transplantation without recurrence in the majority of aHUS patients with terminal renal failure [5, 6]. It has previously been assumed that patients with aHUS will not have evidence of disease progression or haematological recurrence when kidneys are removed or severely dysfunctional resulting in terminal renal failure. One previous report showed, however, that vascular stenosis might occur even in the absence of viable renal tissue [7]. We will describe the investigation and treatment of a child with aHUS and several complement mutations with a clinical course indicating advanced vascular stenosis years after nephrectomy. This case demonstrates that patients may develop severe systemic vascular damage even in the absence of recurrences of disease.

**Materials and Methods**

**Patient**

A currently 12-year-old girl presented at 17 months of age with a transient episode of anaemia associated with infection. One month later she developed HUS and after a second recurrence within 3 months, in spite of weekly plasma exchange, developed end-stage renal failure treated with peritoneal dialysis and weekly plasma exchange. A renal biopsy showed glomerular endothelial cell swelling, arteriolar intimal thickening and mesangial proliferation. Immunofluorescence exhibited deposits of IgM, fibrinogen and C3 in glomerular capillaries.

She underwent deceased-donor renal transplant and nephrectomy of her native kidneys at the age of 3 years, before the advent of eculizumab treatment. She was treated with plasma infusions once weekly and plasma exchange also once weekly but, in spite of these treatments, developed three HUS recurrences related to septic episodes caused by *Staphylococcus aureus*. The HUS episodes were associated with malignant hypertension leading to generalized seizures with transient focal neurological symptoms including increased right-sided muscle tone. Computed tomography of the brain was normal at that time. The hypertensive crisis resulted in removal of the transplant at 4 years of age. She was thereafter treated with haemodialysis through an arteriovenous fistula 5 days a week for 7 years. Treatment with plasma exchange and infusions was terminated after transplant nephrectomy and no haematological recurrences of HUS (haemolytic anaemia and thrombocytopenia) occurred during this period.

At the age of 10 years she developed a transient ischaemic attack (TIA) with severe neurological symptoms including sudden onset of headache and vomiting followed by aphasia, salivation, ataxia, weakness in both arms and confusion. These neurological symptoms resolved within a few hours. Imaging of her carotid arteries demonstrated total occlusion of the right carotid artery and near-occlusion of the left carotid artery (see detailed description below). Echocardiography ruled out left ventricular hypertrophy. Ophthalmological examination was normal. Measurement of C3 and the C3 degradation product C3dg indicated that there was ongoing complement activation (Table 1). Eculizumab (Alexion, Cheshire, Conn) treatment was initiated in the absence of HUS manifestations and viable renal tissue. Doses were 600 mg per week for 3 weeks followed by 600 mg every other week. On this treatment, the patient underwent a successful deceased-donor kidney transplant 11 months after the TIA without recurrence of HUS 1 year later. The current eculizumab dose is 900 mg every other week adjusted for the patient’s weight. No progression of vascular occlusion was noted within 1 year after the TIA by repeated imaging.

The glomerular filtration rate was 79 mL/min/1.73 m² 1-year post-transplantation. She developed transient mild proteinuria 8 months post-transplantation with urinary albumin/creatinine ratio of 30 g/mol (reference value <3.8 g/mol), which normalized afterwards. Protocol renal biopsy was not performed. ADAMTS13 activity was measured twice and found to be normal, once at presentation, assayed by the collagen binding assay [8] and the second time by the FRETS-VWF73 assay [9] 1 year after the second transplantation. The homocysteine level, assayed twice, was 12 µmol/L (reference <10 µmol/L) while on haemodialysis, before the start of eculizumab treatment and 1 year after the second transplantation. This study was performed with the approval of the Ethics Committee of the Medical Faculty at Lund University and the informed consent of the patient’s parents.

**Mutation analysis**

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) [10] and exons of CFI [11], CFB [12], C3 [13], CFH [14] and MCP [15] were sequenced. The presence or deletion of the CFHR3/1 genes was analysed as described [16]. Sequencing of the thrombomodulin gene was not performed.

**CFH antibody analysis**

Analysis of anti-CFH antibodies was performed by ELISA according to a previously described method [3].

**In silico analysis of CFB and C3b**

The atomic coordinates of the C3 convertase C3bBb [17] were used to visualize aHUS-associated mutations using the PyMol software. In this study, amino acid residue numbering of CFB included the 25-residue leader peptide.

**Recombinant CFB constructs**

The CFB mutation L433S was introduced into a CFB-containing plasmid by site-directed mutagenesis, as previously described [18]. The construct was completely sequenced to assure the introduction of the mutation and the lack of additional genetic changes. The recombinant wild-type or mutant (L433S) and previously published aHUS gain-of-function mutation D279G [18] CFB proteins were transiently expressed in human embryonic kidney (HEK-293T) cells.
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<tbody>
<tr>
<td>C3 (0.77–1.38 g/L)</td>
<td>0.49</td>
<td>0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62</td>
<td>0.57</td>
<td>0.96&lt;sup&gt;a&lt;/sup&gt;, 0.59</td>
<td>0.98</td>
<td>1.00</td>
<td>0.64</td>
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<tr>
<td>C3dg (&lt;5 mg/L)</td>
<td>11.5</td>
<td>12.4</td>
<td>13.6</td>
<td>10.4</td>
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<td>Quantitative complement function via the alternative pathway&lt;sup&gt;b&lt;/sup&gt; (30–113%)</td>
<td>41</td>
<td>&lt;1</td>
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<td>Factor H (69–154%)</td>
<td>79</td>
<td>180</td>
<td>69</td>
<td>78, 102</td>
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<tr>
<td>Factor I (60–152%)</td>
<td>91</td>
<td>145</td>
<td>97</td>
<td>98, 118</td>
<td>88, 100, 98</td>
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<tr>
<td>Factor B (59–154% or 90–320 mg/L)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75%</td>
<td>64 mg/L</td>
<td></td>
<td>44, 60%</td>
<td>118%, 90%, 59%</td>
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HD, haemodialysis.

<sup>a</sup>These values were obtained within 6 weeks after removal of the transplant (on HD) or after transplantation.

<sup>b</sup>Assayed by ELISA (Wieslab, Euro Diagnostica, Malmö Sweden).

<sup>c</sup>Factor B analysed in Lund is given in % and in Paris given in mg/L.
on 19 February 2018

Mg\textsuperscript{2+}-containing Hepes buffer (10 mM Hepes pH 7.4, 50 mM NaCl, 10 mM MgCl\textsubscript{2}). A blank activated/deactivated flowcell served as a control with and without injected CFB. Data were analysed using the ProteOn Manager software and the data from the blank flowcell were subtracted.

**Endothelial cell assay**

Primary human umbilical cord veins cells (HUVECs) in the third passage were activated overnight with tumour necrosis factor-alpha (TNF\textalpha, 10 ng/mL) and interferon-gamma (IFN\gamma, 10 U/mL, both from Peprotech, Rockyhill, NJ, USA), as described [18, 20]. After washing with PBS, adherent cells were incubated with 50 µL CFB-depleted serum (CompTech), and with 100 µL recombinant wild-type or mutant CFB supernatants, containing an equal amount (10 µg/mL) of CFB. Alternatively, the cells were incubated with sera of normal anonymous blood donors (n = 55, obtained with INSERM IRB authorization for research purposes) or sera from the patient’s mother or father. Sera were diluted to 33% in M199 medium (Gibco) as described [18, 20]. After a 30-min incubation at 37\textdegree C and washing, the cells were detached, labelled with monoclonal anti-C3c (Quidel, San Diego, CA, USA) or control mouse IgG1, followed by phycoerythrin (PE)-labelled secondary antibody (Beckman Coulter, Roissy, France). Cells were analysed by flow cytometry using Becton Dickinson FacsCalibur (Mountain View, CA, USA), and CellQuest and FCS express software (for acquisition and analysis, respectively).

**RESULTS**

**Imaging of the vascular lesions**

At the time of the TIA magnetic resonance imaging and angiography (MRI/MRA) as well as digital subtraction angiography (DSA) were carried out. MRI demonstrated an older frontal infarct in the left hemisphere as well as small frontal nodular and subcortical vascular lesions bilaterally (Figure 1A). More recent ischaemic lesions were not present. MRA of the cervical and intracranial arteries revealed total occlusion of the right carotid artery (the artery is therefore not visible in Figure 1B) and near-occlusion of the left carotid artery (Figure 1B and C). This was confirmed by DSA (Figure 1D–F). Collateral flow to the right hemisphere from the right posterior communicating artery (Figure 1C and F) and retrograde supply through an organized pial network (Figure 1F) were noted. The following MRI and MRAs 2 and 16 months after the TIA (the latter 6 months after the renal transplantation) showed no change (Figure 1G–I).

**Complement analysis**

Complement analyses carried out on samples from the patient and her parents are summarized in Table 1. C3 was low and C3dg was elevated at debut suggesting complement consumption. Low C3 and elevated C3dg were also detected after removal of all renal tissue, before and during eculizumab treatment while the patient was on haemodialysis. After the second transplantation, C3 levels increased initially to normal range but later fell to below the normal range with subsequent C3dg elevation. Quantitative complement function via the alternative pathway was totally blocked during eculizumab treatment, as expected. The CFI concentration was normal during the entire follow-up. Increased levels of C3dg indicated adequate degradation of C3 by functionally active CFI. The CFB concentration was normal at debut but was decreased or in the lower normal range during haemodialysis and after the second transplantation. CFH and C4 levels were normal at all times.

**Genetic investigation of the patient**

Two complement mutations, in the genes encoding CFI and CFB, as well as the CFH H3 disease-associated haplotype, were found (Table 2). The heterozygous G261D mutation in the CFI gene was found before removal of the first graft but no functional defect of CFI was demonstrated, as previously reported [21, 22]. A novel heterozygous mutation in CFB, L433S, was found, as characterized below. The CFB mutation was not found in the normal population (www.1000genomes.org). The finding of normal CFB levels in certain samples indicated that the mutated protein was normally secreted. In addition to the mutation, a rare polymorphism was found in exon 13 (E566A), and at amino acid position 7 (exon 2) the patient was homozygous for the R7 polymorphism, which was previously shown to exhibit better binding capacity to C3 than other allele variants [23]. No mutations in the genes encoding CFH, C3 or MCP and no deletion of the CFHR3/1 genes were detected. Serum antibodies to CFB were not detected.

**Characterization of the CFB mutation**

A novel heterozygous mutation was found in the CFB gene 4 years after the CFI mutation was detected and before the second transplantation. This mutation is located in the von Willebrand factor-like A domain, near the Mg\textsuperscript{2+} metal-ion adhesion site (MIDAS) and in proximity to, but not directly in...
the area that binds C3b to form the C3bBb convertase (Figure 2A). It is close to three other previously published aHUS CFB mutations [12, 18].

To characterize the secretion and function of the CFB mutation, recombinant wild-type CFB, L433S and D279G mutations (the latter is a gain-of-function aHUS mutation in CFB used as a positive control [18, 19]) were expressed in HEK-293T cells. The level of CFB secreted in the supernatant was similar between the wild-type and the two mutants tested. No CFB was detected in the supernatant of the mock-transfected cells (SN0) (Figure 2B).

The interaction of C3b with the wild-type or mutant CFB variants was tested by ELISA and surface plasmon resonance as previously described [18, 20]. Increased binding was observed for the positive control, D279G, but binding of the L433S mutant was similar to, or even weaker than, the wild-type CFB (Figure 2C and D).

The capacity of the different CFB variants to induce C3 deposition was tested using TNFα/IFNγ-activated HUVECs. D279G, the gain-of-function CFB mutant, showed increased C3 deposition compared with the wild type but no increase was detected for L433S (Figure 2E).

To further address the role of the mutations in CFB and CFI for complement activation, sera from the patient’s parents were applied to TNFα/IFNγ-activated HUVECs and tested for C3 deposition as described for other complement mutations [18, 20]. The serum from the patient was obtained when she was on dialysis and had low C3 and CFB levels, and could
as in this and one other case [7], is absent. This low-grade complement activation would be expected to progressively damage the endothelium consequently leading to vascular stenosis. Thus, even patients with reduced, or lack of, renal function could benefit from complement inhibition.

Eculizumab inhibits C5 and thereby the terminal complement pathway. Complement activity proximal to C5 remains functional. This may explain why the patient exhibited low levels of C3 even after treatment with eculizumab was initiated. The CFI mutation was not shown to promote complement activation via the alternative pathway [21, 22]. Surprisingly, the novel CFB mutation described here did not exhibit enhanced C3b binding and complement activation on endothelial cells, despite its close proximity to other previously described gain-of-function CFB mutations [12, 18]. This is not the only case of complement mutations with no associated functional change. Recently, other aHUS-associated genetic changes, CFH variants I890 and L1007, were found to lack functional significance, despite strong association with aHUS [25]. Nevertheless, the finding that the patient had complement consumption and that both parents’ sera induced excessive C3 deposition on endothelial cells indicates a clear role for complement in the disease process of this patient. When the mutations in CFB, CFI as well as the CFH risk-associated haplotype, and possibly as yet unidentified factors, are combined in vivo the effect may lead to an over-activation of the alternative pathway that cannot be accounted for by each mutation alone. The beneficial effect of eculizumab in preventing disease recurrence after the second transplant is the ultimate evidence for the role of complement activation in this patient.

In addition to vascular damage induced by complement activation, other factors may also contribute to the development of vascular stenosis in our patient, such as a prolonged period of dialysis and elevated homocysteine levels. Children on haemodialysis are at higher risk for developing cardiovascular disease due to uremia-related risk factors, dysregulated calcium/phosphate and parathyroid hormone, dyslipidemia, hypertension and chronic inflammation associated with protein-energy malnutrition [26]. Furthermore, paediatric patients with chronic kidney disease were shown to have increased carotid intima-media thickness [27]. High levels of homocysteine are frequently detected in children with chronic renal failure [26] and correlate with arterial stiffness [28]. Accumulation of homocysteine in patients with inborn cobalamin defects may trigger HUS [29]. Although the patient described in this study did not have particularly high homocysteine levels, the mild elevation, together with other risk factors, could enhance a prothrombotic propensity.

Stenosis and occlusion of large arteries has not been reported as a complication in paediatric dialysis patients suggesting that renal replacement therapy and chronic kidney failure per se could not account for the findings. Complement can be activated during haemodialysis. Several reports have demonstrated generation of activation products C3a and C5a after exposure of plasma or whole blood to haemodialysis filters, both in vitro and vivo (reviewed in Ref. [30]). C3 is adsorbed to the biomaterial surface upon contact with blood and

### Table 2. Genetic work-up of the patient

<table>
<thead>
<tr>
<th>Complement factor</th>
<th>Genetic analysis</th>
<th>Inheritance</th>
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<tbody>
<tr>
<td>CFI</td>
<td>G261D (Gly261Asp), c.782G&gt;A, exon 6 mutation [21]</td>
<td>Paternal</td>
</tr>
<tr>
<td>CFB</td>
<td>L433S (Leu433Ser), c. 1298T&gt;C, exon 10 mutation</td>
<td>Paternal</td>
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<tr>
<td></td>
<td>E566A (Glu433Ala), exon 13, rare polymorphism</td>
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<tr>
<td></td>
<td>R7 Arg exon 2, polymorphism</td>
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<tr>
<td>CFH</td>
<td>H3 haplotype (tgtgt) [20]</td>
<td>Maternal</td>
</tr>
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therefore not be reliably used in this assay. The unaffected parents carried the same CFI and CFB mutations (father) and CFH haplotype (mother) but had normal complement levels at the time of sampling, thus their samples were used. Results from this assay are presented in Figure 2F and show that serum from both the father and the mother exhibited increased C3 deposition on the cytokine-activated endothelial cells compared with sera from 55 normal donors.

### DISCUSSION

We describe a patient with aHUS and mutations in CFB and CFI as well as a risk-associated CFH haplotype in whom we found evidence for complement activation after all renal tissue was removed and no overt haematological recurrences of aHUS occurred. We suggest that the advanced occlusion and stenosis in the patient’s carotid arteries was due to ongoing complement-induced vascular injury. This provided the rationale for treatment with eculizumab in the absence of renal function. Treatment was efficient in blocking complement activation via the alternative pathway, although other end points indicating the prevention of aHUS activity, such as maintaining a normal platelet count and preventing haemolysis, were not deranged even before the initiation of treatment. No further progression of vascular injury occurred during an 18-month period from when the treatment with eculizumab commenced and the patient was successfully transplanted during this time.

Haematological recurrences of aHUS cease to occur when renal function is very low. This clinical observation has led to the notion that viable renal tissue is required for overt relapses to occur. The kidney is the major organ affected during aHUS, although the central nervous system may be involved [24]. It is, as yet, unclear how the disease is activated in the kidney. However, limited evidence suggests that low-grade disease activity can proceed even when renal function diminishes or,
can thus trigger the alternative pathway [31, 32]. Dialysis-related complement activation could possibly contribute to advanced vascular injury in the setting of aHUS and uninhibited complement activation due to mutations. Stenosis of large arteries has been described in aHUS patients on long-term dialysis. One child with a CFH mutation (Ser1191Leu) who was on long-term dialysis after loss of two renal grafts developed stenosis of the middle and anterior cerebral arteries [33, 34]. Her younger monozygous twin sisters, bore the same CFH mutation and also developed aHUS. One was dialysed for 2 years after which she underwent renal transplantation and both were treated with continuous prophylactic plasma...
exchange for 7 and 9 years, respectively, and exhibited normal cerebral vasculature. Another child with aHUS and a CFB mutation (Lys350Asp) developed progressive stenosis of multiple large arteries including thoracic and abdominal aorta branches as well as pulmonary and coronary arteries [7] after several years of dialysis. These cases together with our case suggest that both large and small vessel wall injury is inherent to the course of disease and that progression of extra-renal vascular lesions occurs even in the absence of overt recurrences, a process that may be exacerbated by long-term dialysis.

Increasing evidence indicates that activation of the alternative pathway of complement may be proatherogenic within the vessel wall (reviewed in Ref. [35]). Early atherosclerotic changes have been demonstrated in internal iliac artery samples obtained during kidney transplantation of paediatric haemodialysis patients [36]. In a patient with multiple complement mutations, such as described here, complement activation in atherosclerotic lesions could be enhanced. Our patient exhibited continuous complement activation as indicated by increased complement degradation products in the circulation. This suggests a pathogenetic mechanism whereby low-grade constant complement activation will lead to vascular occlusion. We therefore propose that extra-renal vascular lesions may progress even during symptom-free intervals and that imaging for vascular changes, particularly cerebral, should be monitored. For this reason transplantation should be considered as early as possible in aHUS patients with end-stage renal failure. Eculizumab protection, during periods of dialysis and after renal transplantation, might prevent serious vascular damage in patients with aHUS.

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CONFLICT OF INTEREST STATEMENT

V.F.B. is a member of the Alexion Pharmaceuticals (Cheshire, Conn) national advisory board in France. D.K. was the national coordinator of the eculizumab multi-centre trial in Sweden during 2009–2010. The other authors have no disclosures and no competing financial interests. The results presented in this paper have not been published previously in whole or in part. This study was presented in poster form at the Fourth International Conference ‘HUS-MPGN-TTP & related disorders’ Innsbruck, Austria, 9–11 June 2013.

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