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Eculizumab in an anephric patient with atypical hemolytic uremic syndrome and advanced vascular lesions

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Abstract

Background

Atypical hemolytic uremic 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, underwent bilateral nephrectomy and renal transplantation but lost the transplant due to recurrences. She was on hemodialysis for 7 years. At 10 years of age she developed a transient ischemic 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’s being anephric treatment was initiated with Eculizumab, humanized anti-C5 antibody that blocks terminal complement activation. She underwent a successful kidney transplant nine months later and has not developed a recurrence or progression of vascular stenosis one 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.
Keywords: complement, eculizumab, factor B, factor I, hemolytic uremic syndrome, transient ischemic attack

Short summary
This study describes the clinical course of a child with recurrent episodes of atypical HUS and complement mutations in factor B and factor I. Her native kidneys and kidney transplant were removed and she was on dialysis for 7 years at which point she developed a transient ischemic attack. Imaging demonstrated advanced carotid artery lesions bilaterally. Evidence of ongoing complement activation led to the initiation of eculizumab (anti-C5) treatment while the patient was anephric, and on this treatment the patient was successfully transplanted and did not exhibit progression of arterial lesions.
Introduction

Patients with atypical hemolytic uremic syndrome (aHUS) develop symptomatic disease and recurrences (defined as acute episodes of non-immune hemolytic anemia, 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 [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 hematological 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.
Subject and Methods

Patient

A currently 12 year-old girl presented at 17 months of age with a transient episode of anemia 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 hemodialysis through an arteriovenous fistula five days a week for 7 years. Treatment with plasma exchange and infusions was terminated after transplant nephrectomy and no hematological recurrences of HUS (hemolytic anemia and thrombocytopenia) occurred during this period.
At the age of 10 years she developed a transient ischemic 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 three weeks followed by 600 mg every other week. On this treatment the patient underwent a successful deceased-donor kidney transplant eleven months after the TIA without recurrence of HUS one 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 one year after the TIA by repeated imaging.

The glomerular filtration rate was 79 ml/min/1.73 m² one year post-transplant. She developed transient mild proteinuria eight months post-transplant 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] one year after the second transplantation. The homocysteine level, assayed twice, was 12 µmol/L (reference <10 µmol/L) while on hemodialysis, before the start of eculizumab treatment, and one 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 analyzed 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 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 (ATCC). The cells were
cultured for 3 days in DMEM+glutaMAX™-I with 4.5g/l D-glucose and pyruvate (Gibco, Paisley, UK) without fetal calf serum. The integrity of the proteins was verified by immunoblotting and quantified by ELISA, as previously described [19].

**CFB binding to C3b**

*Enzyme-linked immunosorbent assay (ELISA)*

CFB binding to C3b was measured by ELISA as described previously [18]. Plates were coated with C3b (CompTech, Tylor, TX) and various concentrations of recombinant wild-type or mutant CFB were added in the fluid phase. The binding of the mutant protein was expressed as percent of the wild-type CFB binding in the plateau.

*Surface plasmon resonance*

The interaction of wild-type and mutant CFB with C3b was also analyzed in real-time using surface plasmon resonance technology on ProteOn XPR36 equipment (BioRad, Hercules, CA). C3b was coupled to the GLC biosensor chip, using standard amide-coupling technology, according to the manufacturer’s instructions. Recombinant CFB were injected as an analyte at concentrations 40.625, 81.25, 162.5, 325, 650 pM at a flow of 50 μl/min in Mg²⁺- containing Hepes buffer (10mM Hepes pH 7.4, 50mM NaCl, 10mM MgCl₂). A blank activated/deactivated flowcell served as a control with and without injected CFB. Data were analyzed using ProteOn Manager software and the data from the blank flowcell were subtracted.
**Endothelial cell assay**

Primary human umbilical cord veins cells (HUVEC) in the third passage were activated overnight with tumor necrosis factor-alpha (TNFα, 10 ng/ml) and interferon-gamma (IFNγ, $10^3$ U/ml, both from Peprotech, Rockyhill NJ), 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-minute incubation at 37°C and washing, the cells were detached labeled with monoclonal anti-C3c (Quidel, San Diego, CA) or control mouse IgG1, followed by phycoerythrin (PE)-labeled secondary antibody (Beckman Coulter, Roissy, France). Cells were analyzed by flow cytometry using Becton Dickinson Facs-calibur (Mountain View, CA), 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 ischemic 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 1C). This was confirmed by DSA (Figure 1D-F). Collateral flow to the right hemisphere from the right posterior communicating artery (Figure 1C and 1F) and retrograde supply through an organized pial network (Figure 1F) were noted. The following MRI and MRAs two and 16 months after the TIA (the latter six 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 hemodialysis. After the 2nd 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 hemodialysis and after the 2nd 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 CFH were not detected.

**Characterization of the CFB mutation**

A novel heterozygous mutation was found in the CFB gene four 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$^{2+}$ 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 HEK293T 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,D).

The capacity of the different CFB variants to induce C3 deposition was tested using TNFα/IFNγ-activated HUVEC. D279G, the gain-of-function CFB mutant, showed increased C3 deposition compared to 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 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 to 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 hematological 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 endpoints indicating the prevention of aHUS activity, such as maintaining a normal platelet count and preventing hemolysis, were not deranged even before the initiation of treatment. No further progression of vascular injury occurred during an 18-month period from when treatment with eculizumab commenced and the patient was successfully transplanted during this time.

Hematological 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, 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 hemodialysis 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, pediatric 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 to 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 pediatric dialysis patients suggesting that renal replacement therapy and chronic kidney failure per se could not account for the findings. Complement can be activated during hemodialysis. Several reports have demonstrated generation of activation products C3a and C5a after exposure of plasma or whole blood to hemodialysis filters, both in vitro and vivo (reviewed in [30]). C3 is adsorbed to the biomaterial surface upon contact with blood and 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 dialyzed for two 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 [35]). Early atherosclerotic changes have been demonstrated in internal iliac artery samples obtained during kidney transplantation of pediatric hemodialysis 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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Transparency declaration

VFB is a member of the Alexion Pharmaceuticals (Cheshire, Conn) national advisory board in France. DK was the national coordinator of the Eculizumab multi-center trial in Sweden during 2009-2010. The other authors have no disclosures and no competing financial interests.
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<td>Factor I (60-152 %)</td>
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<td></td>
<td>98</td>
<td>88</td>
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<td>44, 60 %</td>
<td>118 %</td>
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<td>59 %</td>
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HD: hemodialysis. <sup>a</sup>, Assayed by ELISA (Wieslab, Euro Diagnostica, Malmö Sweden). <sup>b</sup>, Factor B analyzed in Lund is given in %, and in Paris given in mg/L. <sup>c</sup>, These values were obtained within 6 weeks after removal of the transplant (on HD) or after transplantation.
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Figure 1. Imaging of the brain, cerebral and carotid vasculature.

A-C: Magnetic resonance imaging and angiography MR/MRA at the time of the transient ischemic attack (TIA). A) MR, T2-Flair imaging showing an old infarct in the frontal corona radiate area of the left hemisphere (arrow) and two small subcortical vascular lesions (arrowheads). MRA frontal (B) and lateral (C) projection illustrates a tight stenosis of the left internal carotid artery ICA (thick arrow). The large right posterior communicating artery (PComA) supplying the right hemisphere (arrow) is visible. The right internal carotid artery (ICA) is occluded and therefore not visible. D-F: Digital subtraction angiography DSA at time of TIA presentation. D) Injection via the right common carotid artery (CCA) exhibits good filling of the external carotid artery and occlusion of ICA at the level of the dural entrance (arrow). E) Injection via the left CCA exhibited moderate stenosis of the left ICA at the dural level (arrow). No filling of the anterior cerebral arteries was noted. F) Injection via the left vertebral artery demonstrated good collateral filling to the right anterior circulation from the right PComA (arrow). A pial vascular network was established giving retrograde supply to parieto-occipital and deep vessels (arrowheads). G-I: MR and MRA carried out 16 months after TIA presentation and 6 months after renal transplantation. G) T2- Flair imaging did not exhibit new lesions. The previously noted infarct (arrow) and small vascular lesions (arrowhead) were unchanged. In this image only the right-sided lesion is demonstrated. H-I, MRA of the right ICA that was still occluded (not visible) demonstrating the stenosis of the left ICA (thick arrow) with no progression. The arrow points to the agenesis of the left ACA (this is a normal variant), and to both pericallosal arteries (arrowhead) that are supplied from the right side.
Figure 2. Characterization of the factor B mutation.

A) The position of the aHUS CFB mutations in proximity to the MIDAS and C3b binding region. C3b is presented in surface, CFB in cartoon. The residues affected by the aHUS-associated mutation presented here, L433S, as well as the previously published CFB mutations in this region D279G, F286L and K350N [12, 18] (are indicated by dark spheres).

B) The secretion level of the L433S mutant compared to the wild-type (WT) CFB, SN0 (supernatant from mock-transfected cells as the negative control) and D279G (positive control), as analysed by ELISA. Results are presented as % of the wild-type in mean ± SD of three independent experiments.

C) C3b binding tested by ELISA. The interaction of wild-type or mutant CFB to plate-coated C3b was measured by ELISA. The results are presented as % of the binding of the wild-type in the plateau of the dose response curve, mean ± SD from 5 different experiments.

D) C3b binding tested by SPR. The interaction of biosensor chip-immobilized C3b with CFB as an analyte was studied in real time. One representative sensorogram out of 3 independent experiments is presented.

E) C3 deposition on cytokine-activated HUVECs in CFB-depleted serum reconstituted with recombinant wild-type CFB, L433S or D279G mutants evaluated by flow cytometry. The results are presented as fold difference of the relative fluorescence intensity (RFI) of the C3 deposition in the presence of a mutant CFB, compared to the wild-type (mean ± SD from 3 experiments).

F) Cytokine-activated HUVECs were incubated with sera from 55 normal donors, CFH-depleted serum (as a positive control), and serum from the patient’s parents, and tested for C3 deposition by flow cytometry. The results are expressed as RFI. The normal range was determined as the average of the C3 deposition from the normal donors ± 2SD, depicted as the horizontal dashed bar. Patient serum had low C3 and CFB levels and could not be used in this test, as it requires normal C3 and CFB levels.
Figure 2