CLINICAL TRIALS AND OBSERVATIONS

TITLE: Eculizumab reduces complement activation, inflammation, endothelial damage, thrombosis, and renal injury markers in aHUS

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Running Title: Biomarkers of aHUS

Word Counts: Text – 3914; Abstract – 200; Figures/Tables – 5/2; References – 86

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Statement of Prior Presentation

Aspects of these data were presented at the following congresses:


Key points

- This exploratory study describes the effect of eculizumab on multiple physiologic pathways impacted by complement dysregulation in aHUS.
- The results highlight the importance of sustained terminal complement blockade even in patients with improved clinical laboratory values.
Abstract

Atypical hemolytic uremic syndrome (aHUS) is a genetic, life-threatening disease characterized by uncontrolled complement activation, systemic thrombotic microangiopathy (TMA), and vital organ damage. We evaluated the impact of terminal complement blockade with the anti-C5 monoclonal antibody eculizumab on biomarkers of cellular processes involved in TMA in patients with aHUS longitudinally, during up to 1 year of treatment, compared with healthy volunteers (HV). Biomarker levels were elevated at baseline in most patients, regardless of mutational status, plasma exchange/infusion use, platelet count, or lactate dehydrogenase or haptoglobin levels. Eculizumab reduced terminal complement activation (C5a and sC5b-9) and renal injury markers (clusterin, cystatin-C, β2-microglobulin, and liver fatty acid binding protein-1) to HV levels, and reduced inflammation (soluble tumor necrosis factor receptor-1), coagulation (prothrombin fragment F1+2 and D-dimer), and endothelial damage (thrombomodulin) markers to near normal levels. Alternative pathway activation (Ba) and endothelial activation markers (soluble vascular cell adhesion molecule-1) decreased but remained elevated, reflecting ongoing complement activation in aHUS despite complete terminal complement blockade. These results highlight links between terminal complement activation and inflammation, endothelial damage, thrombosis, and renal injury and underscore ongoing risk of systemic TMA and progression to organ damage. Further research regarding underlying complement dysregulation is warranted.

[www.ClinicalTrials.gov Identifier: NCT01194973]

Keywords: atypical hemolytic uremic syndrome, eculizumab, biomarkers, complement
Introduction

Atypical hemolytic uremic syndrome (aHUS) is a genetic, life-threatening disease of chronic, uncontrolled complement activation that leads to platelet, leukocyte, and endothelial cell activation, systemic thrombotic microangiopathy (TMA), and subsequent end organ damage or failure.\textsuperscript{1,2} aHUS is almost always caused by inherited or acquired defects in the activation of complement via the alternative pathway (AP),\textsuperscript{1} although rare mutations in \textit{DGKE} and plasminogen have also been described.\textsuperscript{3,4} Historically, prognosis of patients with aHUS has been poor—up to 79% of patients die, require dialysis, or have permanent renal damage within 3 years of diagnosis.\textsuperscript{5}

The role of the AP in the pathophysiology of aHUS is well described.\textsuperscript{1,6} The AP, an integral part of the innate immune response, is continuously active at low levels but amplified under conditions of infection or endothelial stress or damage.\textsuperscript{1} Under normal conditions, complement activation is tightly controlled by regulatory proteins, either soluble (e.g., complement factors H or I) or membrane-bound (e.g., decay accelerating factor [DAF; CD55], protectin [CD59], membrane cofactor protein [MCP; CD46], thrombomodulin [TM; CD141], complement receptor 1 [CR1; CD35]).\textsuperscript{7} In aHUS, complement-associated gene mutations cause permanent loss of complement regulatory control. Gain-of-function mutations of genes encoding complement components and inhibitory auto-antibodies directed at complement regulatory proteins can also lead to chronic AP overactivation.\textsuperscript{1}
Devastating clinical effects observed in patients with aHUS likely result from the impact of uncontrolled complement activity on multiple physiologic pathways. C5a is postulated as a key link between inflammatory and thrombotic pathways and has potent proinflammatory effects. Additionally, sublytic C5b-9 levels are associated with platelet, leukocyte, and endothelial activation and damage, all of which can further increase AP activation via a positive feedback loop.

The kidney is particularly vulnerable to complement-mediated inflammatory injury occurring from deposition of circulating active complement fragments in the glomeruli and local (renal) complement production and activation. Increased C5b-9 deposits have been observed on glomerular endothelium and circulating platelets, both affected in aHUS. The proximal tubule also has been identified as a focus of necrosis in aHUS-related TMA. The renal microvasculature is a common site for TMA, but aHUS may also lead to extra-renal manifestations in the central nervous, pulmonary, gastrointestinal, or cardiovascular systems in ≤50% of patients.

Chronically activated complement has devastating clinical effects and effective management of patients with aHUS through terminal complement blockade prevents TMA and end-organ damage. Eculizumab (Soliris®, Alexion Pharmaceuticals, Inc., Cheshire, CT, USA) is a humanized monoclonal antibody that blocks the cleavage of terminal complement protein C5 into the inflammatory C5a protein and C5b, a precursor of the lytic C5b-9 complex, and is the only approved treatment for aHUS. The efficacy and safety of eculizumab in aHUS was demonstrated in 2 prospective clinical
trials.\textsuperscript{21} Subsequently, an open-label, single-arm, multicenter, multinational, clinical trial (www.ClinicalTrials.gov Identifier: NCT01194973) was conducted in adult patients with aHUS (C10-004).\textsuperscript{24} Inhibition of complement-mediated TMA with eculizumab led to rapid hematological improvements, significant improvements in renal outcomes, and discontinuation of plasma exchange/plasma infusion (PE/PI) and dialysis in most patients.\textsuperscript{24,25}

The effect of terminal complement blockade with eculizumab on upstream AP activation or other markers of disease activity is unclear. We conducted a pre-specified exploratory analysis as part of the C10-004 trial to investigate the impact of terminal complement blockade on biological markers associated with proximal and terminal complement activation,\textsuperscript{22,23,26} inflammation,\textsuperscript{27,28} endothelial cell activation and damage,\textsuperscript{28-35} coagulation,\textsuperscript{5,33,36,37} and renal injury\textsuperscript{38-40} in patients with aHUS.
Materials and methods

Clinical trial

This was a 26-week, open-label, nonrandomized, single-arm, multicenter, trial of eculizumab in patients with aHUS (NCT01194973); patients could continue to receive eculizumab in an extension phase. Adult patients (≥18 years of age) with a diagnosis of aHUS were enrolled at 23 centers in North America and Europe. Eligible patients had platelet counts <150 x 10^9/L, hemoglobin ≤ lower limit of the normal range (LLN), lactate dehydrogenase (LDH) ≥ 1.5 x upper limit of normal (ULN), and serum creatinine levels ≥ ULN at screening, ADAMTS13 activity ≥ 5%, and no positive Shiga toxin-producing Escherichia coli test. An identified complement gene mutation, polymorphism, or factor H autoantibody was not required. Patients were vaccinated against Neisseria meningitidis and received appropriate antibiotics for 14 days if vaccination occurred <14 days before the first eculizumab dose. Eculizumab was administered intravenously at dosages of 900 mg once a week for 4 weeks, 1200 mg at week 5, and then 1200 mg every 2 weeks thereafter. The protocol was approved by the institutional review board at each participating center or by an independent ethics committee and was conducted in accordance with International Conference on Harmonisation Good Clinical Practice Guidelines and the Declaration of Helsinki. All patients provided written informed consent before study entry.

PE/PI

Patients were categorized according to whether or not they received PE/PI during the pretreatment period, defined as beginning on the start date of the current aHUS
manifestation up to the first dose. The median PE/PI intensity was 0.5 sessions/day. Potential correlations between biomarker levels at baseline and the PE/PI rate/day or timing of the last PE/PI session during the pretreatment period were evaluated.

**Biological samples**

Serum, ethylenediaminetetraacetic acid (EDTA) plasma and urine samples (N=26–38, depending upon sample matrix) were obtained from patients with aHUS at baseline, before eculizumab treatment, and at visits 3 (weeks 1–3), 6 (weeks 4–6), 12 (weeks 12–17), 17 (weeks 26–33), 18 (weeks 38–42), and 19 (weeks 49–54) during eculizumab treatment. For simplicity, visit 17 is referred to as week 26 and visit 19 as week 52 of treatment. Plasma samples were processed on ice as quickly as possible to preserve complement activity. Currently employed measures to prevent ex vivo complement activation, as well as to preserve complement, such as the addition of futhan or use of BP100 tubes during blood acquisition, were not in place at the time this study was initiated and therefore some complement assessments in plasma (C5a and sC5b-9) could not be reliably performed. C5a and sC5b-9 were reliably quantitated in urine, however. Freshly obtained urine was mixed (9:1) with 10 mM Tris buffer containing 0.05% Tween 20, 0.01% NaN₃, and protease inhibitors (10 mM benzamidine, 10 mM ε-aminocaproic acid, 20 mM EDTA, and 100 U/mL aprotinin) before centrifugation at 4°C. All samples were aliquoted and stored at -80°C until analysis, without subsequent freeze-thaw. Samples were not available from every patient at every time point, but all available samples at each time point were evaluated. Samples from patients who discontinued eculizumab therapy were not available.
Serum samples from clotted whole blood from 20 healthy volunteers (HV; 10 males, 10 females) were purchased (BioreclamationIVT, Westbury, NY, USA). Ten plasma samples (4 females, 6 males) and 10 urine samples (8 females, 2 males) were freshly obtained from HV selected randomly from a donor pool, according to institutional guidelines. All donors were between the ages of 18 and 68 years. Pregnant donors or those with recent surgery or illness were excluded. Plasma and urine samples from HV were processed and stored under similar conditions as described above for patient samples.

**Biomarker detection**

Levels of plasma and serum markers were evaluated from HV and patients with aHUS at baseline and at regular intervals during eculizumab treatment over a 1-year period; markers in urine were evaluated over 26 weeks of eculizumab treatment. Detailed methods for measurement of levels of plasma complement Ba, serum soluble tumor necrosis factor receptor-1 (sTNFR1), plasma prothrombin fragment F1+2, plasma TM, and urinary cystatin C, tissue inhibitor of metalloproteinases-1 (TIMP-1), β2-microglobulin (β 2-M), liver fatty acid binding protein (L-FABP-1), creatinine, C5b-9, and C5a are described in the **Supplementary Methods**.

**Mutation/polymorphism analysis**

Patient samples were categorized according to the absence (no identified mutation) or presence (identified mutation) of complement gene mutations/polymorphisms detailed
from the medical record during screening. Mutational status of patients who enrolled in the study with no previously identified complement gene mutations/polymorphisms was confirmed by central laboratories (University of Iowa and Assistance Publique–Hôpitaux de Paris, Hôpital Européen Georges Pompidou), and included whole gene sequence analyses of \textit{CFH}, \textit{CFI}, \textit{CD46 (MCP)}, \textit{CFB}, \textit{C3} genes, including exon-intron junctions and multiplex ligation-dependent probe amplification analyses to detect deletions or duplications of the \textit{CFHR3-CFHR1} region.

\textbf{Statistical analysis}

Patient biomarker levels at baseline, week 26, and week 52 were compared with HV biomarker levels using Wilcoxon rank sum test. Baseline biomarker levels among HV and patients receiving PE/PI of varying intensities were compared using the Kruskal-Wallis test. The impact of PE/PI on baseline biomarker levels was also evaluated by linear regression analysis, evaluating potential correlations between either the PE/PI intensity rate or the day of last PE/PI session relative to baseline biomarker levels measured on day 0. Changes in biomarker levels with ongoing eculizumab treatment are described using box-whisker graphs showing median, 25th, and 75th percentiles, and range. Change from baseline in biomarker levels at each post-dose time point were statistically analyzed using a restricted maximum likelihood-based, repeated measures approach (mixed-effect analysis of variance model) among patients with elevated biomarkers at baseline.
Results

Patients

Forty-one adult patients with aHUS were treated; 38 (93%) completed the initial 26-week clinical study period, and 21 (51%) continued treatment for 1 year during the optional extension period. Twenty patients (49%) had ≥1 identified complement gene mutation and/or factor H autoantibody. Thirty patients (73%) enrolled during their first identified clinical TMA manifestation. At screening, mean ± standard deviation platelet count was 119.1 ± 66.1 x 10⁹/L, mean haptoglobin was 0.6 ± 0.40 g/L, and mean LDH level was 492.9 ± 500.9 U/L. Thirty-five patients (85%) received PE/PI before eculizumab. Patients who received PE/PI averaged 9.6 sessions (range, 1–26) during the pretreatment period, with a median rate of 0.5 sessions/day. Twenty-four patients (59%) were on dialysis at baseline and 9 (22%) had a history of prior renal transplantation. Thirty-three patients (80%) had chronic kidney disease stage 4 or 5 (i.e., estimated glomerular filtration rate <30 mL/min/1.73 m²). Additional patient demographics and disease characteristics were described previously. Adult HV from whom control serum, plasma, and urine were obtained are described in the Methods section.

Biomarker levels at baseline

Evaluated markers of proximal and terminal complement activation, inflammation, endothelial cell activation and damage, and coagulation and renal injury are detailed in Table 1. At baseline, all markers were significantly elevated in the majority of patients with aHUS compared with levels measured in adult HV (Table 2).
Plasma Ba levels were elevated in all (35/35) patients at baseline, with median levels 6-fold higher than levels in HV, indicating systemic AP activation. Terminal complement activation markers (urine [U-] C5a and U-sC5b-9) were elevated by 45- and 305-fold, respectively, in >85% of patients with aHUS, and only 1 patient did not demonstrate elevated C5a or sC5b-9. All patients showed elevated serum sTNFR1 levels at baseline (19-fold higher than levels observed in HV), demonstrating evidence of systemic inflammation. Serum sVCAM-1 and plasma TM levels were elevated by 2- and 4-fold in >94% of patients, reflecting endothelial activation and injury, respectively. Baseline plasma F1+2 and D-dimer levels were also 6- and 10-fold higher than levels observed in HV in >94% of patients, indicating thrombin generation and fibrinolysis (Table 2). At baseline, 69%–83% of patients with aHUS also showed significantly elevated levels of candidate renal injury biomarkers associated with acute kidney injury, ischemic injury and nephrotoxicity in rodent models and human disease, including those associated with proximal tubular injury (U-cystatin-C,\textsuperscript{38,53-55} clusterin,\textsuperscript{38,58,59} and β 2-M\textsuperscript{38,60}), interstitial injury (TIMP-1\textsuperscript{40,56}) and deteriorating renal function (U-L-FABP-1\textsuperscript{39,57}); levels of these markers were 9- to 48-fold higher than levels measured in HV (Table 2).

During the 7-day period from screening/enrollment to baseline, platelet, LDH, or haptoglobin levels returned to normal values in some patients, allowing evaluation of markers among patients with normal baseline hematologic or biochemical laboratory values. Patients with normal platelet count, LDH, or haptoglobin levels at baseline demonstrated elevated levels of Ba, sTNFR1, sVCAM-1, D-dimer, U-cystatin C (Figures 1A and 1B) and all other markers (U-C5a, U-C5b-9, F1+2, TM, U-clusterin, U-
TIMP-1, U-β 2-M, U-L-FABP-1 not shown). In addition, patients receiving repeated PE/PI before eculizumab treatment also showed elevated levels of every marker evaluated (Figure 2; U-C5a, U-C5b-9, F1+2, TM, U-clusterin, U-TIMP-1, U-β 2-M, U-L-FABP-1 not shown). Patients showed elevated biomarker levels at baseline regardless of less frequent (≤0.5 sessions/day) or more frequent PE/PI (>0.5 sessions/day) (P<0.0027 for all compared with HV; see Supplementary Figure), and linear regression analysis confirmed that intensity of pretreatment PE/PI was not correlated with biomarker levels at baseline (correlation coefficient [CC] = -0.0873 to 0.3218; P≥0.10 for all). Although the timing of baseline blood collection relative to the last PE/PI session was variable, there was no correlation between timing of the last PE/PI session and baseline biomarker levels (CC = -0.0094 to 0.3611; P≥0.0830 for all).

**Biomarker levels during eculizumab treatment**

All biomarkers described were reduced with eculizumab. Blockade of terminal complement activation led to sustained reductions in levels of markers of complement activation (Figure 3A). Patients demonstrated immediate and sustained reductions in U-C5a and U-C5b-9 following the first dose of eculizumab (P<0.0001 versus baseline by 2.5 weeks and at all later time points) to levels consistent with those of HV (P=0.3694 and P=0.2552, respectively, versus HV, after the first eculizumab dose), with up to 100% reduction observed at week 26 (mean % reduction of 90% ± 7.48 and 98% ± 1.39, respectively). The rapid decrease in terminal complement activation was followed by a reduction at week 6 in plasma Ba levels (Figure 3A; P=0.0039 versus baseline levels) and at all later time points (P≤0.0001 for all) (mean % reduction of 30% ± 14.05...
at week 52). Ba remained significantly elevated compared with levels in HV (3-fold versus HV at week 52; \( P \leq 0.0001 \)).

Ongoing treatment with eculizumab also led to reduced levels of biomarkers of inflammation, endothelial activation and damage, and coagulation (Figure 3B). sTNFR1 levels were significantly reduced from baseline levels by week 6 and at all later time points (\( P < 0.0001 \) versus baseline). By week 52, sTNFR1 levels decreased by up to 94% (mean % reduction of 60\% \pm 14.27) but remained elevated (5-fold; \( P < 0.0001 \) compared with HV). Median sVCAM-1 and TM levels were significantly decreased from baseline by week 17 (\( P < 0.0001 \) for both) and reduction was sustained at all later time points (\( P \leq 0.005 \)). While median sVCAM-1 levels remained elevated (1.7-fold compared with HV \( P < 0.0001 \)) at week 52, TM levels decreased by up to 77\% (mean % reduction of 60\% \pm 10.32) to levels near those of HV (1.2-fold higher than HV; \( P = 0.022 \)) at that time point. With eculizumab, median F1+2 and D-dimer levels both decreased by week 2.5 (\( P < 0.0001 \)) and at all time points thereafter (\( P \leq 0.05 \)). F1+2 levels decreased by up to 89\% (mean % reduction of 26\% \pm 27.39) by week 52, and D-dimer levels decreased by up to 99\% (mean % reduction of 47\% \pm 58.34). At week 52, levels of both coagulation markers remained modestly elevated (\( P \leq 0.007 \) for both) at 2.6- and 1.8-fold above the levels observed in HV, respectively.

Eculizumab treatment significantly reduced levels of all renal injury markers by week 6; reduction was sustained at all later time points (\( P \leq 0.0005 \) for all) and markers were
decreased to levels consistent with HV ($P>0.1309$ for all) by week 26 with the exception of U-TIMP-1, which remained 2.5-fold higher ($P=0.0188$; Figure 3C).

**Analysis by complement genetic mutation/polymorphism**

Marker levels were evaluated before and during eculizumab treatment among patients with and without identified complement regulatory gene mutations/polymorphisms. All markers (Table 2) were elevated at baseline relative to those observed in HV regardless of presence or absence of an identified complement gene mutation and all markers were reduced during 1 year of treatment with eculizumab (representative results for Ba, sVCAM-1, and TM shown in Figure 4).
Discussion

Results of this exploratory analysis of the C10-004 trial more fully characterize the physiologic pathways impacted by complement dysregulation and provide insight into the effect of eculizumab on these pathways in aHUS. At baseline, patients had significantly elevated levels of markers associated with proximal and terminal complement activation, systemic inflammation, endothelial cell activation and damage, coagulation, and renal injury. Elevated marker levels at baseline were observed in the majority of patients, even in patients with normal baseline platelet, LDH, or haptoglobin levels, and regardless of prior use of PE/PI or mutation status.

All patients presented with significant proximal AP activation as evidenced by elevated Ba, confirming the fundamental complement-mediated pathophysiology of aHUS. The continued elevation of Ba after 1 year of eculizumab treatment demonstrates that unregulated AP activation in aHUS patients is persistent despite complete inhibition of terminal complement activity by eculizumab. This finding is not unexpected, since AP activation occurs upstream of C5 and would be expected to continue even with downstream, complete terminal complement C5 blockade. Consistent with baseline AP activation, patients with aHUS also demonstrated significant terminal complement activation at baseline, as high levels of both urine C5a and sC5b-9 were observed for most patients, including those with no identified complement gene mutations. While a potential study limitation is the absence of plasma C5a and sC5b-9 level measurements, urine sampling to determine terminal complement activation is well established. Furthermore, urine levels may better reflect local complement activation;
indeed, urinary sC5b-9 levels have been shown to correlate highly with increased tubulointerstitial C3 synthesis,\textsuperscript{62} local deposition in the glomerular and tubular epithelium, and disease severity in patients with membranous nephropathy.\textsuperscript{63} In our study, both urine C5a and sC5b-9 levels decreased immediately following the first eculizumab dose, quickly reaching levels observed in HV. Our results in this large cohort of patients with aHUS contrast with those of a recent single case report in which eculizumab therapy resolved HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), decreased both plasma and urine sC5b-9 levels, reduced complement hemolytic activity, but did not appear to reduce plasma C5a/C5a(desArg) levels.\textsuperscript{64} Additional studies may reveal if these disparate results are due to differences in assay methodology or reagent specificity; many, if not all C5a/C5a(desArg) antibodies cross react with C5 (and potentially with C5 bound to eculizumab).

Elevated baseline sTNFR1 levels also were observed in all patients, highlighting the systemic inflammation present in aHUS. sTNFR1 levels were reduced significantly by week 6 and profoundly within 1 year of treatment with eculizumab. These results suggest that elevated terminal complement activation in aHUS is a major contributor to inflammation, shown to play a central role in the pathophysiology of both acute kidney injury\textsuperscript{65,66} and progressive renal disease.\textsuperscript{67} Interestingly, although Ba levels remained elevated during 1 year of treatment, median levels were decreased by 30%, suggesting that C5 blockade with eculizumab may mitigate upstream AP activation. Potential mechanisms are suggested by data showing that sublytic C5b-9 induces multiple effects
on endothelial cells, including apoptosis, cellular retraction, and exposure of extracellular matrix,\textsuperscript{13,68} which in turn may activate the alternative pathway directly.\textsuperscript{13,69}

Inflammation in aHUS also has been shown to contribute to endothelial cell activation and damage, evidenced by significant elevations in baseline sVCAM-1 and TM levels, respectively.\textsuperscript{11} After 1 year of treatment, sVCAM-1 remained elevated relative to HV levels, while TM reduced to near normal levels. Importantly, levels of markers associated with renal injury were reduced and became indistinguishable from levels in HV by 26 weeks. These biomarker results are consistent with the renal impairment and improvement observed in the trial, and indicate that terminal complement blockade with eculizumab interrupts progression from inflammation to renal injury and organ dysfunction despite evidence of persistent AP and endothelial cell activation.\textsuperscript{70}

Virtually all patients had elevated F1+2 and D-dimer levels at baseline, indicating thrombin generation and fibrinolysis. Increased F1+2 levels preceded renal injury in other TMA studies in Shiga toxin-producing \textit{Escherichia coli} HUS.\textsuperscript{71,72} Reduction of F1+2 and D-dimer levels with eculizumab suggests that prevention of C5a and C5b-9 formation and bioactivity, through terminal complement blockade, down-regulates the coagulation cascade. This is consistent with a study of paroxysmal nocturnal hemoglobinuria, which demonstrated that eculizumab treatment results in a significant decrease in thrombosis\textsuperscript{73} and in markers associated with thrombin generation and reactive fibrinolysis, including plasma D-dimer, thrombin-anti-thrombin complexes, interleukin-6, soluble P-selectin, and microparticles with prothrombotic activity.\textsuperscript{33}
Reduction in F1+2 and D-dimer with continued terminal complement blockade in patients with aHUS is also consistent with clinical findings from this study demonstrating rapid and complete inhibition of TMA after eculizumab treatment initiation.\textsuperscript{25}

In aHUS patients both with and without identified complement mutations, markers were significantly elevated at baseline and ongoing eculizumab therapy significantly reduced levels in both subsets of patients. A recent retrospective study among a subset of patients enrolled in an aHUS/thrombotic thrombocytopenic purpura registry that fulfilled the diagnostic criteria for aHUS reported elevated levels of Bb, plasma C5a, and sC5b-9 among patients with and without complement regulatory gene mutations.\textsuperscript{74} Although differences in methodology and matrices evaluated preclude a direct comparison of biomarker levels, similar trends were observed, and together, these results confirm recent recommendations that an identified complement gene mutation is not required for aHUS diagnosis or initiation of eculizumab therapy.\textsuperscript{16,20}

It should be stressed that the biomarkers characterized in our study have not yet been validated to confirm aHUS diagnosis or to make other clinical decisions in dosing or treatment. Indeed, there is disagreement on the utility of using individual markers to monitor disease activity in aHUS and no single marker has been validated for this purpose. While some have described using CH50 assays to monitor patients receiving eculizumab,\textsuperscript{75-77} others have shown the insensitivity of CH50 or plasma C5a and sC5b-9 assays to fully assess ongoing aHUS disease activity compared with assays that reflect endothelial complement deposition.\textsuperscript{78} Regulatory guidance includes reference to
potential risk of TMA manifestations following eculizumab discontinuation.\textsuperscript{22,23} Recent evidence from case studies\textsuperscript{79-84} and an observational study\textsuperscript{85} suggest that while certain individual patients discontinued therapy without safety concerns, 6 of 16 patients (38\%) had TMA manifestations following eculizumab discontinuation.

It is remarkable that even patients with normal baseline laboratory values for platelet count, LDH, and haptoglobin or use of PE/PI before eculizumab had strong evidence of complement activation, vascular inflammation, coagulation, endothelial activation, and damage and renal injury. Other studies also have shown that C3 consumption and complement deposition on platelets and endothelium occurs during periods when a clinical manifestation is not evident.\textsuperscript{78,86} The current study extends these observations, demonstrating that underlying complement dysregulation continues even with PE/PI management and in the absence of overt symptoms.

Taken together, these results point to associations between biomarkers and disease progression and organ damage in aHUS: chronic AP activation leads to C5a and C5b-9 formation and TNF-\(\alpha\) elaboration,\textsuperscript{28,51} which directly activates and damages endothelial cell surfaces, inducing VCAM-1 expression\textsuperscript{29,30} and TM shedding.\textsuperscript{31,32} C5a-driven VCAM-1 expression is also increased,\textsuperscript{41} while C5b-9 deposition on endothelial cell surfaces further induces endothelial activation and damage,\textsuperscript{12,43,45} provoking additional AP activation, which is improperly regulated.\textsuperscript{13} Thus, chronic AP overactivation and ensuing elaboration of potent inflammatory mediators leads to increased endothelial cell
activation, injury, and thrombosis, resulting in the clinical phenotype of TMA and progression to extra-renal injury and end-stage renal disease.

Ongoing terminal complement inhibition with eculizumab treatment markedly reduces inflammation and coagulation, and decreases endothelial activation and damage. Though significantly reduced during 1 year of treatment with eculizumab, persistently elevated Ba and sVCAM-1 levels in patients with aHUS reflect the effect of genetic dysregulation of AP and ongoing activation upstream of C5, and subsequent propensity for endothelial cell activation. By blocking terminal complement activity, eculizumab reduces ongoing endothelial damage and renal injury even in the presence of chronic AP activation. Although limited by low numbers of patients, these data underscore the ongoing risk of systemic TMA and progression to organ damage, and highlight the need for further research into underlying complement dysregulation in aHUS patients with or without clinical TMA manifestations.

Acknowledgments
The authors thank Kristen W. Quinn, PhD, of Peloton Advantage, LLC, John F. Kincaid, MD, MA, and Erin Harvey, MSc, of Alexion Pharmaceuticals, Inc., for assistance with manuscript preparation; Rong Lin, MD, MPH, Jimmy Wang, PhD, Joseph Yen, PhD, and Marcia Wang, PhD, for biostatistical analyses; and Alexion Pharmaceuticals clinical data management and operations teams for study operations and data integrity. The authors are deeply indebted to the patients, patients’ families, and clinicians who
participated in the C10-004 study. This study was sponsored by Alexion Pharmaceuticals, Inc.

Authorship contributions
R.C., A.K., C.B., and S.F. were responsible for study design, data analysis, interpretation, and wrote the manuscript; R.C., A.K., and K.B. designed biomarker panels, validated the assays, and performed the research; S.F. and C.B. directed the project, interpreted the data, and wrote the manuscript; M.O. and C.B. provided essential input on patient data; Y.Y. and A.M. collected, processed, and organized patient and control samples, and assisted with data organization and review. All authors have reviewed and approved the manuscript.

Disclosure of conflicts of interest
All authors are employees and stock shareholders of Alexion Pharmaceuticals, Inc.
References


### Table 1. Markers of Complement Activation, Vascular Inflammation, Endothelial Activation and Damage, Coagulation, and Renal Injury

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<th>Disease Process</th>
<th>Biomarker</th>
<th>Function/Association With Complement</th>
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<td><strong>Complement Activation</strong></td>
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| AP activation                 | Ba            | • Alternative pathway biomarker upstream of C5<sup>26</sup>  
|                               |               | • Alternative pathway is stimulated by damaged endothelial cells<sup>13</sup> and activated platelets<sup>61</sup> |
| Terminal complement           | C5a           | • Marker of C5 activation<sup>22,23</sup>  
|                               |               | • Proinflammatory<sup>42</sup>  
|                               |               | • Mediates chemotaxis, activates endothelial cells, upregulates TNF-α and VCAM-1<sup>28,41</sup> |
| Terminal complement           | sC5b-9        | • Marker of C5 activation<sup>22,23</sup>  
|                               |               | • Mediates endothelial cell activation,<sup>12</sup> glomerular injury,<sup>43</sup> and ischemic injury leading to organ damage<sup>44</sup>  
|                               |               | • Stimulates vWF multimer secretion,<sup>45</sup> endothelial cell prothrombinase activity,<sup>50</sup> and tissue factor expression<sup>46</sup> |
| **Vascular Inflammation/Damage and Coagulation** | sTNFR1 | • Surrogate, more stable marker for TNF-α<sup>27</sup>  
|                               |               | • TNF-α is pro-inflammatory; associated with vascular<sup>49</sup> and chronic renal inflammation and progression of renal failure<sup>27,47,48</sup>  
|                               |               | • TNF-α upregulated by complement activation<sup>51</sup> |
| Endothelial activation        | sVCAM-1       | • Adhesion molecule released by activated endothelial cells<sup>29</sup>  
<p>|                               |               | • Upregulated by TNF-α and terminal complement&lt;sup&gt;29,30&lt;/sup&gt; |</p>
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<th>BIOMARKERS OF aHUS</th>
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| **Endothelial cell damage** | Thrombomodulin | • Protective against thrombotic risk, inflammation, and complement activation when membrane-bound<sup>52</sup>  
• Released in soluble form by damaged endothelial cells<sup>31</sup>  
• TNF-α down-regulates membrane form and increases release of soluble form<sup>32</sup> |
| **Coagulation** | Prothrombin fragment F1+2 | • Direct marker of thrombin generation<sup>36</sup>  
• Generated by cleavage of prothrombin following tissue factor–induced coagulation<sup>10</sup> |
| | D-dimer | • Fibrin degradation product indicating fibrinolysis<sup>37</sup> |
| **Renal injury** | Urine cystatin-C | • Proximal tubular injury<sup>38,53-55</sup> |
| | Clusterin | • Proximal tubular injury<sup>38,58,59</sup> |
| | β2-microglobulin | • Proximal tubular injury<sup>38,60</sup> |
| | TIMP-1 | • Interstitial tubular injury<sup>40,56</sup> |
| | L-FABP-1 | • Deteriorating renal function<sup>39,57</sup> |

AP, alternative pathway; L-FABP-1, liver fatty acid binding protein-1; sTNFR1, soluble tumor necrosis factor receptor-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TIMP-1, tissue inhibitor of metalloproteinases-1; TNF-α, tumor necrosis factor-α; vWF, von Willebrand factor.
**Table 2.** Baseline Levels of Markers of Complement Activation, Vascular Inflammation, Endothelial Activation and Damage, Coagulation, and Renal Injury in HV and in Patients with aHUS

<table>
<thead>
<tr>
<th>Disease Process</th>
<th>Biomarker</th>
<th>Range (Min-Max)</th>
<th>Median Baseline Level in aHUS Patients (range, <em>P Value</em>)</th>
<th>n/N (%) With Elevated Baseline Level</th>
<th>Median Fold Increase Over HV at Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complement Activity</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AP activation</td>
<td>Plasma Ba</td>
<td>388.0-588.0 ng/mL</td>
<td>2676.4 (935.0–3668.0; &lt;0.0001)</td>
<td>35/35 (100)</td>
<td>5.5</td>
</tr>
<tr>
<td>Terminal complement</td>
<td>U-C5a</td>
<td>0.0-0.7 ng/mg U-creatinine</td>
<td>9.0 (0.3–76.6; 0.0001)</td>
<td>26/29 (89.7)</td>
<td>45.0</td>
</tr>
<tr>
<td>Terminal complement</td>
<td>U-sC5b-9</td>
<td>0.0-0.6 ng/mg U-creatinine</td>
<td>30.5 (0.2–665.7; &lt;0.0001)</td>
<td>23/27 (85.2)</td>
<td>305.0</td>
</tr>
<tr>
<td><strong>Vascular Inflammation/Damage and Coagulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>sTNFR1</td>
<td>407.3-1391.3 pg/mL</td>
<td>17616.9 (4008.5–54158.2; &lt;0.0001)</td>
<td>38/38 (100)</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>Biomarkers</td>
<td>Mean</td>
<td>95% CI</td>
<td>P-value</td>
<td>Percentage (n)</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
<tr>
<td><strong>Endothelial</strong></td>
<td>sVCAM-1</td>
<td>659.8</td>
<td>(375.4–1865.5; &lt;0.0001)</td>
<td>2.0</td>
<td>36/38 (94.7)</td>
</tr>
<tr>
<td><strong>activation</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Endothelial</strong></td>
<td>Thrombo-</td>
<td>10</td>
<td>(3.4–24.1; &lt;0.0001)</td>
<td>3.6</td>
<td>33/34 (97.1)</td>
</tr>
<tr>
<td><strong>cell damage</strong></td>
<td>modulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coagulation</strong></td>
<td>Prothrombin</td>
<td>1017.6</td>
<td>(217.7–5774.0; &lt;0.0001)</td>
<td>5.5</td>
<td>36/38 (94.7)</td>
</tr>
<tr>
<td><strong>fragment</strong></td>
<td></td>
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<td></td>
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<tr>
<td><strong>F1+2</strong></td>
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</tr>
<tr>
<td><strong>D-dimer</strong></td>
<td></td>
<td>2735.0</td>
<td>(330.0–44100.0; &lt;0.0001)</td>
<td>9.8</td>
<td>34/36 (94.4)</td>
</tr>
<tr>
<td><strong>Renal</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Renal injury</strong></td>
<td>U-clusterin</td>
<td>1232.3</td>
<td>(129.9–6091.2; &lt;0.0001)</td>
<td>8.6</td>
<td>24/29</td>
</tr>
<tr>
<td></td>
<td>U-creatinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U-TIMP-1</strong></td>
<td></td>
<td>23.8</td>
<td>(1.4–230.4; &lt;0.0001)</td>
<td>39.7</td>
<td>22/29</td>
</tr>
<tr>
<td></td>
<td>U-creatinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U-L-FABP-1</strong></td>
<td></td>
<td>58.0</td>
<td>(3.7–1309.8; &lt;0.0001)</td>
<td>48.3</td>
<td>22/29</td>
</tr>
<tr>
<td>Biomarker</td>
<td>Normal Range</td>
<td>Median</td>
<td>Proportion of Patients</td>
<td>U-creatinine</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
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<td></td>
</tr>
<tr>
<td>β2-microglobulin</td>
<td>0.0-2.7 µg/mg</td>
<td>18.4</td>
<td>20/28</td>
<td>(0.4–127.7; 0.0002)</td>
<td></td>
</tr>
<tr>
<td>U-cystatin C</td>
<td>0.3-301.3 ng/mg</td>
<td>1256.9</td>
<td>18/26</td>
<td>(14.3–7189.6; 0.0005)</td>
<td></td>
</tr>
</tbody>
</table>

*Normal ranges were evaluated in healthy volunteers (HV; n=20 for serum analytes, n=10 for plasma analytes, and n=9 for urine analytes).

*Baseline biomarker levels between groups were compared using the Wilcoxon rank sum test. Units are as indicated for HV levels.

*Proportion of patients out of the total available samples evaluated showing levels >95th percentile of HV levels.

*Plasma C5a and sC5b-9 samples were not obtained under complement-preserving conditions and could not be assessed.

AP, alternative pathway; HV, normal human volunteers; L-FABP-1, liver fatty acid binding protein-1; sTNFR1, soluble tumor necrosis factor receptor-1; sVCAM-1, soluble vascular cell adhesion molecule 1; TNF-α, tumor necrosis factor-α; U, urine; U-TIMP-1, urine tissue inhibitor of metalloproteinases-1.
Figure Legends

Figure 1. Biomarker levels in patients with normal platelet counts and normal LDH and haptoglobin levels at baseline. Median levels of markers of complement activation, vascular inflammation/damage and coagulation, and renal injury were elevated in aHUS patients with and without: (A) normal platelet count; and (B) normal LDH and haptoglobin levels at baseline. Error bars represent 95% confidence intervals. Abbreviations: aHUS, atypical hemolytic uremic syndrome; HV, healthy volunteers; LDH, lactate dehydrogenase; sTNFR1, soluble tumor necrosis factor receptor-1; sVCAM-1, soluble vascular cell adhesion molecule-1; U, urine.

Figure 2. Biomarker levels in patients receiving PE/PI at baseline. PE/PI does not affect biomarkers of complement activation, vascular inflammation/damage and coagulation, and renal injury in patients with aHUS compared with HV at baseline. Median levels and 95% confidence intervals are represented. Abbreviations: aHUS, atypical hemolytic uremic syndrome; HV, healthy volunteers; PE/PI, plasma exchange/plasma infusion; sTNFR1, soluble tumor necrosis factor receptor-1; sVCAM-1, soluble vascular cell adhesion molecule 1; U, urine.

Figure 3. Biomarker levels during terminal complement blockade with eculizumab. Longitudinal decreases in median levels of biomarkers of: (A) complement activation; (B) vascular inflammation/damage and coagulation; and (C) renal injury were demonstrated with eculizumab therapy in patients with aHUS compared with HV.
Changes in biomarker levels with ongoing eculizumab treatment are displayed using box-whisker graphs showing median, 25th, and 75th percentiles, and range. *Levels were significantly reduced compared with baseline; the $P$ value of reduction at the first significant time point is shown. Abbreviations: aHUS, atypical hemolytic uremic syndrome; HV, healthy volunteers; sTNFR1, soluble tumor necrosis factor receptor-1; sVCAM-1, soluble vascular cell adhesion molecule 1; TM, thrombomodulin; U, urine; U-\(\beta\) 2-M, urine \(\beta\)2-microglobulin; U-Clu, urine clusterin; U-FABP-1, urine fatty acid binding protein-1; U-TIMP-1, urine tissue inhibitor of metalloproteinases-1.

**Figure 4. Biomarker levels in patients with or without an identified complement regulatory gene mutation or polymorphism.** Biomarker profiles were elevated at baseline and were reduced during eculizumab treatment in patients with aHUS with or without an identified complement regulatory gene mutation/polymorphism, compared with HV. Longitudinal median biomarker levels are shown with $P$ values relative to HV levels indicated. Abbreviations: aHUS, atypical hemolytic uremic syndrome; HV, healthy volunteers; sVCAM-1, soluble vascular cell adhesion molecule 1; TM, thrombomodulin.
Figure 2
Figure 4
Eculizumab reduces complement activation, inflammation, endothelial damage, thrombosis, and renal injury markers in aHUS

Roxanne Cofiell, Anjli Kukreja, Krystin Bedard, Yan Yan, Angela P. Mickle, Masayo Ogawa, Camille L. Bedrosian and Susan J. Faas

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