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ORIGINAL ARTICLE

Temporal changes in complement activation in haemodialysis patients with COVID-19 as a predictor of disease progression

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ABSTRACT

Background. Complement activation may play a pathogenic role in patients with severe coronavirus disease 2019 (COVID-19) by contributing to tissue inflammation and microvascular thrombosis.

Methods. Serial samples were collected from patients receiving maintenance haemodialysis (HD). Thirty-nine patients had confirmed COVID-19 and 10 patients had no evidence of COVID-19. Plasma C5a and C3a levels were measured using enzyme-linked immunosorbent assay.

Results. We identified elevated levels of plasma C3a and C5a in HD patients with severe COVID-19 compared with controls. Serial sampling identified that C5a levels were elevated prior to clinical deterioration in patients who developed severe disease. C3a more closely mirrored both clinical and biochemical disease severity.

Conclusions. Our findings suggest that activation of complement plays a role in the pathogenesis of COVID-19, leading to endothelial injury and lung damage. C5a may be an earlier biomarker of disease severity than conventional parameters such as C-reactive protein and this warrants further investigation in dedicated biomarker studies. Our data support the testing of complement inhibition as a therapeutic strategy for patients with severe COVID-19.

Keywords: complement, COVID-19, haemodialysis

INTRODUCTION

As of 1 August 2020, >18 million people worldwide had been infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19). Around 15% of patients develop severe disease with a clinical picture of an atypical acute respiratory distress syndrome and cytokine storm [1]. Severe disease appears to be associated with a marked host inflammatory response to virally infected tissue. Patients with severe disease have increased

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levels of pro-inflammatory cytokines and evidence of microvascular thrombosis [2].

Complement is a major component of the innate immune response and elevated levels of plasma C5a, indicating complement C5 activation, have been identified in patients with severe COVID-19 [3, 4]. Vasculitis-like lesions in the lungs with fibrin thrombi of small vessels and infiltrates of neutrophils and monocytes have been identified in post-mortem lung tissue from patients with severe COVID-19 [5]. C5b-9 deposition has been shown along the micro- and macrovasculature of the lung in patients with COVID-19 and, in one study, these deposits co-localized with staining for the SARS-CoV-2 spike protein [4, 5]. Patients with a vasculitic-type skin rash with C5b-9 and C4d deposition in the skin microvasculature have also been described [5].

Activation of coagulation and microvascular thrombosis are a feature of severe COVID-19 that may be mediated by complement activation. The interaction of the complement and coagulation systems is complex, however, activation of complement is well described to be pro-thrombotic [6]. There are multiple mechanisms through which complement activation can result in a procoagulant phenotype. Complement C3a and C5a acting on neutrophils and monocytes can trigger pro-inflammatory cytokine production, which in turn decreases levels of anticoagulant factors, induces tissue factor release and activates the extrinsic coagulation pathway. Activated complement components can also directly interact with endothelial cells, activate coagulation cascades and induce platelet aggregation and adhesion [6, 7].

Mouse models support a role for complement in contributing to tissue damage during coronavirus and other viral infections. In a mouse model of SARS-CoV infection, C3-deficient mice had less severe lung pathology and lower levels of pro-inflammatory cytokines [8]. Similar results have been described with the use of an anti-C5aR antagonist in mice with Middle East respiratory syndrome coronavirus (MERS-CoV) lung disease [9]. However, it is important to note that complement can exert important antiviral effects and, in keeping with this, many viruses encode genes that enable them to go undetected by the complement system [10]. During H1N1 and H5N1 influenza infection in mice, complement C3 is required for viral clearance and C3-deficient mice developed more severe lung injury [11].

Here we report an analysis of serial measurements of plasma levels of C3a and C5a as markers of complement C3 and C5 activation, respectively, in maintenance haemodialysis (HD) patients with both non-severe and severe COVID-19.

MATERIALS AND METHODS

Study participants

All participants (patients and controls) were recruited from the Imperial College Renal and Transplant Centre, London, and provided written informed consent prior to participation. The study was approved by the Health Research Authority, Research Ethics Committee (reference 20/WA/0123: The impact of COVID19 on patients with renal disease and immunosuppressed patients).

Samples

Samples were collected from patients receiving maintenance HD with confirmed SARS-CoV-2 infection by reverse transcription polymerase chain reaction (RT-PCR). Patients were recruited both from their outpatient HD unit and from the inpatient renal wards. Ethylenediaminetetraacetic acid (EDTA) blood samples were collected and plasma stored at (−80°C prior to analysis. Plasma C3a and C5a were measured using enzyme-linked immunosorbent assay [C3a plus enzyme immunoassay (EIA) and C5a EIA; Quidel, San Diego, CA, USA] according to the manufacturer's instructions. Samples were incubated at 37°C until just thawed then placed on ice to prevent *ex vivo* activation of the complement system. Information on clinical parameters and biochemical and haematological results were collected from the clinical records.

We divided our COVID-19 patients into severe and non-severe disease groups. All cases with severe disease were hospital in-patients and fulfilled the World Health Organization (WHO) criteria for severe disease [respiratory rate ≥30/min, blood oxygen saturation ≤90%, arterial oxygen partial pressure (PaO_2):fractional inspired oxygen (FiO_2) ratio <300 or infiltrates affecting 50% of the lung field within 24–48 h] [1]. Notably, we identified five individuals who were admitted and had samples collected before meeting the WHO severe disease criteria. We refer to this period as the pre-deterioration phase. All patients with non-severe disease were managed in an outpatient setting and did not meet the WHO criteria for severe disease or require respiratory support. In severe disease, recovery was defined as a significant improvement in oxygenation such that supplemental oxygen was no longer required. In non-severe disease, three samples were available for each patient and these were categorized as presentation (median 2 days from positive PCR test), mid-point (median 11 days from positive PCR test) and recovery (median 17 days from positive PCR test). Control HD patients were asymptomatic with no clinical evidence of COVID-19 and negative for the presence of SARS-CoV-2 immunoglobulin M (IgM) and IgG using a lateral flow immunoassay that utilizes the SARS-CoV-2 antigen MK201027, which is located in the spike region glycoprotein (BioMedomics, Morrisville, NC, USA) [12].

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All data are reported as median per group with interquartile range unless stated otherwise. Comparison between groups was by Mann–Whitney U test or for multiple variables, by Kruskal–Wallis test with Dunn's *post hoc* test. Repeated measures correlation was calculated using the rmcorr R package (R Foundation for Statistical Computing, Vienna, Austria) [13, 14].

RESULTS

Plasma C3a and C5a levels were measured in 39 HD patients with confirmed SARS-CoV-2 infection (PCR positive) and 10 asymptomatic HD controls (antibody negative; Figure 1). Twenty of the patients with COVID-19 were classified as having non-severe disease (all managed in the outpatient setting) and 19 had evidence of severe disease (all hospitalized). In the group of patients with severe disease, 21.1% ($n = 4$; C3a and C5a levels indicated in red in Figure 1A and B) died. The remainder ($n = 15$) survived to hospital discharge. The four patients who died developed worsening respiratory function with high oxygen requirements. Three were managed in a ward-based setting. One was admitted to the intensive care unit for non-invasive ventilation (NIV). This individual used domiciliary nocturnal NIV for chronic interstitial lung disease. Median age, gender and

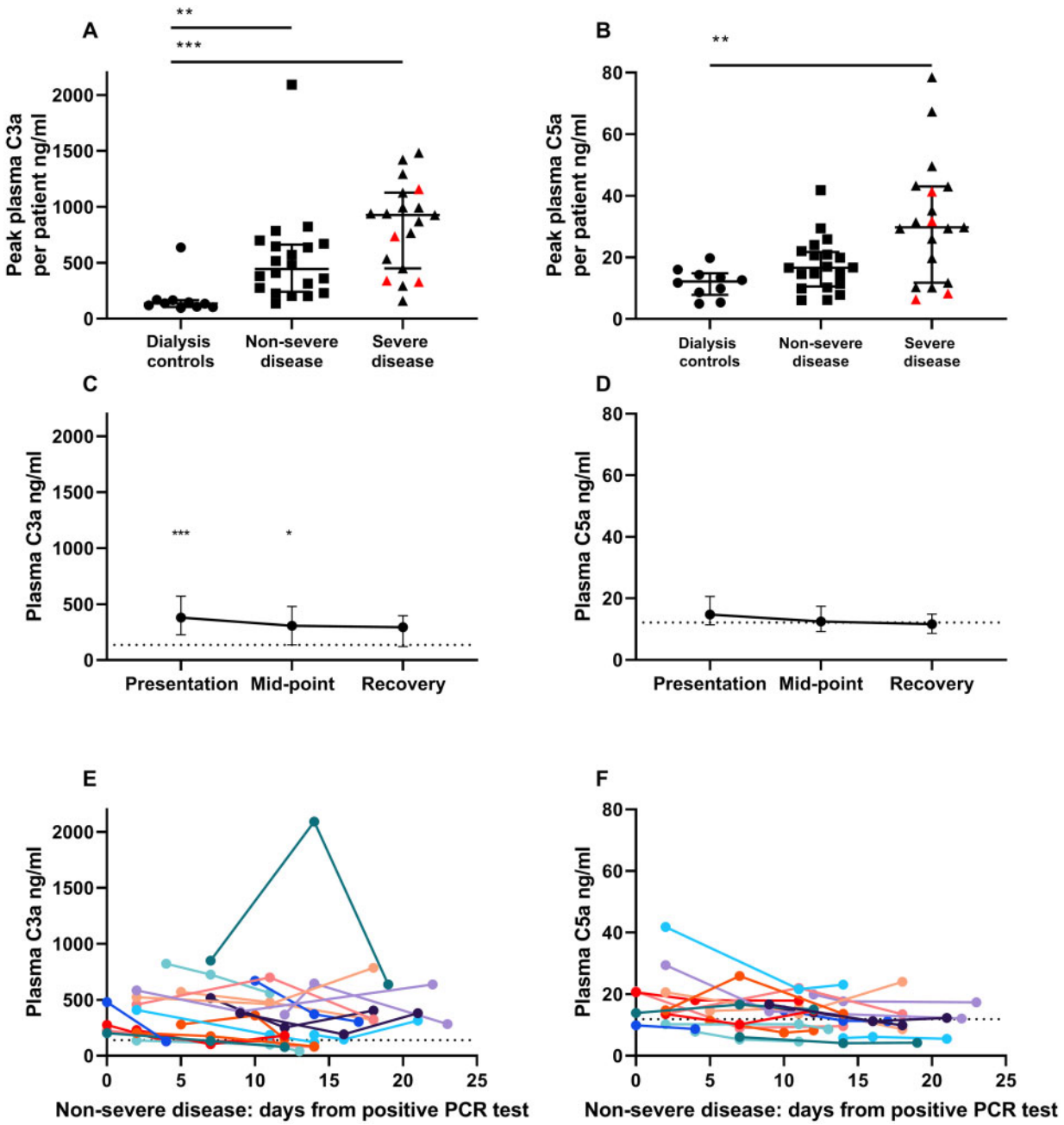

FIGURE 1: Plasma levels of C3a and C5a are elevated in HD patients with severe COVID-19. Peak plasma (A) C3a and (B) C5a levels per patient with either non-severe or severe COVID-19. Patients who died are indicated in red. The horizontal bar denotes the median and whiskers denote the interquartile range. ***$P < 0.001$, **$P < 0.01$ derived from Kruskal–Wallis test with Dunn's *post hoc* correction. Serial measurements of (C) C3a and (D) C5a for each patient with non-severe disease grouped at presentation, mid-point of illness and recovery. The dotted line represents the median value of dialysis controls. The horizontal bar denotes the median and whiskers denote the interquartile range. ***$P < 0.001$, *$P < 0.05$ derived from Mann–Whitney test. Serial measurements of (E) C3a and (F) C5a for each patient with non-severe disease ($n = 20$). The timing of the samples is denoted by days from the positive PCR test. The dotted line represents the median value in dialysis controls.

ethnicity were similar between the three groups (Table 1). Peak values of C-reactive protein (CRP), D-dimers and ferritin were significantly higher in patients with severe compared with non-severe disease (Table 1). In contrast, nadir lymphocyte counts were significantly lower in patients with severe disease (Table 1).

Serial samples were available for all patients with COVID-19; three samples for patients with non-severe disease and between two and nine samples for patients with severe disease. This resulted in a total sample size of 184. A single sample was available from each of the COVID-negative HD control patients.

We first compared the peak values of C3a and C5a for each patient between groups (Figure 1A and B). Peak plasma C3a levels were significantly higher in patients with either severe or non-severe disease compared with HD controls (Figure 1A; median C3a 136.7, 444.9 and 926.6 ng/mL for HD controls, non-

Table 1. Demographics of patients included in the study

Characteristics	All SARS-CoV-2 positive ($n = 39$)	Non-severe disease ($n = 20$)	Severe disease ($n = 19$)	Dialysis controls ($n = 10$)	P-value (non-severe versus severe)
Age (years), median (range)	71.6 (39.7–84.7)	72.3 (39.7–83.6)	65.9 (48.0–84.7)	68.0 (23.8–85.2)	
Male/female, *n*/*n*	26/13	12/8	14/5	7/3	
Ethnicity, % (*n*)					
Caucasian	38.4 (15)	35.0 (7)	42.1 (8)	20 (2)	
Indoasian	35.9 (14)	45.0 (9)	26.3 (5)	70 (7)	
Black African	10.2 (4)	10.0 (2)	10.5 (2)	10 (1)	
Black Caribbean	7.7 (3)	5.0 (1)	10.5 (2)		
Other	7.7 (3)	5.0 (1)	10.5 (2)		
Baseline IS, % (*n*)	15.4 (6)	15.0 (3)	15.7 (3)	0 (0)	
Timing of the first sample from symptoms (days), median (range)	6 (0–17)	5 (0–12)	8 (0–18		
Timing of the first sample from positive PCR (days), median (range)	4 (0–17)	2 (0–12)	4 (0–18)		
Timing of the first sample from severe disease (days), median (range)	1 (−7–11)		1 day ((−7–11)		
CRP (mg/L), median (range)	125.6 (0.6–415.5)	33.2 (0.6–176.9)	220.0 (45.8–415.5)		<0.0001
Lymphocyte count ($\times 10^9$/mL), median (range)	0.7 (0.1–4)	0.9 (0.4–3.2)	0.4 (0.1–1)		<0.0001
D-dimer (μg/L), median (range)	1986 (319–14403)	1898 (319–5144)	2360 (574–14403)		0.05
Ferritin (μg/L), median (range)	1270 (195–10440)	573 (195–2786)	2609 (638–10440)		<0.0001
Outcome, % (*n*)					
Death	10.2 (4)	0 (0)	21.1 (4)		0.04
Recovered	89.7 (39)	100 (20)	78.9 (15)		

Laboratory values represent peak or nadir value for each patient's disease course.
IS, Immunosuppression.

severe and severe disease, respectively). Peak plasma C5a levels were also significantly higher in patients with severe disease compared with HD controls, but levels between non-severe disease and controls were not statistically different (Figure 1B; median C5a 12.19, 16.65 and 29.78 ng/mL for HD controls, non-severe and severe disease, respectively).

We next looked at serial changes in plasma C3a and C5a in non-severe COVID-positive patients (Figure 1C–F). In patients with non-severe disease ($n = 20$), the median C3a level was significantly elevated above the median level of the dialysis controls at both presentation and the mid-point in the illness, but not in the recovery samples (Figure 1C). In contrast, the C5a levels did not differ from the median level of the dialysis controls at any of these time points (Figure 1D). Only one patient with non-severe disease had elevated levels of C3a similar to those seen with severe disease, which peaked 14 days after positive PCR testing and improved by day 19 (Figure 1E). Review of the clinical course of this patient did not reveal any features of severe disease or other explanation for this. C5a was not elevated in this patient and C5a levels were below the median level seen in control HD patients at all three time points.

We next looked at serial changes in plasma C3a and C5a in our cohort with severe disease (Figure 2). In 5 of the 19 patients who were hospitalized, samples were available prior to development of features of severe disease as assessed by WHO criteria. In the samples taken during this pre-deterioration phase the median C5a, but not C3a level, was significantly elevated (Figure 2A and B). During the severe phase, the median levels of both C3a and C5a were significantly elevated compared with the respective median level in the dialysis controls (Figure 2A and B). C3a levels remained elevated throughout this phase whereas the median C5a level started to decline from Day 7. Notably, five patients with severe disease had consistently normal plasma C5a levels during the severe phase, indicating that severe COVID-19 is not invariably associated with elevated plasma C5a (Figure 2D). Two of these five patients died, and in these patients peak levels of C3a, although elevated, were in the lower range seen in patients with severe disease (329 and 372 ng/mL). Three of the patients survived to hospital discharge, and in these patients peak levels of C3a were elevated to a greater degree (926, 994 and 1422 ng/mL). There were no significant differences in biochemical parameters between the groups of patients with and without elevated C5a.

Elevated CRP and a decrease in lymphocyte counts are associated with COVID-19 severity. Both C3a and C5a positively correlated with CRP levels (Figure 3A and B) and negatively correlated with lymphocyte count (Figure 3C and D). There was also a positive correlation between levels of C5a and C3a (Figure 3E).

The temporal relationship between C3a, C5a and CRP was further examined in the five patients with measurements

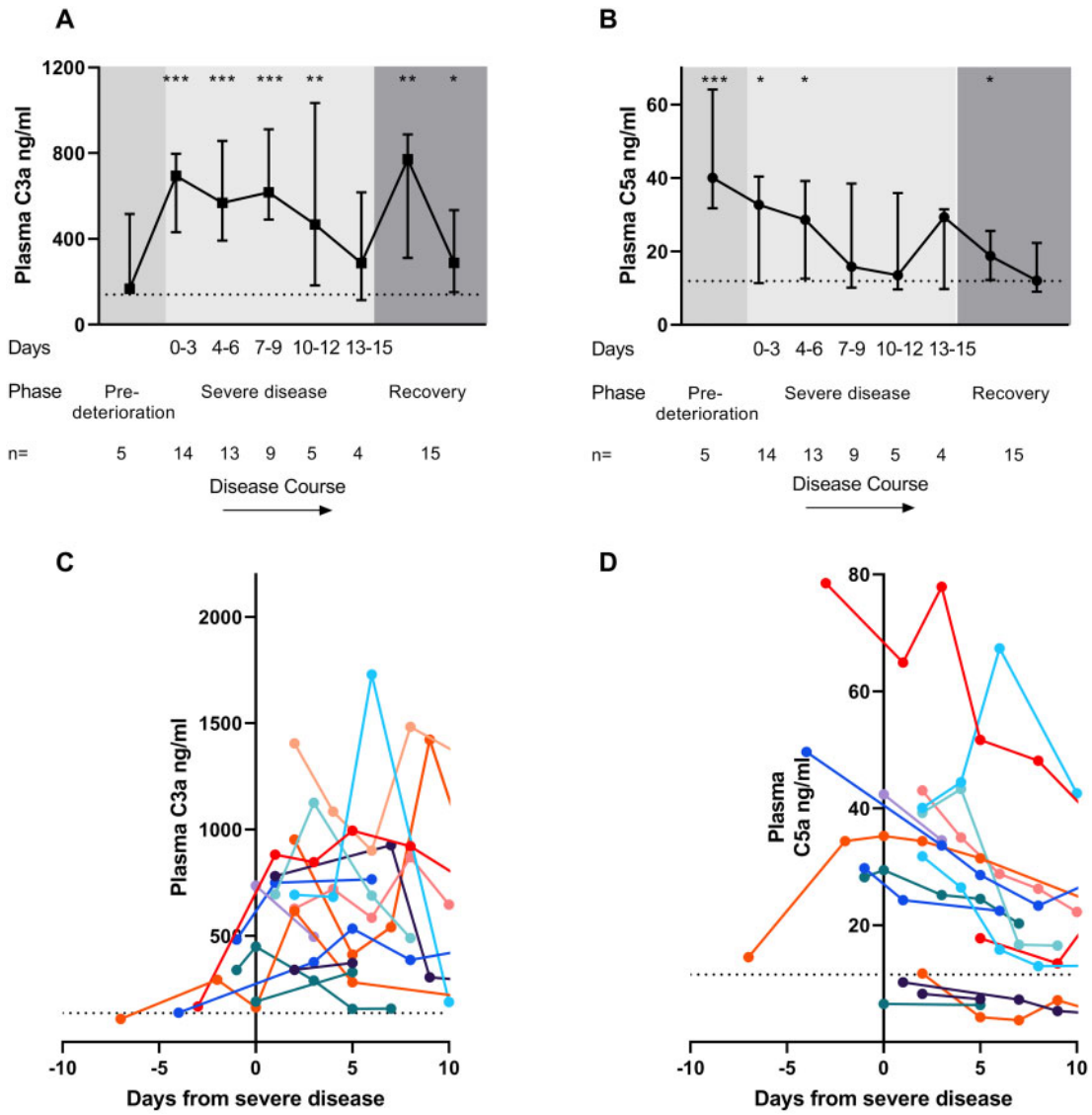

FIGURE 2: Plasma C5a but not C3a is elevated prior to clinical deterioration in HD patients with severe COVID-19. Serial measurements of (A) C3a and (B) C5a for each patient with severe disease ($n = 20$). The timing of the samples is denoted by clinical course and consists of the pre-deterioration phase ($n = 5$), severe phase (number as indicated) and recovery phase ($n = 15$). The dotted line represents the median value in dialysis controls. The horizontal bar denotes the median and whiskers denote the interquartile range. *P < 0.05, **P < 0.01, ***P < 0.001 derived from Mann–Whitney test. Serial measurements of (C) C3aand (D) C5ain patients with severe disease for whom a sample were available early (≤2 days) in their disease course. This represents 15 of the 19 patients with severe disease. The dotted lines represent the median value in dialysis controls.

available prior to disease deterioration (Figure 4). In these patients, C5a increased prior to an increase in CRP, whereas C3a levels appeared to correlate more closely with CRP and clinical status.

DISCUSSION

Our study shows evidence of complement activation in patients with severe COVID-19. Other studies have also reported elevated C5a levels in patients with severe and critical disease [3, 4] but, to our knowledge, this is the first study to utilize multiple serial samples to track the progression of complement activation throughout the disease course. In addition, by investigating a cohort of maintenance HD patients we were able to obtain samples from a group of patients with mild disease who, if they had not been attending for HD sessions, would likely not have had contact with healthcare services. This group, with mild symptoms, is likely to be underrepresented in other studies. Disease course in our patients receiving maintenance HD was comparable to that of other reported patient groups in terms of clinical features and biochemical parameters [15]. Our results are therefore likely to be translatable to other groups of patients with COVID-19 without a background of renal disease.

In the group of patients who developed severe disease but for whom samples were available prior to clinical and biochemical deterioration, C5a was already elevated at this early time point whereas C3a levels more closely correlated with other known parameters of disease severity (Figure 5). These data indicate that elevation of C5a may be a more sensitive predictor of

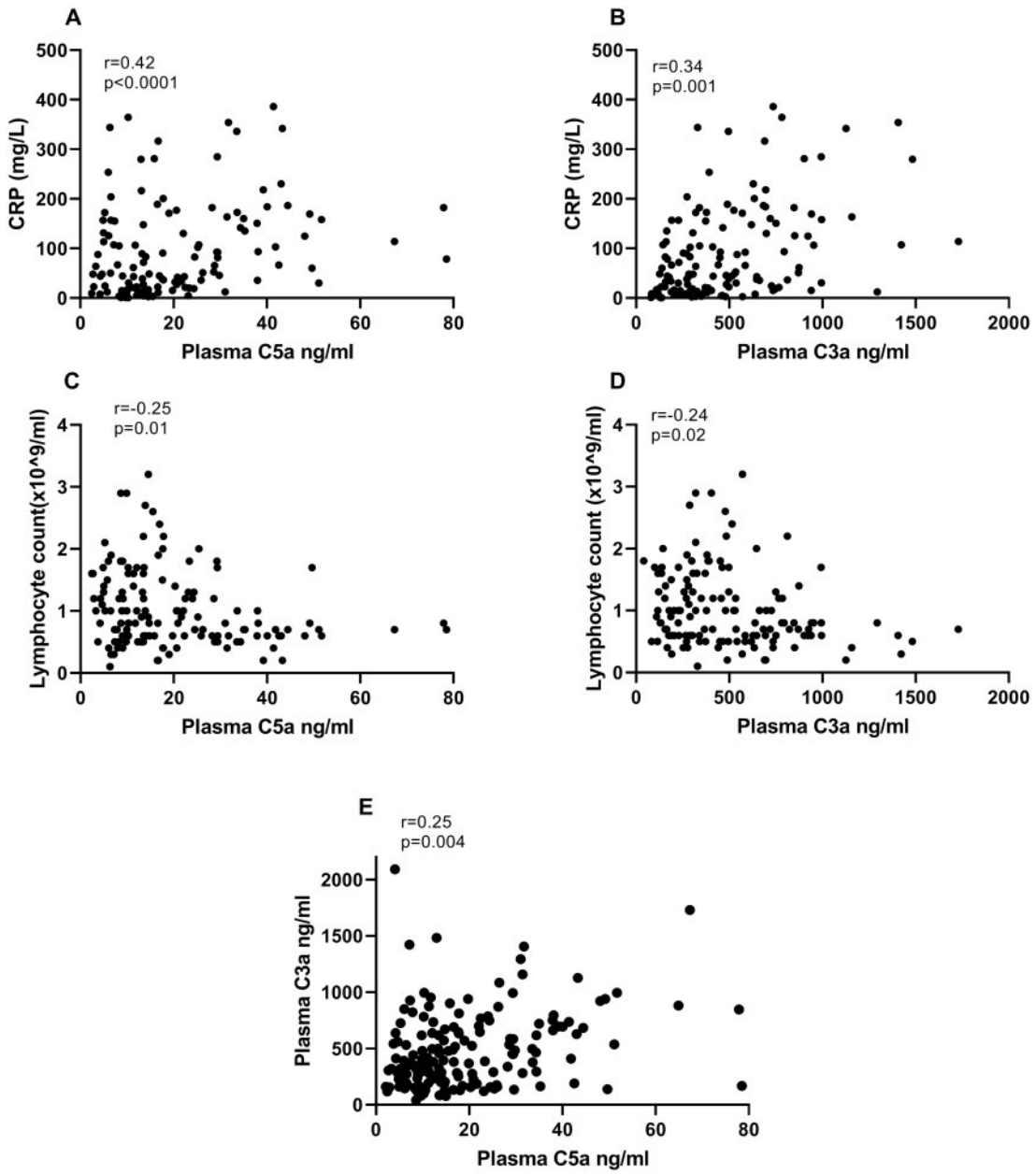

FIGURE 3: Correlation between C5a, C3a, lymphocyte count and CRP. Correlation between (**A**) C5a and CRP, (**B**) C3a and CRP, (**C**) C5a and lymphocyte count, (**D**) C3a and lymphocyte count and (**E**) C5a and C3a. Statistical analysis was performed using repeated measures correlation, as the data included multiple samples from the same patient.

clinical deterioration than CRP or other clinical parameters and future studies designed to assess it as a biomarker may be warranted.

Our findings suggest that activation of complement plays a role in the pathogenesis of COVID-19, leading to endothelial injury and lung damage. It is likely that C5a and C3a are contributing to cytokine storm, leucocyte recruitment/activation and amplification of the inflammatory cascade. Complement activation may also be mediating endothelial cell damage, causing leucocyte–platelet aggregates to form and activating coagulation pathways leading to microthrombi. Further studies are indicated to investigate both the mechanisms by which SARS-CoV-2 induced complement activation and how this then leads to severe disease. One study has suggested a role for the lectin pathway as the initial mechanism for complement activation, showing binding of SARS-CoV-2 N protein to (mannan-binding lectin serine protease-2) [16].

The use of eculizumab to inhibit C5 activation has been described in a series of four patients who were admitted to the intensive care unit and recovered. However, these patients all received antiviral therapy and hydroxychloroquine in addition to eculizumab [17]. There are other isolated case reports of patients treated with alternative anti-C5a and anti-C3 agents under compassionate use and these agents were apparently well tolerated [16, 18]. *In vitro* studies have shown that avdoralimab, a monoclonal antibody targeting C5aR1 can inhibit C5a-mediated myeloid cell activation in cells isolated from patients with COVID-19 [4]. There are currently three registered clinical

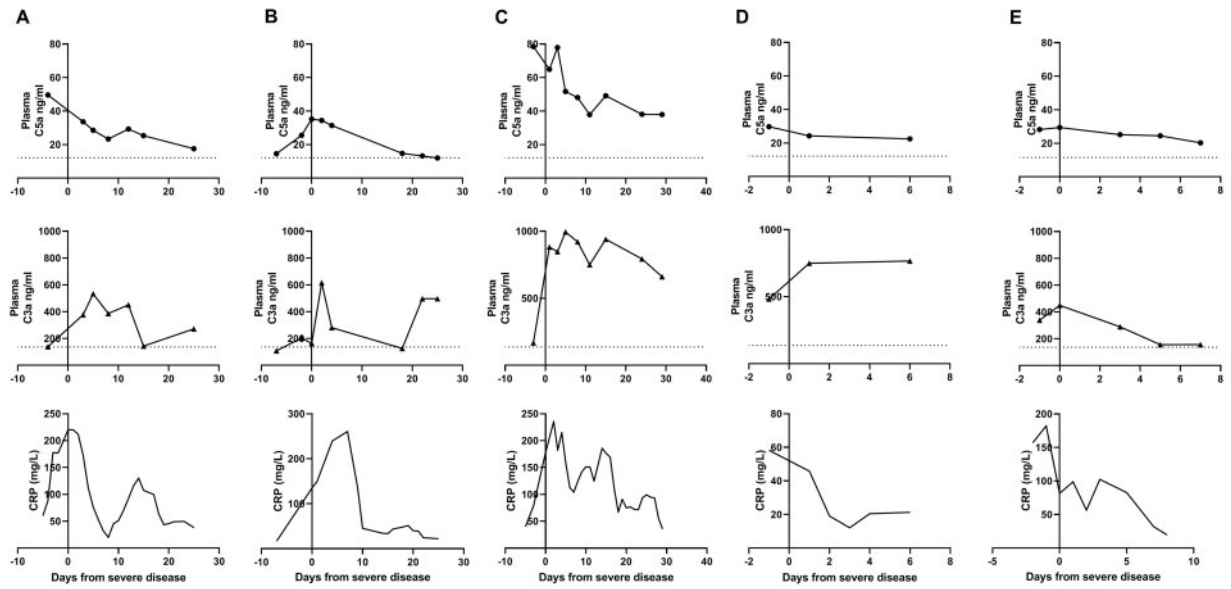

FIGURE 4: Temporal relationship between C5a, C3a and CRP in patients with samples available prior to developing severe disease. Serial C3a, C5a and CRP measurements for the five patients with samples available prior to developing severe COVID-19. Each panel (**A**–**E**) of three graphs represents an individual patient.

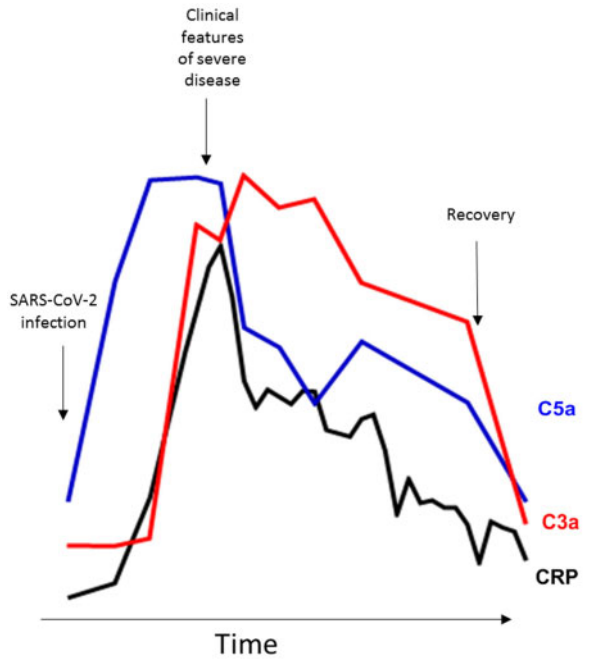

FIGURE 5: Conceptual diagram of the putative temporal relationship between C5a, C3a, CRP (and other markers of disease severity) and clinical COVID-19.

trials for off-label use of eculizumab and one for the use of IFX-1 (an alternative anti-C5a agent) in COVID-19. Our results, together with other reports showing deposition of complement components at sites of tissue injury [5], strongly advocate that inhibition of complement activation may be a viable therapeutic strategy for the treatment of severe COVID-19. As a small number of patients did not have elevated plasma C5a despite severe disease features, it is possible that this may predict response to treatment with C5a inhibitors. As such, measurement of plasma C5a could be used to stratify patients to appropriate treatment. In addition, our analysis of serial samples has uncovered that C5 activation may precede clinical deterioration, raising the possibility that it could be used as a predictive marker of disease progress.

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CONFLICT OF INTEREST STATEMENT

M.C.P. has received personal fees from Apellis, grants and personal fees from Achillion Pharma and personal fees from Alexion Pharma.

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