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C3:CH50 ratio as a proposed composite marker for Eculizumab monitoring in atypical hemolytic uremic syndrome: Preliminary results

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Abstract

Treatment of atypical hemolytic uremic syndrome (aHUS) by the complement C5 inhibitor Eculizumab (Soliris®) is highly effective but unfortunately, associated with an economic pressure on the health care systems even in high incomes countries. Despite spacing infusions has been proposed as the unique solution to minimize this economic impact, no reliable laboratory assays are available to tailor such therapy optimization. To propose and evaluate a complement composite marker for eculizumab efficacy monitoring in aHUS. We have retrospectively analyzed complement profiles data of eight aHUS patients under eculizumab from the International Registry of HUS/Thrombotic Thrombocytopenia Purpura, and calculated a novel marker *'C3:CH_{50} ratio'* by dividing C3 value by CH_{50} one for each sample during induction and maintenance periods. The results significance was compared to the currently used biomarkers for

eculizumab tailoring. In contrary to the current biomarkers used for eculizumab efficacy monitoring like CH_{50} and soluble or deposit membrane attack complexes, 'C3:CH_{50} ratio' seems to be the most interesting one since its value at pre-Eculizumab dosage equaled 0.92± 0.2 while the post-Eculizumab one increased significantly to reach 24.54± 10.7; p< 0.001. Furthermore, this ratio correlated negatively with platelets count (r=- 0.722, p= 0.018) whilst no correlation was found within the thrombotic microangiopathy (TMA) biomarkers and complement blockade for the other parameters that change in pre and post-eculizumab therapy. As far as we know, this is the first study that suggests a post-Eculizumab parameter correlating simultaneously with drug's activity (complement inhibition) and disease activity (platelets counts). Nonetheless, the limited number of patients enrolled in this study should be considered in larger studies to guide Eculizumab optimization by indicating the time when subsequent withdrawal or infusion spacing is allowed or recommended.

Keywords: eculizumab, aHUS, alternative complement pathway, composite marker, drug monitoring.

Introduction

Hemolytic uremic syndrome (HUS) is an important group of renal disorders and an ultra-rare form of thrombotic microangiopathy (TMA) which affects patients of all ages (1). Since the early 2000s, the complement alternative pathway (AP) dysregulation has emerged as the major cause of the atypical HUS (aHUS), responsible for 60–70 % of cases (1-4). A persistent cleavage of complement protein C5 by AP leads to generation of proinflammatory C5a and lytic C5b proteins, and the formation of the membrane attack complex (C5b–9), which in turn results in endothelial cell activation, injury, and death (Figure 1). By contrast to other immunodeficiencies,

aHUS shows no particular relationship with repetitive infections but is associated with lifelong risk of thrombocytopenia, hemolysis, and organ damage (renal, neurological, cardiovascular, pulmonary, and gastrointestinal impairment) (5).

The past decade's successful challenges in aHUS science were essentially (i) the great advances in aHUS pathogenesis understanding and (ii) the availability of Eculizumab (Soliris®), a monoclonal antibody that binds C5 and prevents the AP activation and organ damage (5). Noteworthy, the European Medicines Agency (EMA) and Food and Drug Administration (FDA) have approved eculizumab according to the following scheme: the initial or induction phase (up to four weeks for patients with ≥40 kg body mass) by infusion of 900mg every week initially and then followed by the maintenance phase using 1200 mg every second week on maintenance, which costs US$ 573,720 per patient per year (6). Consequently, the issue to minimize the economic impact starts to challenge heavily the medical and scientific communities. Recently, the concept of "the optimization of eculizumab administration" was introduced, based on the two following pillars: (i) define the best choice for each individual patient, and (ii) Tailor this therapy on the basis of scientific evidences (7).

In other hand, complete complement blockade by eculizumab occurs *in vivo* with serum concentrations higher than 35µg/mL (8) and the expected concentration required to inhibit the complement-mediated hemolysis has been reported to be from 50 to 90µg/mL. However, the inter-individual variability influences largely this plasma bioavailability. Philippe Gatault et *al.* have found that when someone respect the recommended schedule regimen, several patients may reach more than 4 folds of the target concentration (100% of patients reach eculizumab plasma levels >50µg/mL, 88.9% of patients>100 µg/mL and 55.6% of patients >300 µg/mL) (9). According to these evidences, a pharmacokinetics model was needed for drug tailoring to adjust

eculizumab serum concentrations and minimize the economic impact. In fact, a one-compartment model with both first-order and Michaelis–Menten ratesable was developed with respect to the patient's weight to satisfactorily describe eculizumab pharmacokinetics. A weight-based schedule to maintain permanent and fully blocking complement activity was developed as follow: spacing infusions by (i) 2 weeks for patients of 90 to 120 kg, (ii) 4 weeks for 70 to 90 kg, and (iii) 6 weeks for less than 70 kg (9). Nevertheless, in daily clinical practice this weight-based approach lacks precision and requires a reliable biomarker for the best clinical response and most economical use of this expensive drug. Obviously, the Italian medical team of Prof. M. Cugno has succeeded to space infusions at intervals of 3 or 4 weeks in stable patients by measuring the degree of C5 blockade using several hemolytic assays like C5 functional assay, classical pathway functional assay CH_{50} and the alternative pathway functional assay AH_{50} (*7*).

Later, the International Consensus of aHUS Management has recommended for eculizumab optimization to assess the drug's activity, expressed by CH_{50}, rather than disease activity (serum Hemoglobin (Hg), lactate dehydrogenase (LDH), and platelet count). Thus, only the patient who maintains a complete complement inhibition with CH_{50} activity less than 10% is concerned by longer intervals or lower doses (10). Nonetheless, the optimal or individualized treatment schedule has not yet been established because of the lack of reliable tests for treatment monitoring (11).

In this way, very recently, Marina Noris et al. found that the complete complement inhibition assessed by CH_{50} and/or by soluble C5b-9 could not be reliable to monitor Eculizumab therapeutic efficacy in aHUS (6). These exciting breakthroughs drew our attention to look for a novel tool for eculizumab monitoring which would correlate with disease activity. Basically, our hypothesis was based on the following evidences (Fig.1). Briefly, the AP's organ damage is

initiated by C5 convertase, the enzyme that cleave C5. It is composed of C3 convertase itself associated with high amount of C3b (n_{C3b}>2). The intensity of C3 cleavage and plasma C3b availability determine whether the AP stops at the level of C3 convertase (low amount of C3b) or continues to form lytic complexes of C5b-C9 (in the case of high amount of C3b) (12). Innate or acquired deficiency in the complement regulatory proteins, that block C3 and/or C5 convertase and neutralize C3b, constitutes the biochemical abnormality of aHUS. It leads to an intense formation of C5 convertase enzyme and consequently high consumption of C3. Using eculizumab dramatically reduces the plasma C5 and C3 convertases, complement lytic capacity (CH_{50}) and cause changes in C3 levels (12, 13). The plasma C3 consumed by CH_{50} is translated mathematically into an arithmetic division; $C3/CH_{50}$. Since each sample could be assayed for its CH_{50} and C3levels, it would be easily to calculate this ratio. Thus, the composite ratio '*$C3{:}CH_{50}$ ratio'* is calculated by dividing C3 level to CH_{50} one of a given sample to evaluate eculizumab efficacy in complement blockade.

Material and Methods

To examine our composite marker we have conducted a retrospective study based on the International Registry of HUS data published by M Noris et *al.* (6). We have used the criteria for eculizumab efficacy and safety that contain the primary end points, based on TMA complications like platelets count, and the secondary end points, based on hematologic outcomes like lactate dehydrogenase (LDH), serum creatinine and hemoglobin (Hg) levels (5).

A followed up study of eight (08) patients for a cumulative observation period were collected. During the periods of pre- and post-Eculizumab therapy, laboratory parameters for each sample were obtained at various time intervals to measure TMA laboratory markers (Hg,

LDH and platelet count) and complement activity parameters (CH_{50}, C3, C5a, Soluble C5b-9 and deposites C5b-9) (6).

Briefly, cases 1 to 4 started eculizumab treatment at 13, 8, 8, and 30 days after aHUS onset, respectively. Cases 1, 2, and 4 received 4 infusions 900 mg weekly, then 1200 mg fortnightly; case 3 received 2 infusions 300 mg weekly, then 300 mg every 2-3 weeks. Two samples were analyzed for each of them in pre-eculizumab therapy and few weeks in post-therapy. We analyzed samples' data taken in acute phase (pre-Ecu) for the four cases and at 9 days, 14 d, 21 d and 14 d of post-Ecu periods for Case1 to Case4, respectively.

Case 5: a 7-year-old child, four samples were analyzed for this patient: at remission (pre-Ecu), 8d post-Ecu, 12 d and 11 d post-Ecu 600 mg. Eight days after the former eculizumab infusion indicated almost complete circulating C5 inhibition, the eculizumab dose was doubled (from 300 to 600 mg every other week). After 6 months of 600 mg eculizumab, sC5b-9 and CH_{50} levels measured in parallel did not differ from values obtained under 300 mg eculizumab.

Case 6: an 8 year-old boy in whom eculizumab was started (600 mg weekly for 2 infusions and then every 12/14 days), and then increased to 900 mg. Three plasmas were used for this patient: in acute (pre-Ecu), 6 d post-Ecu 600 mg, 6 d post-Ecu, 900 mg.

Case 7: A 35 years male who received 9 eculizumab infusions (600-1200mg) during the first month and then 1200mg fortnightly. The following 18 months eculizumab treatment was spaced every 3 weeks. This study included the analysis's data of three samples of this patient; acute (pre-Ecu), 14 d post-Ecu 1200 mg, 21 d post-Ecu 1200 mg.

Case 8: A woman with recurrent aHUS since 34 years of age, Eculizumab was given (900 mg weekly, 5 infusions), and then 1200 mg eculizumab fortnightly. Eighteen months later,

eculizumab was spaced to 1200 mg once a month. Data of two samples' analysis were collected: acute (pre-Ecu), 14 d post-Ecu 1200 mg, 1 mo post-Ecu 1200 mg.

After data collection, statistical analyses were performed using one-way ANOVA and unpaired two-tailed t-test. Pearson rank test was used for correlation testing while U Man Whitney test was used for mean comparisons. Unfortunately, plasma eculizumab depends on body weight being (9) and neither free eculizumab nor plasma sC5b-9-eculizumab complexes were available in the International Registry of HUS/thrombotic thrombocytopenia purpura (TTP) to proceed to further statistical analysis(*6*).

Results

TMA markers were measured during induction and maintenance periods. The complement profiles monitored during therapy were available for each sample of the eight patients. Our data analysis obtained from the database published by M. Noris et *al.* confirmed the efficacy of eculizumab in aHUS by the improvement of all the biological disease activity markers (Fig.2). There were a significant difference in serum CH_{50} (% of control) (113.85± 22.21 vs.12.3± 3.69); and C5b-9 deposits levels (% of control) (442± 90 vs.85± 9.25) for patients under eculizumab while serum C3 and plasma SC5b-9 levels don't change between pre- and post-eculizumab periods (Fig.3). Interestingly, using our hypothesis, we have found that C3:CH_{50} ratio changed more significantly in post-Eculizumab (0.92± 0.2 *vs.* 24.54± 10.7) $p<.001$ compared to serum CH_{50} and C5b-9 deposits levels. Of note, this ratio correlated negatively with platelets increasing (r= -0.722, p= 0.018) (Fig.4A&B) while no correlation could be found within TMA biomarkers and the complement blockade for the other marker that change in pre and post-Ecu periods (Table 1). Thus, this proposed ratio may be of help to monitor eculizumab efficacy.

Discussion

The therapeutic complement blockade by eculizumab was proposed for the conditions where complement regulatory proteins are insufficient or when the complement activation overwhelms physiological regulation. Eculizumab (Soliris®, manufactured and marketed by Connecticut-based Alexion Pharmaceuticals) is the unique approved treatment for aHUS. It is a humanized monoclonal antibody that binds with high affinity the human C5 complement protein and blocks the formation of proinflammatory C5a and lytic C5b. Consequently, it prevents deleterious properties of the amplification and terminal complement pathways activation with respect to immunoprotective functions of proximal complement proteins. This human hybrid carries out immunological features of both IgG2 and IgG4 constant regions. IgG2 fails to activate cellular immunity by Fc receptors while IgG4 does not activate complement in purpose to reduce the pro-inflammatory potential of this molecule and increase its plasma half life to ~11 days (14). Importantly, the current guidelines on eculizumab treatment prescribe the same dose regimen for all adults (≥18 years of age) regardless their inter-individual variability; and consequently, a majority of aHUS patients receive substantially more drug than needed for complete C5 inhibition (15). An individualized approach to dose regimen would optimize the needed drug quantity and redefine intervals between doses (7).

Noteworthy, the International Consensus of aHUS Management has postulated that the therapy tailoring has to be based on drug's activity (complement blockade) rather than disease activity (10). While the lectine pathway assay are not suitable for eculizumab monitoring, regarding the high frequency of MBL deficiency, a very close correlation for the classical pathway (CP) and alternative pathway (AP) was seen (11). Once the assessment of the CP was recommended as the monitoring tool for eculizumab therapy, several assays were proposed like

the quantification of membrane attack complexes (sC5b-9) in its two forms, the plasmatic and the deposit one (6). However, Eculizumab engineering was performed to eliminate any bioactivity of this monoclonal antibody except C5-blockade, because it contains a CH2 domain of IgG2 and CH3 of IgG4 to avoid clearance by the complement system and phagocytosis, respectively. Thus, serum sC5b-9 has no interest because it forms clinically inactive Eculizumab-sC5b-9 complexes with slow plasma clearance that will accumulate and variable concentrations will be obtained independently of the drug activity or complement blockade.

The current study has shown that changes in C3 levels between pre-and post-ecu were not significant; therefore no interest could be expected for it as therapeutic monitoring tool. Moreover, previous report emphasize that plasma C3 levels cannot be recommended for therapy monitoring because a low C3 level such as seen in several aHUS patients is not expected to normalize under eculizumab (6). Despite our work found that CH_{50} change significantly in pre and post-ecu and correlate with platelets count, several research stress that it still not reliable to tailor eculizumab optimization (6, 7). M. Noris et *al.* have demonstrated that neither CH_{50} nor sC5b-9 deposits parallel with disease activity recorded the same days (6). Furthermore, M. Cugno et *al.* have demonstrated that CH_{50} don't necessary correlate with C5 blockade assessed by C5 functional assay. R. Peffault de Latour *et al.* explained that by the *batch-to-batch* variation in quality of sheep erythrocytes, and the fact that the traditional hemolytic CH_{50} assay is prone to variability in lower detection range, which might be clinically significant (16). Therefore CH_{50} is not sufficiently reliable for eculizumab blockade assessment and our conception for a composite parameter that combines both C3 and CH_{50} of a given sample is proposed as an alternative tool. Our proposed '*C3:CH50 ratio'* correlates significantly with the platelets count increases (Fig.4). By contrast to M. Noris et *al*. who have concluded that C5b-9 deposits test is not just a surrogate

of platelet count measurement because serum-induced endothelial C5b-9 deposits (% of controls) under eculizumab treatment did not correlate negatively with platelet counts (r=0.009) (6), we have found that our composite marker change negatively significantly with platelets count (fig.4.B). Consequently, this ratio would be a useful tool to monitor eculizumab efficacy and personalize eculizumab therapy on the basis of the drug's activity (CH_{50}) and disease activity markers (Platelets). Furthermore, this ratio may find all interest in situations that preclude the use of CH_{50} or other complement biomarkers for complement blockade monitoring, like patients underlying hypocomplementemia who maintain low C3 and low CH_{50} independently of the therapy responsiveness. To link CH_{50} activity with the first protein of the amplification pathway, complement C3, within a ratio would reflect exactly the *in vivo* situation (Figure1).

Recently, EB.Volokhina et *al.* have developed a sensitive, reliable and easy-performed assay which can be used to design individualized eculizumab dosage regimens. This assay, as independent on personal skills, can be performed at any hospital where ELISA equipment is established (11). Another study suggested the use the standardized assay based on Wieslab® complement system as a screening test for eculizumab responsiveness (17). Thus, it is possible that in the future only one of these is needed, but more data are needed in order to compare these different assays and parameters and decide which test should be chosen.

One of the present study limits is the lack of validation of this ratio in other AP-mediated damage conditions like paroxysmal nocturnal hemoglobinuria (PNH). Nevertheless, for PNH, a recent study over a two-year period was conducted in patients treated with eculizumab with serum analysis of CH_{50} and free eculizumab (16).

Conclusion

As far as we know, this is the first study that suggests a composite marker that changes significantly during induction and maintenance periods by eculizumab and correlates with the disease activity primary marker platelets count. However, retro- and prospective studies in a larger number of patients are needed to prove the sensitivity of this C3:CH50 ratio in purpose to guide eculizumab dosage and spacing in aHUS patients. At the end, we stress that eculizumab administration's optimization according to a scientific-based therapy tailoring still an unmet need in aHUS research and management.

Declaration of interest

K.E.K & D.B state that they have no conflict of interest.

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FIGURES & TABLES

Figure 1. The alternative complement cascade in aHUS. In aHUS, the pathogenic cascade of the alternative complement pathway, the complement amplification loop, is responsible of the *in vivo* damage (glomerular endothelial cell) (A). It can be reproduced *in vitro* by using sheep erythrocytes-based immune complexes (CH50 assay) (B). The complement lytic capacity depends the membrane attack complex amount (MAC, C5b-9) which depends on the quantity of cleaved C5, which depends itself on the amount of plasma C3. The introduction of Eculizumab (50-90 µg/mL) blocks this cascade *in vivo* and *in vitro* from the C5 point.

CD55, CD59, CD46 (MCP = Membrane Cofactor Protein) are the cell membrane regulatory proteins that block the AP activation to prevent tissue injury. aHUS: atypical hemolytic and uremic syndrome, MAT: microangiopathy thrombotic, CH_{50}: complement hemolytic 50.

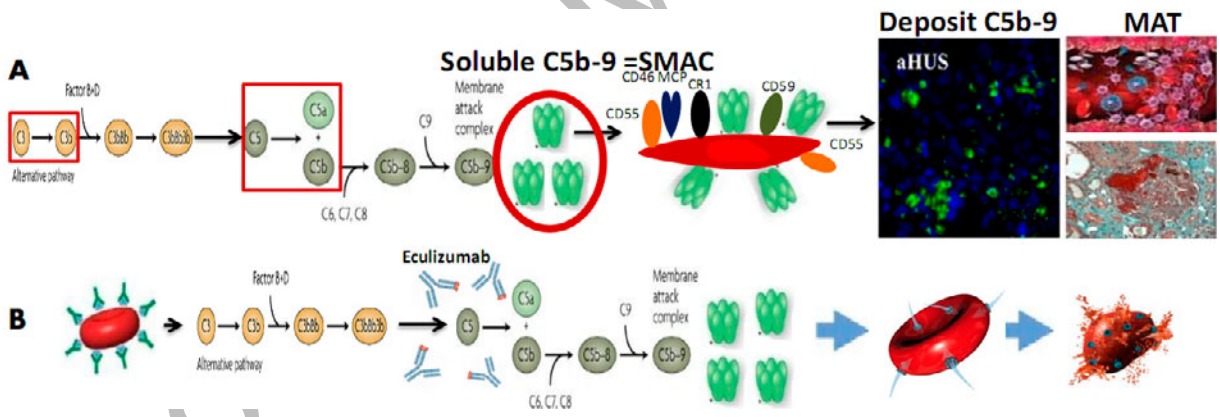

Figure 2. Eculizumab efficacy. In post-Ecu period, all the micro-angiopathy (MAT) biomarkers tend to amelioration. Platelets ($x10^3/\mu L$), lactate dehydrogenase [LDH] (IU/L), hemoglobin (g/dL) and serum creatinine (mg/dL).*P at < .05.

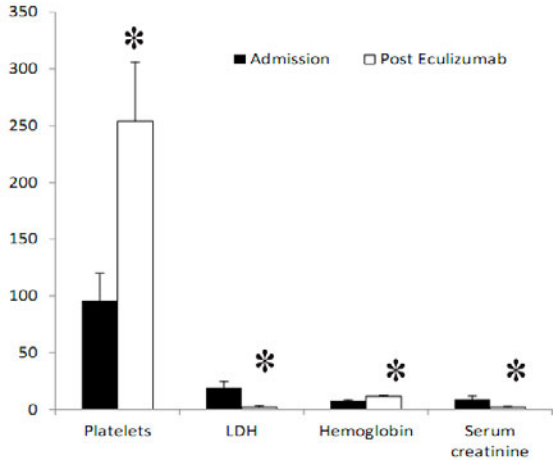

Figure 3. Complement blockade biomarkers in pre- and post-Eculizumab periods. Only the serum CH_{50} level and endothelial cell deposits of membrane attack complexes decrease in response to eculizumab regimen. Serum C3 (mg/dL), Plasma soluble membrane attack complexes [sC5b-9] (Eq/mL), Serum complement hemolytic 50 [CH50] (U Eq/mL) and membrane attack complexes deposits on cell surface [C5b-9 deposits] (% of control). *P at < .05, NS: no statistical significance.

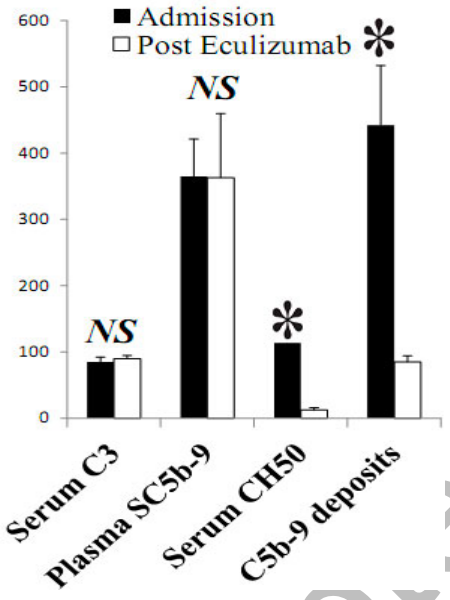

Figure 4. C3:CH50 ratio as a marker for Eculizumab efficacy. The most significant difference of the complement markers in post-Ecu was obtained for C3:CH50 ratio (A). Furthermore, C3:CH50 is the unique parameter that parallel with disease biomarkers (B). **P at < .001.

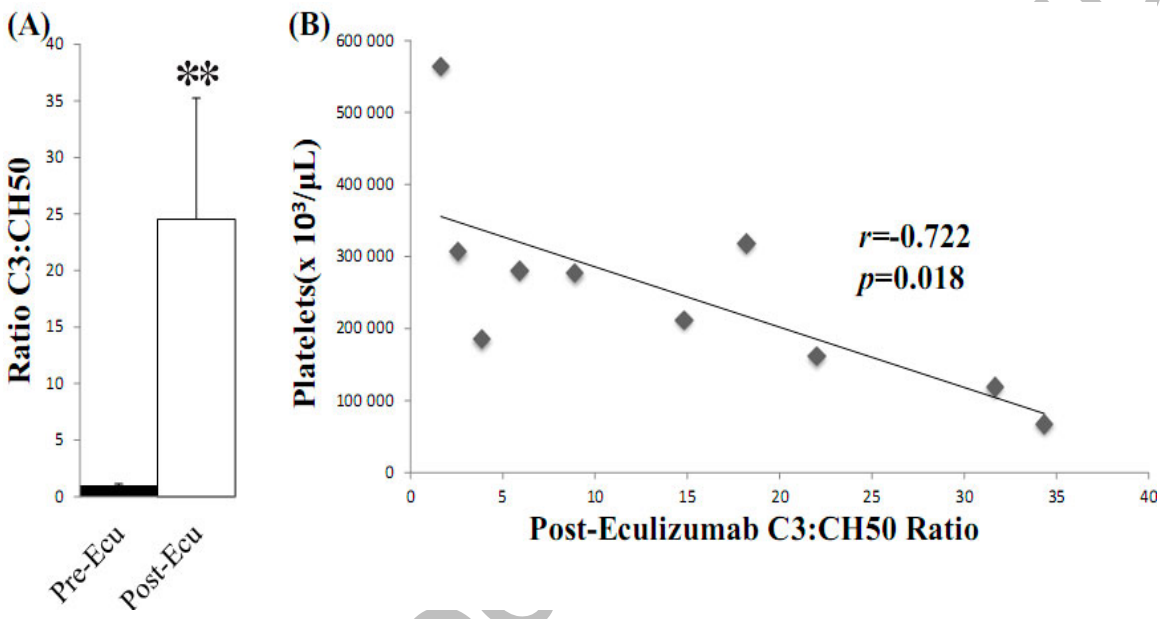

Table 1. Pearson correlations among disease and complement biomarkers.

Correlation	Pearson coefficient *r*	*P*
C3		
vs. Platelets	- .543	.083
vs. LDH	.759*	.01
vs. Hemoglobin	- .537	.088
vs. Serum creatinine	.433	.182
vs. Serum CH_{50}	*- .530*	*.094*
Plasma SC5b-9		
vs. Platelets	.065	.831
vs. LDH	-.212	.507
vs. Hemoglobin	.039	.897
vs. Serum creatinine	- .0231	.94
Serum CH_{50}		
vs. Platelets	.796**	.001
vs. LDH	-.592*	.042
vs. Hemoglobin	.152	.619
vs. Serum creatinine	-.326	.276
C5b-9 deposits		
vs. Platelets	.009	.975
vs. LDH	.229	.472
vs. Hemoglobin	.086	.777
vs. Serum creatinine	-.113	.712

C3: Serum C3 (mg/dL), Platelets (x 10^3/µL), LDH: lactate dehydrogenase (IU/L), Hemoglobin (g/dL), sC5b-9: Plasma soluble membrane attack complexes (Eq/mL), CH50: Serum complement hemolytic 50 (U Eq/mL), C5b-9 deposits: membrane attack complexes deposits on cell surface (% of control). *$P < .05$, **$P < .01$.