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The complement component C5 is not responsible for the alternative pathway rabbit erythrocyte hemolytic assay activity during eculizumab treatment

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Running title: C5 is fully blocked during eculizumab treatment

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Dear Editors,

Eculizumab is a therapeutic complement C5 inhibitor, approved for the treatment of atypical hemolytic uremic syndrome (aHUS), paroxysmal nocturnal proteinuria (PNH), generalized myasthenia gravis and neuromyelitis optica spectrum disorder. Correct dosing of eculizumab is important to ensure complete complement blockade. Complement activity is often monitored by measuring either formation of the C5 activation product C5b-9 *in vitro* using an ELISA, independent of red blood cells, or by hemolytic assays measuring hemoglobin release from erythrocytes. Both approaches allow analysis of complement activation via classical (CP) and alternative (AP) pathways.

Reported degree of complement inhibition varies in patients that attain target drug concentrations (>100 µg/mL), depending on the assays used. Studies with ELISA-based CP and AP assays[1-5], demonstrate full blockade in patient samples containing target drug concentrations. However, studies with hemolytic assays report various degree of hemolysis in the presence of eculizumab, which could be interpreted as incomplete C5 blockade by the drug. When CP hemolytic assay is used, complete eculizumab blockade is usually observed[1, 6-9]. Nevertheless, considerable AP hemolytic activity is frequently reported as ongoing under eculizumab treatment even when eculizumab should be in excess[1, 6, 10].

The question of possibly incomplete blockade becomes especially relevant in cases where a patient does not respond well to eculizumab therapy. Then, it is important to know whether lack of response may be related to residual C5 activity in order to determine further treatment strategy by increasing dosage or choosing other treatment options. Thus, in this study we investigated whether the residual hemolysis of erythrocytes detected by the complement AP assay in the presence of eculizumab may be caused by incomplete C5 blockade by the drug or could be explained by C5-independent mechanisms.

We analyzed samples from patients treated with eculizumab, in erythrocyte-based hemolytic assays. Because serum concentration used in hemolytic assays varies between the protocols of different laboratories, we tested samples in broad range of serum concentrations.

Samples from five aHUS patients, containing 207-367 µg/mL of eculizumab, were analyzed. In the AP erythrocyte assay high degree of hemolysis was observed, increasing to 73% at the highest serum concentration (50%) (Figure 1A). In CP hemolytic assay, all patient samples gave results comparable to heat-inactivated normal human serum (HI-NHS), used as a negative control (Figure S1A); i.e. < 5% hemolysis even at 50% serum concentration. Furthermore, normal human serum (NHS)

control spiked with increasing amounts of eculizumab showed the same high lytic activity in AP with complete blockage of CP in hemolytic assays (Figure S1B and S1C).

Further, we tested whether hemolytic activity in eculizumab-containing samples can be caused by the residual formation of C5b-9. We chose the commercial Wieslab CP and AP assays, as they are widely used. No C5b-9 formation was detected in patient samples or NHS spiked with eculizumab at target values in AP (Figure 1B and Figure S2A) or CP Wieslab assay (Figure S2B and S2C). Moreover, no soluble C5b-9 (sC5b-9) was formed in NHS activated by zymosan A in the presence of eculizumab, while formation of a C3 activation markers C3bBbP, C3bc and C3s was unaffected by the drug (Figure 1C, 1D, S3A, S3B). Moreover, serum of a genetically C5 deficient donor produced lysis of erythrocytes identical to that of NHS spiked with 500 µg/mL eculizumab, both reaching 85% lysis at the highest serum concentration in AP hemolytic assay, but not in CP hemolytic assay or Wieslab tests (Figure 1E, Figure S4).

As shown above, C3 activation was not affected by the presence of eculizumab. Thus, we investigated whether hemolysis in AP could be affected by C3 activity. We incubated NHS and C5-deficient serum with the potent C3 inhibitor compstatin Cp40. Presence of compstatin alone inhibited hemolysis in NHS and in C5 deficient serum (Figure 1F).

Erythrocytes remaining after the AP assay (Figure 1G and 1H) were more osmotically fragile with >75% of erythrocytes exposed to NHS with eculizumab or C5 deficient serum already lyse at 0.7% of NaCl, whereas addition of compstatin Cp40 considerably decreases the lysis.

Overall our results indicate that erythrocyte lysis seen in the AP hemolytic assay during eculizumab treatment is not caused by the residual C5 activity. Residual AP hemolysis most likely occurs due to changes in erythrocyte membrane fragility, rendering it susceptible to mechanical lysis mediated by C3 activation and opsonization with C3 activation products. Erythrocytes are live cells with high sensitivity to storage time, buffer conditions and batch-to-batch variation, which is not the case for stable ELISA assays. This is a well-known experience for complement laboratories over decades, including ours. Therefore, we propose that in general the erythrocyte based assays, when possible, should be replaced by robust, reproducible ELISA methods in future complement research.

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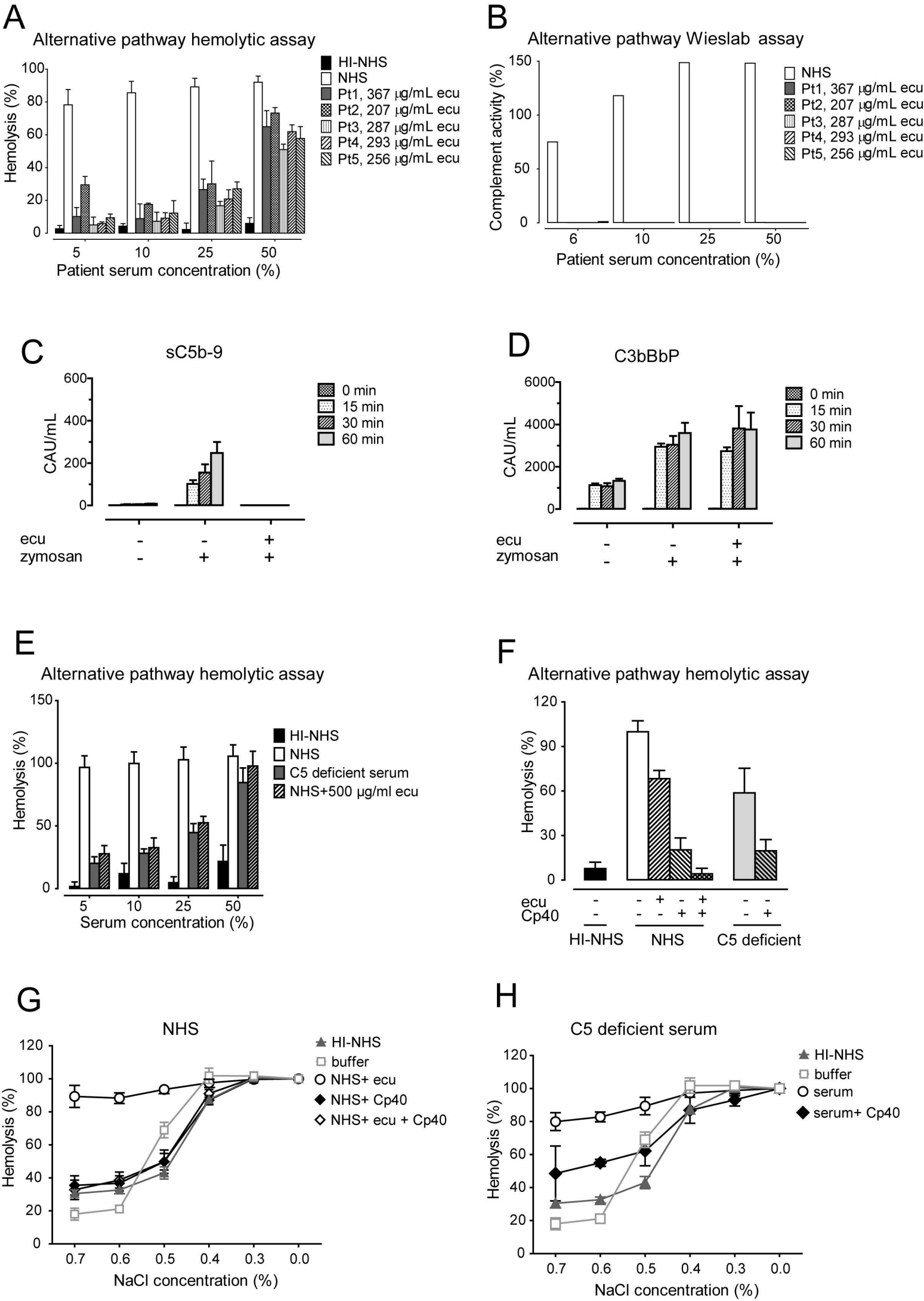
Figure legend

Samples of five aHUS patients containing eculizumab showed high degree of hemolysis in alternative pathway (AP) hemolytic assay (A). No C5b-9, in these samples was formed in AP Wieslab test (B). Moreover, no soluble C5b-9 (sC5b-9, C) was formed during *in vitro* activation of normal human serum control (NHS) with zymosan A when eculizumab was added, however generation of C3 activation product C3bBbP remained unaffected (D). In AP hemolytic assay, serum of C5 deficient donor showed similar lysis as NHS spiked with 500 µg/mL eculizumab (E). This lysis was suppressed in the presence of 40 µg/mL compstatin Cp40 (F). Rabbit erythrocytes pre-exposed to NHS spiked with 500 µg/mL eculizumab (G) or C5 deficient serum (H) at AP permissive conditions showed high osmotic fragility, which was restored by addition of Cp40.


A
Alternative pathway hemolytic assay
Hemolysis (%)
Patient serum concentration (%)
HI-NHS
NHS
Pt1, 367 µg/mL ecu
Pt2, 207 µg/mL ecu
Pt3, 287 µg/mL ecu
Pt4, 293 µg/mL ecu
Pt5, 256 µg/mL ecu
B
Alternative pathway Wieslab assay
Complement activity (%)
Patient serum concentration (%)
NHS
Pt1, 367 µg/mL ecu
Pt2, 207 µg/mL ecu
Pt3, 287 µg/mL ecu
Pt4, 293 µg/mL ecu
Pt5, 256 µg/mL ecu
C
sC5b-9
CAU/mL
0 min
15 min
30 min
60 min
ecu
zymosan
D
C3bBbP
CAU/mL
0 min
15 min
30 min
60 min
ecu
zymosan
E
Alternative pathway hemolytic assay
Hemolysis (%)
Serum concentration (%)
HI-NHS
NHS
C5 deficient serum
NHS+500 µg/ml ecu
F
Alternative pathway hemolytic assay
Hemolysis (%)
ecu
Cp40
HI-NHS
NHS
C5 deficient
G
NHS
Hemolysis (%)
NaCl concentration (%)
HI-NHS
buffer
NHS+ ecu
NHS+ Cp40
NHS+ ecu + Cp40
H
C5 deficient serum
Hemolysis (%)
NaCl concentration (%)
HI-NHS
buffer
serum
serum+ Cp40

Supplementary information

The complement component C5 is not responsible for the alternative pathway rabbit erythrocyte hemolytic assay activity during eculizumab treatment

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Materials and Methods

Sample collection

Serum samples of patients diagnosed with aHUS were collected during the course of regular treatment with eculizumab. C5 deficient serum was obtained from the deficient donor described before (1). This donor has a genetic defect with no secreted C5 protein. An NHS control was prepared by pooling the samples from 15 healthy adult individuals. Ethylenediamineteraacetic acid (EDTA) plasma samples of 17 healthy adults were used to establish reference range in C5 ELISA.

All samples were processed within 1 h of blood collection. Whole blood was allowed to clot at room temperature for 30–45 min (serum samples) or immediately placed on ice (EDTA plasma samples). Serum and EDTA plasma samples were prepared by centrifugation (10 min, 2500 × g, 4 °C), aliquoted and stored at − 80 °C. Heat-inactivated NHS (HI-NHS) was prepared by incubating NHS 30 min at 56˚C.

The study was approved by the institutional review board of the Radboud university medical center and was performed in accordance with the appropriate version of the Declaration of Helsinki. Informed consent of all patients and/or their parents as well as of the healthy volunteers was obtained prior to analysis.

Complement activity assays

CP hemolytic assay was performed using sheep erythrocytes dissolved in Alsever's solution (Håtunalab AB, Håtunaholm, Sweden). Erythrocytes were washed by resuspension and centrifugation in DGVB++ buffer (2.5 mM veronal buffer, pH 7.35, 70 mM NaCl, 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, 140 mM dextrose, 0.1% porcine gelatin) until the supernatant was clear. Then the cells were incubated with 1:1000 amboceptor (Siemens Healthcare, Erlangen, Germany) diluted in DGVB++ for 20 min and washed twice as before. Working concentration of sheep erythrocytes was set to yield 405 nm absorbance of 1.8-2.2 for 1:10 diluted suspension. All absorbance measurements were performed using Spark® multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

AP hemolytic assay was performed using rabbit erythrocytes dissolved in Alsever's solution (Envigo, Venray, The Netherlands). Erythrocytes were washed until clear and resuspended in Mg-EGTA buffer (2.1 mM veronal buffer, pH 7.4, 58 mM NaCl, 7 mM $MgCl_2$, 116 mM dextrose, 10 mM EGTA, 0.08 % porcine gelatin). Working concentration of rabbit erythrocytes was set to yield 405 nm absorbance of 0.8-1.2.

In both hemolytic assays, 10 µL of working erythrocyte suspension was mixed with 40 µL serum diluted in DGVB++ or Mg-EGTA buffers for CP and AP assays, respectively. When needed, eculizumab (Alexion Pharmaceuticals, Cheshire, CT) and/or compstatin Cp40 (a kind gift from

professor John D. Lambris) were added to the reaction mixture. Erythrocytes were incubated with serum for 60 min, after which 50 µL of EDTA-GVB buffer (4.4 mM veronal buffer, pH 7.35, 104 mM NaCl, 40 mM EDTA, 0.1% porcine gelatin) was added to each well. Remaining erythrocytes were pelleted by centrifugation and absorbance of the supernatant was analyzed at 405 nm. Full lysis was determined as absorbance of 10 µL of erythrocyte suspension lysed with 90 µL of H_2O. In CP and AP assays all centrifugation steps were performed 1 min at 800 g at RT and all incubation steps were done in Eppendorf ThermoMixer® C (Eppendorf, Hamburg, Germany) at 37˚C and 600 rpm orbital agitation.

Complement system classical and alternative WIESLAB® assays (2) (SVAR Life Science AB, Malmö, Sweden) were used according to manufacturer's instructions. Where indicated, higher serum concentrations were used in the assays. These assays are based on stable ELISA platform and are completely independent of cells.

In kinetic complement activation analysis, NHS was incubated at 37˚C and 600 rpm orbital agitation for 0, 15, 30 and 60 min in presence or absence of 100 µg/mL eculizumab (Alexion Pharmaceuticals) and 100 µg/mL zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, Zwijndrecht, The Netherlands). To stop complement activation, reactions were places on ice and supplemented with 20 mM EDTA final concentration.

Erythrocyte fragility assay

Rabbit erythrocytes were incubated for 30 min with 30% serum, supplemented with inhibitors as described for the AP hemolytic assay. To stop complement activation, 50 µL of EDTA-GVB was added to each well, remaining erythrocytes were pelleted by centrifugation, resuspended in NaCl solution (0.0-0.7 %) and incubated for 30 min at RT. Lysis was assessed as in hemolytic assays.

Immunodetection methods

Eculizumab concentrations were determined in serum using an ELISA method described before (3).

Complement activation markers C3bc, C3bBbP and soluble C5b-9 (sC5b-9) were measured using in-house ELISA methods as described before (4, 5). Quantification was done in complement activation units per mL (CAU/mL) using International complement standard 2 (ICS 2) (5). C3a was measured by using a commercial C3a Human ELISA kit (HK354, Hycult Biotech, Uden, The Netherlands).

C5 concentration was determined by an in-house ELISA. Polyclonal Antiserum to Human C5 Protein (A306, Quidel, San Diego, CA) diluted 1:10000 in carbonate buffer (pH = 9.6) was coated onto MICROLON®600 high binding 96-well plates (Greiner bio-one, Frickenhausen, Germany) overnight at

4°C. The plates were blocked with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) for 60 min. The wells were washed with PBS supplemented with 0.05% Tween-20 (Sigma-Aldrich) (PBST) after this and between each of the following steps. Serum samples and purified C5 for standard (3.9-150 ng/mL) (Calbiochem, San Diego, CA) were diluted in PBST, supplemented with 0.2% BSA. Samples and standards were added to the wells and incubated for 60 min at RT. C5 detection was performed using incubation with a Monoclonal Antibody to Human C5 (A217, Quidel) diluted 1:10 000 in PBST for 60 min at RT followed by Goat anti-Mouse-HRP (P0447, Dako, Hevelee, Belgium) diluted 1:2000 PBST with 0.2% BSA. O-phenylenediamine dihydrochloride tablets (Dako) were added as a substrate. The reaction was stopped with 0.33 M H_2SO_4, and samples were measured at 492 nm. The assay had lower limit of detection of 3.9 ng/mL; intra-assay and inter-assay variation coefficients of 7% and 14%, respectively. Normal reference range was 42-93 µg/mL as determined by mean±2 standard deviations of 17 healthy adult controls. Serum and EDTA plasma samples gave same results in this assay. C5 deficiency was confirmed by the ELISA (<0.4 µg/mL C5, reference range 42-93 µg/mL). Similar results were obtained by using Human C5 ELISA from Hycult, Uden, The Netherlands, and Human Complement C5 ELISA Kit (ab125963) from Abcam, Cambridge, UK.

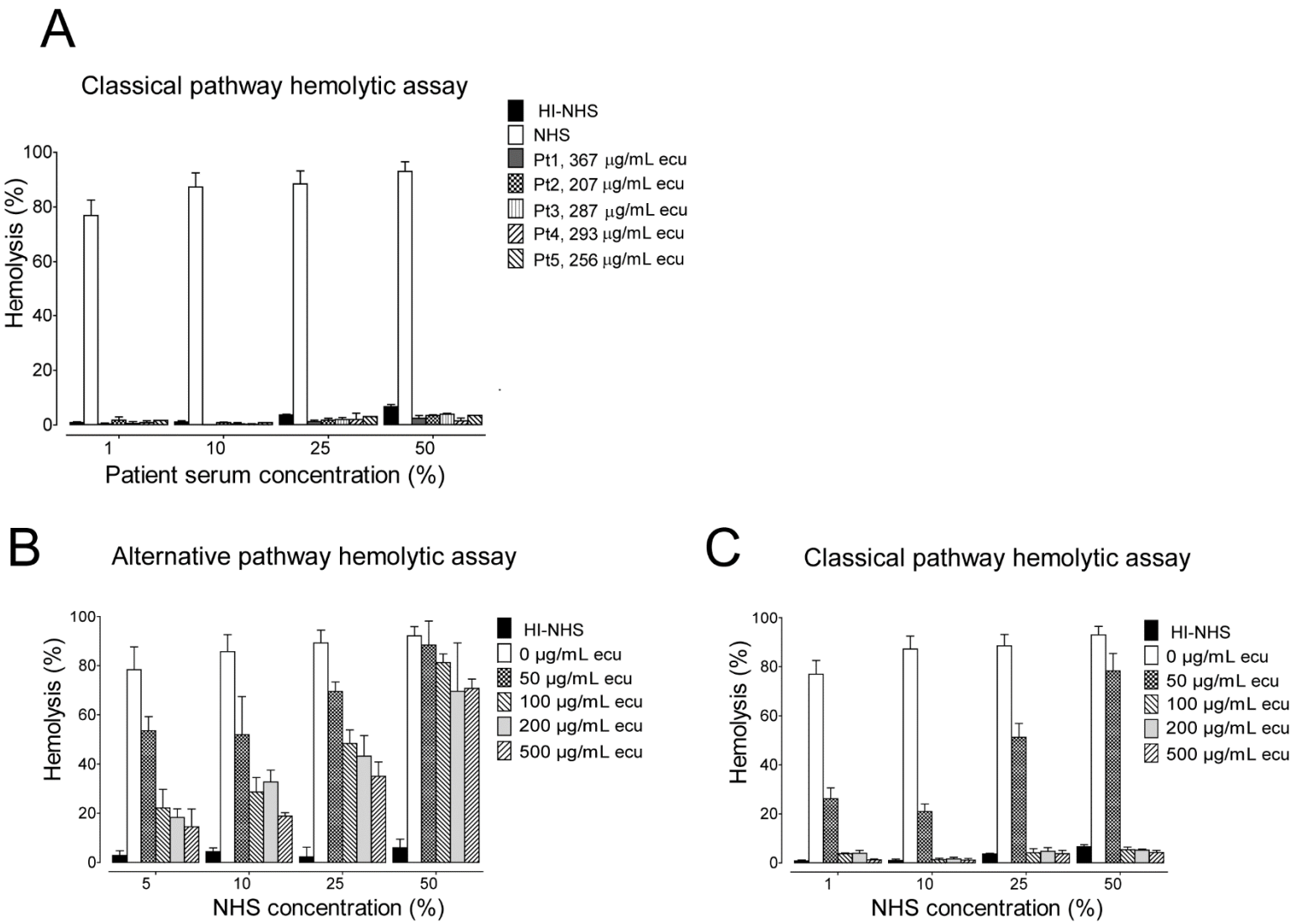

Figure S1

Serum samples of patients with atypical hemolytic uremic syndrome (Pt1-5) undergoing eculizumab treatment (A) and normal human serum (NHS) spiked with eculizumab (C and D) were analyzed by classical and alternative pathway hemolytic assays at various serum concentrations. Eculizumab (ecu) concentrations are indicated in the legends. HI-NHS = heat-inactivated NHS. Data from three independent experiments are shown as means and standard deviations.

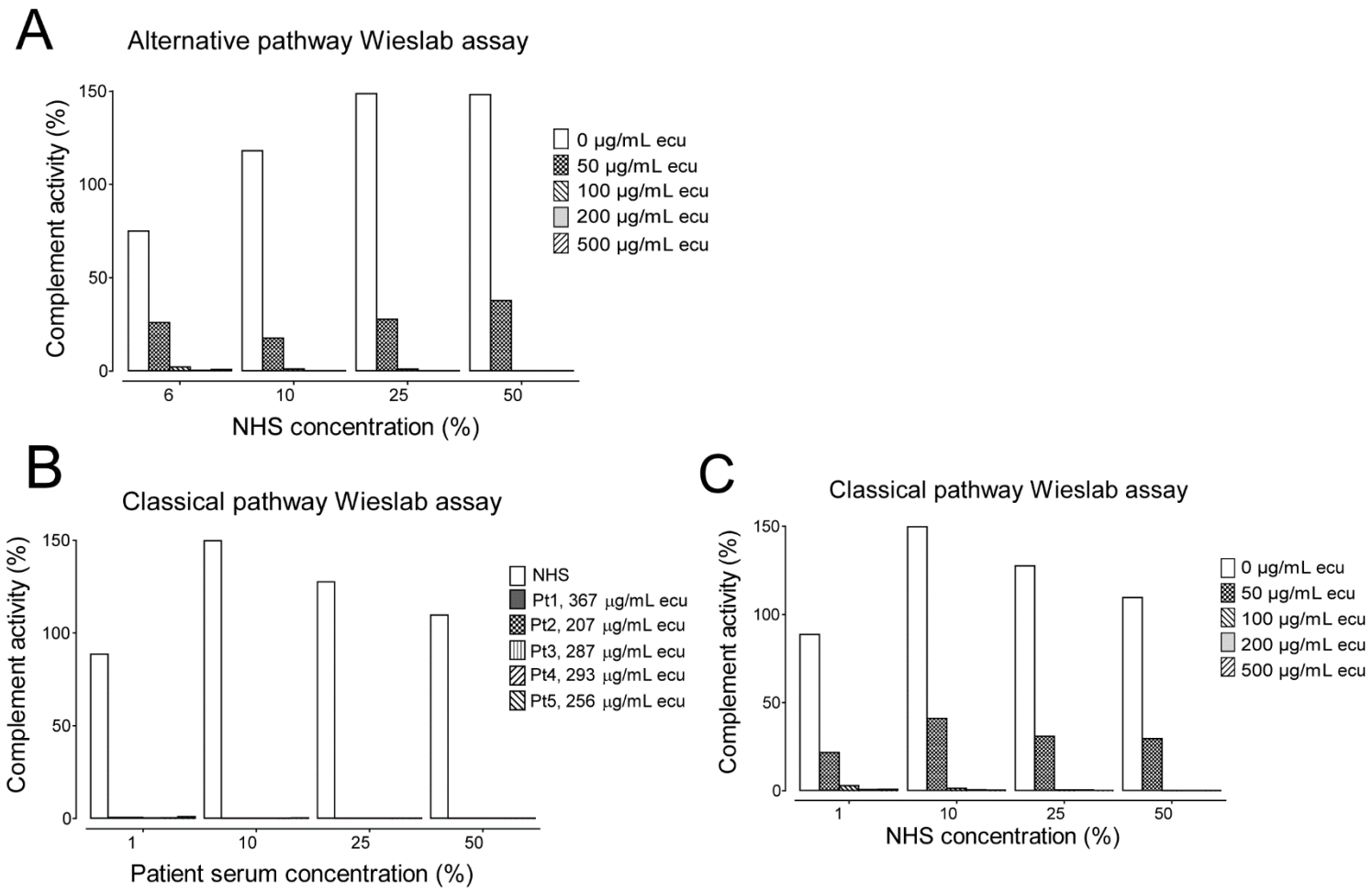

Figure S2

Normal human serum (NHS) spiked with eculizumab (A and C) and serum samples of patients with atypical hemolytic uremic syndrome (Pt1-5) undergoing eculizumab treatment (B) were analyzed by alternative (AP) and classical pathway (CP) Wieslab assays at various serum concentrations, 6% for AP and 1% for CP are standard conditions of the manufacturer's protocol. Eculizumab (ecu) concentrations are indicated in the legends.

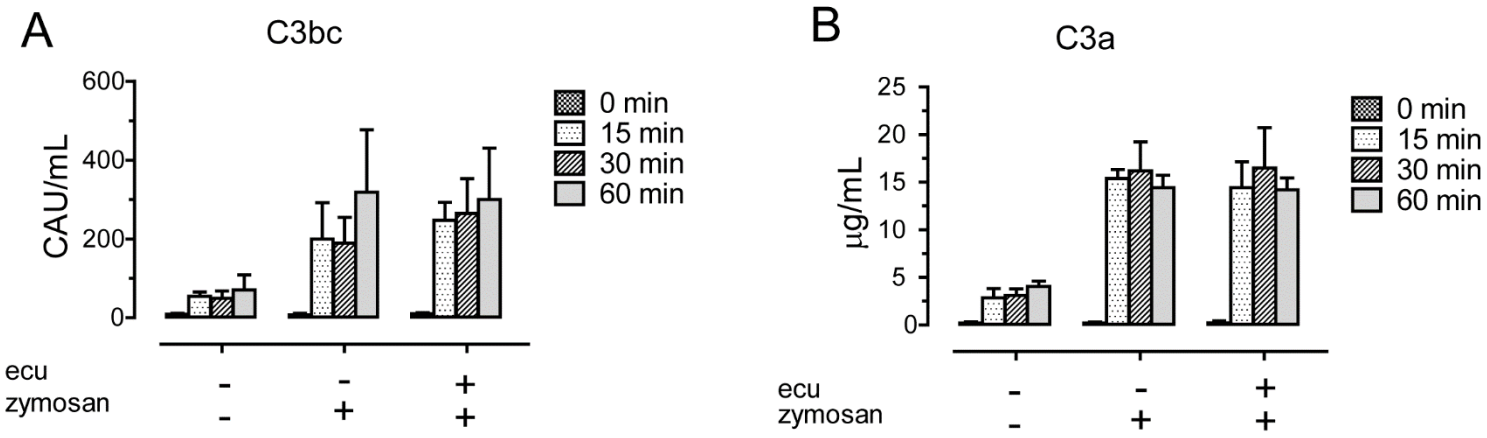

Figure S3

In vitro kinetics of spontaneous complement activation in normal human serum (NHS) incubated with 100 µg/mL zymosan in presence or absence of 100 µg/mL eculizumab (ecu). Samples were incubated at 37°C with gentle agitation for 0, 15, 30 and 60 min and levels of C3bc (A) and C3a (B) were analyzed. Data of C3bc were quantified using international complement standard 2 (ICS 2) and are presented as complement activation units per mL (CAU/mL) (5). Data from three independent experiments are shown as means and standard deviations.

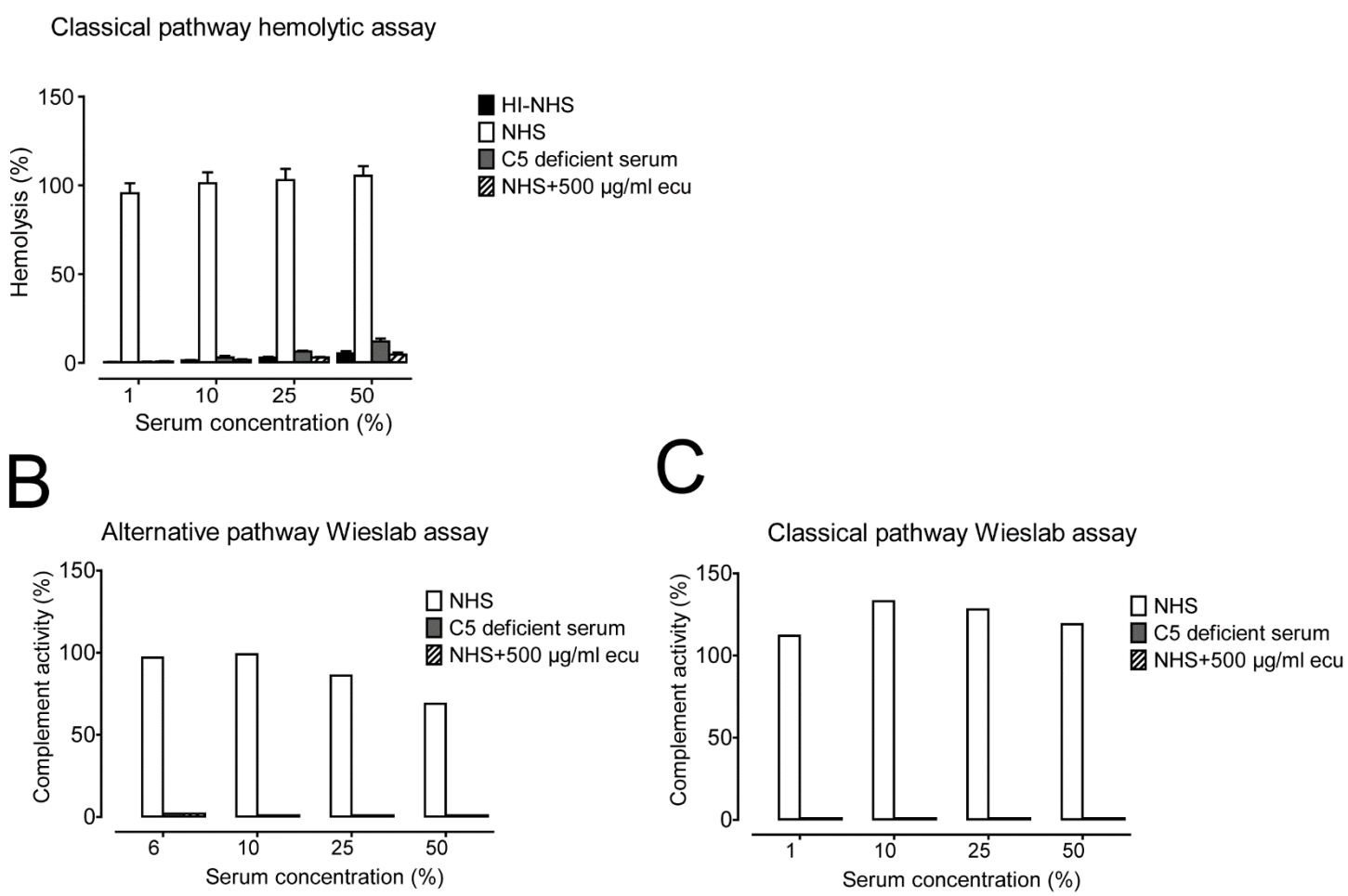

Figure S4. Normal human serum (NHS) spiked with 500 µg/mL eculizumab (ecu) and C5 deficient serum were analyzed by classical pathway hemolytic assay (A) and by alternative (B) and classical (C) pathway Wieslab assays. HI-NHS = heat-inactivated NHS. Data for hemolytic assay are from three independent experiments and shown as means and standard deviations.

Full discussion of the results

In this work we observed substantial rabbit erythrocyte hemolytic activity in AP but not in sheep erythrocyte CP assay in sera from five aHUS patients with saturating eculizumab concentration, in NHS spiked with eculizumab, and in genetically completely C5 deficient serum. The AP hemolysis was strongly dependent on the amount of added serum and was higher in these samples than in HI-NHS. The last indicates that the AP hemolysis was caused by active components present in serum. Our data excludes contribution of C5, as no C5 activation products could be generated by activation *in vitro.* Furthermore, this notion is supported by AP erythrocyte lysis in the C5 deficient serum. C5 deficiency was determined as <1% of the lower value of the normal range, based on the limit of detection. Previously, we have shown that 1% of NHS produces 3% of measurable AP complement activity (6). Thus profound hemolysis seen in AP assay cannot be due to <1% of C5 in C5 deficient serum.

When C5b-9 formation was tested in Wieslab assays, complete inhibition in both CP and AP was seen in the five aHUS patients and in NHS supplemented with eculizumab ≥ 100 µg/mL and in C5 deficient serum. We have shown eculizumab blockade in Wieslab assays not only at the standard serum concentrations, advised by the manufacturer, but also at higher serum concentrations as used in hemolytic assays. This indicates that the complete serum blockade described in these tests before (6-9) is not an artifact of low serum concentration. Consistently, in kinetic assays in this study, sC5b-9 was blocked under eculizumab, while release of C3 activation products remained unaffected. Before, we have already shown that eculizumab blocked release of the C5a, another C5 activation marker (10). Previous studies report eculizumab inhibition of sC5b-9 and C5a release in supernatants of AP hemolytic assay on rabbit erythrocytes (7) and no active C5 detected in PNH patients treated with eculizumab (11). These data altogether present solid evidence of absence of C5 activity under eculizumab.

The AP hemolysis in C5 deficient serum and in eculizumab-spiked NHS was inhibited by the presence of compstatin. This indicates that the hemolysis in AP assay is most likely caused via C3 activation. Previously, we have demonstrated C3 convertase formation on rabbit erythrocytes in the presence of eculizumab (12). Our results indicate that deposition of the C3 convertase (C3bBbP) and its inactivation products (iC3b, C3b, C3d) increases mechanical fragility of erythrocytes (Figure 6), which makes them more prone to lysis during agitation step of the assay. We regard this as a most likely mechanism, since C3 deposition itself cannot lyse the cells and in this assay there are no other possibilities such as opsonization-mediated adherence or phagocytosis with subsequent lysis.

The C3-mediated complement damage under eculizumab may be relevant in pathophysiology of disease as well. More than half of all aHUS patients carry genetic variants or autoantibodies that result in overactive C3 convertase at the cell surface (13). Complement dysregulation in this disorder, however, can be efficiently corrected by blocking C5 with eculizumab (14, 15). Nevertheless, in C3G, a renal disease which is characterized by overactive C3 in the fluid phase, treatment with eculizumab is far less efficient with no response in up to half of the patients (16-18). Similarly, in PNH patients C3 deposition on erythrocytes of patients under eculizumab leads to extravascular hemolysis, which diminishes the benefits of eculizumab blockade (19, 20).

Overall our results indicate that erythrocyte lysis seen in the AP hemolytic assay under eculizumab treatment is not caused by the residual C5 activity. Residual AP hemolysis is most likely to occur due to changes in erythrocyte membrane fragility, rendering it susceptible to mechanical lysis mediated by C3 activation and opsonization with C3 activation products. Erythrocytes are live cells with high sensitivity to storage time, buffer conditions and batch-to-batch variation, which is not the case for stable ELISA assays. This is a well-known experience for complement laboratories over decades, including ours. Therefore, we propose that in general the erythrocyte based assays, when possible, should be replaced by robust, reproducible ELISA methods in future complement research.

Acknowledgements

We thank patients and their parents/guardians for participation in the study.

Conflict of interest

Prof. Dr. Jack F.M. Wetzels is a member of the international advisory board of Alexion and also received a grant from Alexion. Other authors have no conflict of interest.

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