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Extravascular hemolysis and complement consumption in Paroxysmal Nocturnal Hemoglobinuria patients undergoing eculizumab treatment

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Running title: Hemolysis in eculizumab-treated PNH patients

ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia characterized by complement-mediated intravascular hemolysis that is effectively treated with eculizumab. However, treatment responses are reported heterogeneous with some patients presenting residual hemolysis and requiring RBC transfusions. Recent reports have shown that both extravascular hemolysis and incomplete C5 blockade can explain these suboptimal hematological responses. Here we have tested our eculizumab-treated PNH patients (n=12) for signs of hemolysis and assessed complement biomarkers. Patients were also genotyped for complement receptor 1 (CR1, CD35) and C5 polymorphisms and evaluated for free eculizumab in plasma. We report that 10 patients (83%) present parameters suggesting persistent hemolysis, although they did not require additional transfusions. Seven of them (58%) become direct Coombs-test positive as a consequence of treatment, including all patients carrying the low-expression *CR1-L* allele. CH50 and sC5b-9 assays demonstrate that the persistent low-level hemolysis identified in our treated patients is not a consequence of incomplete C5 blockade, supporting that this hemolysis, as has been suggested previously, results from the extravascular removal of C3 opsonized PNH erythrocytes. We also show that continuous alternative pathway activation in eculizumab-treated individuals carrying the *CR1-L* allele results in abnormally decreased levels of C3 in plasma that could, potentially, increase their susceptibility to bacterial infections. Finally, we encourage a routine evaluation of free eculizumab levels and terminal pathway activity to personalize eculizumab administration.

KEYWORDS

PNH, Complement, extravascular hemolysis, Complement receptor 1, eculizumab.

INTRODUCTION

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a disorder caused by the proliferation of hematopoietic stem cells carrying, in most of the cases, a somatic mutation in the gene *PIG-A*, which is necessary for the biosynthesis of the glycosil phosphatidyl-inositol (GPI) anchor. As a consequence, PNH erythrocytes (PNH-E) lack, among other proteins, the complement regulators decay accelerating factor (DAF; CD55) and membrane inhibitor of reactive lysis (MIRL; CD59) and become susceptible to complement-mediated intravascular hemolysis.(Wilcox, et al., 1991, Takeda, et al., 1993, Miyata, et al., 1993) PNH is effectively treated with eculizumab, a monoclonal antibody that blocks C5 cleavage, impeding activation of the lytic pathway and membrane attack complex (MAC) formation.(Thomas, et al., 1996, Rother, et al., 2007, Hillmen, et al., 2004) Eculizumab treatment prevents intravascular hemolysis, which abolishes thrombotic events, the main cause of death in PNH, and results in marked improvement of all clinical parameters and the quality of life of the patients.(Hillmen, et al., 2006, Brodsky, et al., 2008) Despite the unquestionable benefits of eculizumab, the responses to treatment are reported heterogeneous, with most treated patients presenting signs of continuous low-level hemolysis and 25-35% of them still requiring red blood cell (RBC) transfusions.(Brodsky, et al., 2008, Luzzatto, et al., 2011, Kelly, et al., 2011) In addition to lytic pathway dysregulation, absence of CD55 and CD59 in PNH-E also impairs regulation of the complement alternative pathway, a situation that persists under eculizumab treatment and that results in intense deposition of activated C3 fragments on the PNH-E surface.(Logue, et al., 1973) As a consequence of the accumulation of C3 opsonized PNH-E, several eculizumab-treated patients become direct Coombs-test positive.(Roth, et al., 2010, Hill, et al., 2010) C3 opsonized PNH-E are susceptible of extravascular clearance through the reticuloendothelial system in the liver and the spleen.(Jaffe, et al., 1976, Ross and Lambris, 1982, Lin, et al., 2015) This alternative removal mechanism of PNH-E, which is unmasked by the blockade of the lytic pathway-mediated intravascular hemolysis of the PHN-E by eculizumab, is thought

to be the cause of the on-going residual hemolysis that present many treated PNH patients.(Hill, et al., 2010, Risitano, et al., 2009) Critically, it has also been found that the intensity of complement deposition in PNH-E inversely correlates with the levels of the complement regulator CR1 (CD35) on erythrocytes, which are determined by genetic variants at the *CR1* locus;(Rodriguez de Cordoba and Rubinstein, 1986, Wilson, et al., 1986) individuals carrying the low expression allele *CR1-L* being particularly susceptible to experience extravascular hemolysis under eculizumab treatment.(Rondelli, et al., 2014) In addition to this extravascular removal of heavily opsonized PNH-E, one study has recently indicated that the residual low-level hemolysis could, in some patients, be related to incomplete C5 blockade and recommended close supervision of free eculizumab levels and terminal pathway activity to prevent this possibility.(Peffault de Latour, et al., 2015)

Here, we searched for correlations between hemolysis parameters, complement determinations (including *CR1 H/L* genotypes) and plasma levels of free eculizumab in our cohort of eculizumab-treated patients. We show that most eculizumab-treated patients presented signs of low-level hemolysis. Importantly, this residual hemolysis has no clinical manifestations in our patients. We also show that evaluation of the free eculizumab levels and terminal pathway activity may help to improve rationalization of the eculizumab administration and that eculizumab-treated individuals carrying the *CR1-L* allele may require special attention as they may be predisposed to present more severe disease presentations and susceptibility to infections.

PATIENTS, MATERIALS AND METHODS

Subjects.

The studies reported here have Institutional Review Board's approval. Informed consent was provided to all individuals participating in the study, according to the Declaration of Helsinki. The study group included 12 patients who were diagnosed of having PNH using flow cytometry techniques, as reported previously.(Munoz-Linares, et al., 2014) The average age of the patients was 48 years. Eleven were classified as classic-PNH and one as aplasia anemia-PNH, according to the classification of Nakakuma as amended by Parker.(Nakakuma, et al., 1995, Parker, et al., 2005) All patients in our series have received eculizumab because of severe hemolysis, PNH clone granulocytes percentage >45% and severe symptoms attributable to the disease, causing serious impairments in their quality of life. All patients included in the study have been receiving eculizumab for more than one year (ranging between 1yr and 8yr; mean 4.3±2.1yr). Eculizumab was administrated intravenously following standard guidelines. In all cases, transfusion independence was achieved at the beginning of treatment with disappearance of the symptomatology attributable to the disease. Nine patients have always received 900mg of the drug every 14d and three patients, due to a breakthrough hemolysis, required a dosing increase that have been maintained since then at 1200mg every 2wk. All 12 patients have received prophylactic vaccination with meningococcal tetravalent vaccine and are revaccinated every 2yr. Since the beginning of 2013, all patients have received ciprofloxacin 500mg/d orally (or penicillin oral 400mg/12h). In the last months all the patients has been vaccinated with anti-meningococcus B (Bexsero).

We collected samples from all patients treated during 4 weeks (two eculizumab cycles), at three different points during treatment; before and after eculizumab administration and in the intermediate week between two eculizumab cycles. Parameters, such as levels of lactate dehydrogenase (LDH), bilirubin, % of reticulocytes, hemoglobin and Coombs-test data were determined only in the samples

collected before the eculizumab administration and in the intermediate week. None of the patients had clinical complications during the length of the study.

Genotyping

Genomic DNA was prepared from peripheral blood cells of all individuals included in these studies according to standard procedures.(Miller, et al., 1988) Patients were genotyped for the three polymorphisms in *CR1* gene that allow discrimination of the *CR1-H* and *CR1-L* alleles by automatic DNA sequencing of PCR amplified fragments. To amplify a fragment that includes the *Hind*III RFLP (intron 27) we used the forward primer 5´-GGGTTCTTGCTCTTGACTTC-3´ and the reverse primer 5´-GAATGCTGGACTGTCTTGC-3´. To amplify the region that includes the H1208R (exon22) site we used the forward primer 5´-CCTTGTGCTAGGGAGAATTG-3´ and the reverse primer 5´-CCAGAGGTTAATCTCCCTG-3´ and for the P1827R (exon 33) we used the forward primer 5´-TCCAGGAACACTGTCTTTG-3´ and the reverse primer 5´-TGACAGTTACAGCAAAGCC-3´. Patients were also genotyped for polymorphisms in the C5 exon including the R885H polymorphism associated with poor response to eculizumab treatment.(Nishimura, et al., 2014) To amplify this region we used the forward primer 5´-GCAGGAGAATTGCTTGAATC-3´ and the reverse primer 5´-GCACGATTTCAGACTTACAGAA-3´. Automatic sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA.).

Flow cytometry

Erythrocytes from PNH patients were harvested by centrifugation, washed with PBS several times until the supernatant remained clear and stored for up to a week in ACD-A buffer at 4ºC. C3 deposition on erythrocytes and percentage of PNH-E were determined simultaneously by 2-color flow cytometry. Briefly, a 0.4% suspension of erythrocytes was incubated with a rabbit polyclonal anti human C3 antibody (in house)

that recognizes all C3, C3b, iC3b and C3dg at 1µg/mL in PBS for 30 min at room temperature (RT). Then we incubated the erythrocytes with an anti-rabbit IgG antibody labeled with Alexa 488 (Lifetechnologies) 1µg/mL and with a mouse monoclonal anti-CD59 labeled with R-Phycoerythrin (Sigma-Aldrich) used in 1:50 dilution for 30min at RT to identify PNH-E. FLAER detection of the GPI anchor in granulocytes was performed as described previously.(Ahluwalia, et al., 2014)

Free eculizumab levels

Levels of free eculizumab circulating in the plasma of PNH patients were determined by ELISA, essentially as described by Peffault de Latour et al. Wells were coated with C5 (10 µl/mL in PBS) overnight at 4^{o}C. After blocking with Tris-Tween (50mM Tris pH 7.4, 150mM NaCl, 0.2% Tween 20), containing 1% BSA for 1h at room temperature, we added the EDTA-plasma samples diluted 1:8000 in Tris-Tween/1% BSA, and incubated them 90min at 37^{o}C. All samples were tested in duplicate. After several washes with Tris-Tween/1% BSA, we added a peroxidase-labeled (HRP) goat anti-human IgG (Dako) antibody and incubated for 1h at RT. For the detection of the antibody we used OPD substrate. The reaction was stopped with 10% sulfuric acid. Absorbance was measured at 492nm. Sequential dilutions of a known concentration of pure eculizumab were used as the calibration curve.

Hemolytic assay CH50

The CH50 assay was performed using standard procedures. Briefly, sheep amboceptor (Siemens) was used to sensitize sheep erythrocytes. Then, 50µl of sequential dilutions of the patient serum (from 1/10) in GVB^{2+} (2.9mM barbital, 1.7mM sodium barbital, 144mM NaCl, 0.5mM $MgCl_2$, 0.15mM $CaCl_2$, 0.1% gelatin) were mixed with 50µl of sensitized sheep erythrocytes (1 X 10^9/mL) in GVB^{2+} and incubated for 30min at 37^{o}C. Reactions were done in duplicates and with 6 serial dilutions of serum. Reactions were stopped by adding 100µl of GVB/10mM EDTA and centrifuged at

1800rpm for 5min at 4^{o}C. The supernatants were read at 414nm. Erythrocytes with serum in GVB^{2+}/10mM EDTA were used as a blank and erythrocytes with serum and water as 100% lysis. As a calibration curve we used identical dilutions of a normal human serum. In these assays, 1/10 dilution of this NHS lyses 100% of sensitized sheep erythrocytes.

Dose-response curve to eculizumab

To determine the minimal concentration of eculizumab necessary to block completely the C5 hemolytic activity, we added increasing amounts of eculizumab (from 12.5 to 200 µg/mL) to the serum of a healthy donor and performed a CH50 assay as described previously. These serum samples with eculizumab added were analyzed in parallel to detect the free eculizumab levels using the ELISA assay described above. By plotting the levels of free eculizumab against CH50 activity values, we established that the minimum free eculizumab levels required to completely block C5 activity were 110±9µg/mL (Figure 3).

Determination of C3 and sC5b-9 plasma levels

C3 plasma levels were measured by nephelometry as previously described (Siemens AG, Germany). Plasma levels of sC5b-9 were measured by ELISA as follows: 96-well plates were coated with a goat anti-mouse IgG2a in PBS at 4ºC overnight. After blocking the plates with Tris-Tween/1% BSA for 1h at 37ºC, an appropriate dilution of a mouse anti-human TTC antibody (Hycult) was added and the plates incubated for another hour at 37ºC. Then, plasma samples diluted 1/10 in Tris-Tween/1% BSA were added and incubated for 1h at 37ºC. sC5b-9 was detected using a mouse anti-human C5 monoclonal antibody (IgG1) (in house) for 1h at 37ºC, followed by a peroxidase-labeled goat anti mouse IgG1 for 30min at 37ºC temperature. For detection we used OPD substrate. The reaction was stopped with 10% sulfuric acid. Absorbance was measured at 492nm. Sequential dilutions of a human plasma with known

concentrations of sC5b-9 were used for the calibration curve. All samples were tested in duplicate.

Statistical analysis

We presented continuous data as mean and standard deviation. When comparing two groups, we used the Student's t-test for normally distributed data. For proportions, we used the Pearson chi-square test or the Fisher's exact test for scarce data. To estimate a correlation between percentage of PNH-E and percentage of C3 opsonized erythrocytes, correlation pairs were analyzed using Pearson's correlation test. We considered $p<0.05$ as statistically significant; all statistical tests were two-sided. SPSS software, version 21 (SPSS Inc., Chicago, IL, USA) was used for all analyses.

RESULTS

Patient's response to eculizumab treatment

Our cohort consists in 12 PNH Spanish patients treated with eculizumab for more than 1 year (ranging between 1yr and 8yr; mean 4.3±2.1yr; see Patients, Materials and Methods). All patients received 900mg every 14 days, except PNH002, PNH008 and PNH018, who received 1200mg every 14 days. Samples from each patient were collected during 4 weeks, before and after the eculizumab administration and in the intermediate week between two eculizumab cycles. Eculizumab treatment efficiently blocked intracellular hemolysis in all patients, which resulted in a significant increase of hemoglobin and a dramatic reduction of LDH levels in plasma (Tables 1 and 2). Only one of our patients required occasional RBC transfusions during treatment and, notably, this was the only patient in our series presenting aplastic anemia (AA-PNH; PNH002). All our patients declared a great improvement of their quality of life and none failed a single eculizumab administration during the whole length of the treatment. Despite this unquestionable success of eculizumab treatment, most of our eculizumab-treated patients present parameters that suggest persistent low levels hemolysis. Thus, 83% (10/12) of the PNH patients show elevated reticulocyte counts, 58% (7/12) have not completely recovered normal hemoglobin levels, 50% (6/12) have elevated bilirubin and 42% (5/12) present slightly elevated LDH levels immediately before eculizumab administration (Table 1). These data are consistent with that previously reported by Risitano et al.(Risitano, et al., 2009) and Hill et al.(Hill, et al., 2010) in that most eculizumab-treated PNH patients present parameters suggesting persistent low-level extravascular hemolysis. In our series, however, this residual hemolysis does not appear to manifest clinically since our patients, with the exception of the AA-PNH one, have not had RBC transfusion requirements. None of our patients carry the (Arg885His) C5 polymorphism associated with resistance to eculizumab.(Nishimura, et al., 2014)

Four PNH patients in our cohort (PNH006, PNH004, PNH018, PNH008) were genotyped *CR1-H/CR1-L* heterozygotes and the remaining eight *CR1-H* homozygotes. Individuals carrying the *CR1-H/CR1-H* and *CR1-H/CR1-L* genotypes differ in the number of CR1 (CD35) molecules per erythrocyte,(Khera and Das, 2009) which should impact on their capacity to control complement alternative pathway activation and C3 deposition on PNH-E. To offer a figure value to these differences we genotyped three large control pedigrees in which we have determined the numbers of CR1 molecules per erythrocyte and showed that erythrocytes from *CR1-H* homozygotes have twice and ten times more CR1 (CD35) molecules than erythrocytes from *CR1-H/CR1-L* and *CR1-L/CR1-L* individuals, respectively (Supplementary Figure 1). Notably, three of the *CR1-H/CR1-L* heterozygote patients in our series were those requiring the highest numbers of transfusions/year prior to the eculizumab treatment, two required hospitalization due to severe infections and all four *CR1-H/CR1-L* heterozygote patients were included in the group of PNH patients presenting putative extravascular hemolysis (Table 1). Moreover, the group of patients carrying the *CR1-L* allele present, after treatment, higher reticulocyte counts (5.1±4% vs 11.1±4%; p=0.04) and plasma levels of bilirubin (1.8±1mg/dL vs 4.8±3mg/dL; p=0.03) than the group of *CR1-H* homozygotes.

C3 deposition of PNH erythrocytes

As indicated, all four *CR1-H/CR1-L* heterozygote patients in our series were included in the group of PNH patients presenting putative extravascular hemolysis (Table 1). This is consistent with previous findings showing that extravascular hemolysis is a consequence of the uncontrolled C3 activation and progressive C3 opsonization of the PNH-E that characterizes patients under eculizumab treatment. To get further insight into this question, we have studied the PNH-E population and the C3 fragment deposition in the 12 eculizumab-treated PNH patients in our cohort.

The analysis by FLAER of the granulocytes illustrated that in all PNH patients a very large percentage of their granulocytes lack the GPI anchor (ranging between 51% and 99%; mean 82±18%) and therefore are deficient of both, CD55 and CD59 (Table 3). There was, however, no correlation between these FLAER data and the percentage of CD59 negative erythrocytes (PNH-E) determined by flow cytometry using an anti CD59 antibody, although individuals carrying the *CR1-L* allele were those with the highest percentage of PNH-E cells (Table 3). Despite the large variability among patients in the percentage of PNH-E (ranging between 20% and 94%; mean 65±24%), this percentage remained remarkably stable in each individual during the whole period of the study (Figure 1a).

The percentage of cells becoming C3 opsonized in the eculizumab treated patients remained also stable overtime and correlated well (r^2=0.64; p<0.002) with the percentage of PNH-E (Figures 1b and 1c). Seven patients became direct Coombs positive (58%) and with one exception they were those presenting more than 25% of their PNH-E C3 opsonized (Table 3). As expected, all four patients carrying the *CR1-H/CR1-L* heterozygote genotype were those with the highest % of C3 opsonized PNH-E and all become direct Coombs-test positive. Crucially, all *CR1-H/CR1-L* heterozygotes presented decreased C3 plasma levels, indicating that continuous complement alternative pathway activation in these patients results in massive C3 consumption (Figure 2a and 2b, Table 3). Consistent with the hypothesis that eculizumab-treated PNH patients with highest % of C3 opsonized PNH-E are the likely candidates to suffer extravascular hemolysis, reticulocytes counts and bilirubin levels were significantly elevated in PNH patients presenting more than 25% of their PNH-E C3 opsonized compared with those with C3 opsonized PNH-E under 25% (Figure 2c and 2d).

The observation that *CR1-H/CR1-L* heterozygote patients develop a partial C3 deficiency during treatment prompted us to check their medical records for unusual frequency or severity of infections. Interestingly, only three patients have had this type

of complications during treatment (PNH002, PNH008 and PNH018). PNH002 is the AA-PNH patient and have presented with repeated infections likely due to his frequent very low counts of neutrophils. PNH008 and PNH018 are patients carrying the *CR1-L* allele, who required hospitalization for 10 and 4 days, respectively, due to severe infections of unknown origin (*N. meningitides* was excluded). Both patients responded well to broad-spectrum antibiotics, but required supplemental dosing of eculizumab to control hemolysis. Although these data are suggestive that C3 consumption during eculizumab treatment in patients carrying the *CR1-L* allele may render them more susceptible to infections, we are aware of the limitations of our small sample and that larger series of patients are needed to raise definitive conclusions.

Free eculizumab in plasma of eculizumab-treated PNH patients

To rule out that the residual low-level hemolysis detected in these patients was related to incomplete C5 blockade, we evaluated their free eculizumab in plasma and determined their CH50 activity and sC5b-9 levels. Previously, we experimentally established that a minimum concentration of 110±9μg/mL of free eculizumab in plasma is required to confer complete terminal pathway inhibition (Figure 3a). This value is significantly higher than the 35μg/mL serum concentration of eculizumab that the technical note for this drug recommends to assure complete intravascular hemolysis blockade. Notably, our value of 110±9μg/mL fits well with that recently determined by Peffault de Latour et al. to confer complete terminal pathway inhibition.(Peffault de Latour, et al., 2015) We measured the circulating free eculizumab levels and the activity of the terminal pathway (CH50 and sC5b-9) in all 12 patients in our cohort at three time points during two consecutive eculizumab cycles. As expected, levels of free eculizumab in all patients increased after eculizumab infusion and decreased progressively during the following 14 days (Figure 4). Interestingly, in 2 patients (PNH003 and PNH006) levels of free eculizumab fell below the experimentally

determined minimum value of 110±9μg/mL at the time point previous to the next eculizumab administration and in two additional patients (PNH030 and PNH033) the free eculizumab levels were close to this value (Figure 3b and Figure 4). CH50 assays performed in the 12 PNH patients at all time points were all completely negative and patients only occasionally exhibited traces of sC5b-9, indicating that these borderline free eculizumab levels do not compromise the complete blockade of C5 activation (Figure 4). Consistent with this, no differences were observed between these four individuals (free eculizumab levels <150ug/mL) and the rest of the eculizumab-treated patients (free eculizumab levels >150ug/mL) for the hemolysis biomarkers assessed in these studies (Table 4). Therefore, the evaluation of the free eculizumab levels and terminal pathway activity in our cohort demonstrate a complete terminal pathway inhibition in all our patients, which indicates that the residual hemolysis detected likely involve extravascular mechanisms.

DISCUSSION

Eculizumab treatment efficiently blocked intracellular hemolysis in all our patients, which improved significantly their clinical situation. Notably, only one of our patients required occasional RBC transfusions during treatment and likely this was more a consequence of the substantial bone marrow failure that presents this patient than a suboptimal response to the eculizumab treatment. These data, therefore, deviate from earlier reports indicating a heterogeneous response to eculizumab treatment with 25-35% of the eculizumab-treated patients still requiring RBC transfusions.(Brodsky, et al., 2008, Luzzatto, et al., 2011, Kelly, et al., 2011) Because these reported suboptimal hematological responses were attributed to either extravascular hemolysis or incomplete C5 blockade,(Hill, et al., 2010, Risitano, et al., 2009, Peffault de Latour, et al., 2015) we assessed hemolysis and complement biomarkers in our PNH patients looking for signs of hemolysis. These analyses demonstrated that despite our patients have not required RBC transfusions, most of them, in agreement with previous reports, present signs of persistent hemolysis (Table 1). Importantly, evaluation of the free eculizumab levels and terminal pathway activity data (CH50 and sC5b-9) in all our patients demonstrated that this residual hemolysis was not a consequence of incomplete blockade of C5 activation. In fact, the patients with signs of on-going hemolysis in our cohort characterize by presenting a very high percentage of C3 opsonized PNH-E, direct Coombs-test positive, high percentage of reticulocytes, elevated bilirubin and slightly elevated LDH levels, which fulfills the characteristics described earlier by Risitano et al.(Risitano, et al., 2009) and Hill et al.(Hill, et al., 2010) for patients undergoing extravascular hemolysis.

Rondelli et al.(Rondelli, et al., 2014) made the important observation that *CR1* polymorphisms, coding for levels of CR1 (CD35) in erythrocytes, determine the levels in which the PNH-E cells are C3 opsonized and that, therefore, influence their phagocytosis and extravascular clearance through the reticuloendothelial system in the liver and the spleen. Our PNH cohort includes eight *CR1-H* homozygotes and four

CR1-H/CR1-L heterozygotes. We did not found *CR1-L* homozygotes in this small size group. In agreement with previous results,(Roth, et al., 2010, Hill, et al., 2010, Rondelli, et al., 2014) all our *CR1-H/CR1-L* heterozygote patients become direct Coombs-test positive after eculizumab treatment due to intense C3 opsonization. We also observed a positive correlation between the percentage of PNH-E, reticulocyte counts and levels of bilirubin, which is also in agreement with previous reports indicating that carriers of the *CR1-L* allele are particularly predisposed to extravascular hemolysis during eculizumab treatment.(Rondelli, et al., 2014)

Previous studies have reported that the *CR1* genotypes did not affect significantly their clinical features prior to treatment.(Rondelli, et al., 2014) Interestingly, we noticed that *CR1-H/CR1-L* heterozygote patients, in addition to presenting higher percentages of PNH-E in circulation than *CR1-H* homozygote patients carrying similar sizes of granulocyte PNH clones, did receive more transfusions prior to eculizumab treatment. We are aware that our numbers are small to raise definitive conclusions. However, these data suggest that CR1 (CD35) levels may also be an important disease modifier before eculizumab treatment; carriers of *CR1-L* alleles would require more transfusion requirements because they have increased levels of hemolysis.

Our results also revealed that during eculizumab treatment *CR1-H/CR1-L* heterozygote patients present strong activation of the alternative pathway that results in elevated C3 consumption. Unfortunately, we have no data of their plasma C3 levels prior to eculizumab treatment to determine whether this is a permanent condition in these patients. We have determined C3 levels in all untreated PNH patients in our cohort and all presented C3 levels within the normal range (n=16; 128±23mg/dL). This group of untreated patients includes 10 patients who are *CR1-H/CR1-L* heterozygotes. However, most of these untreated *CR1-H/CR1-L* heterozygote patients present PNH granulocyte size clones much smaller than those carried by the *CR1-H/CR1-L* heterozygote PNH treated patients (1% to 61%; 19±24% *vs* 62% to 99%; 88±17%).

The observation that *CR1-H/CR1-L* heterozygote patients develop a partial plasma C3 deficiency as a consequence of the strong alternative pathway activation that take place on the surface of their PNH-E raises the possibility that these patients may have an increased susceptibility to infections than those who are *CR1-H* homozygotes. Three mechanisms are crucial in the complement-mediated host defense against bacterial infections: 1) direct killing by the terminal pathway; 2) recruitment of phagocytic cells at the site of infection by the C5a anaphylotoxin; and 3) C3 opsonization for immune-recognition. In eculizumab-treated patients with complete blockade of the terminal pathway, an intact opsonization mechanism is essential for efficient phagocytosis and intracellular killing of bacteria. This mechanism depends on C3 levels. Not surprisingly, similarly to individuals with primary deficiency of C3, it has been reported that patients with acquired C3 deficiencies have increased predisposition to infectious disease.(Ram, et al., 2010, Homann, et al., 1997)As indicated before, we are aware that further studies with larger cohort are needed to raise definitive conclusions. However, our observation that the only patients in our cohort requiring hospitalization due to infections were two *CR1-H/CR1-L* heterozygote carriers may be a sign that special attention should be given to PNH patients carrying the *CR1-L* allele when they are treated with eculizumab.

In conclusion, our study confirms that despite complete C5 blockade, most eculizumab-treated patients show persistent signs of low-level hemolysis, which likely corresponds to the extracellular removal of the heavily C3 opsonized PNH-E that generate during the eculizumab treatment. The clinical relevance of this residual hemolysis is however contentious; in our patients, this residual hemolysis has no clinical manifestations as illustrated by the fact that the only patient requiring additional RBC transfusions presents aplastic anemia. Notably, we also show that on-going alternative pathway activation in eculizumab-treated PNH patients carrying the *CR1-L* allele results in an acquired partial C3 deficiency that could, potentially, increase their susceptibility to infections. Finally, we provide further evidence that the evaluation of

the free eculizumab levels and terminal pathway activity data offers useful information to personalize eculizumab administration.

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DISCLOUSURE OF CONFLICTS OF INTEREST:

SRdeC, AV and EO have received honoraria from Alexion Pharmaceuticals for giving lectures and participating in advisory boards. None of these activities has had any influence on the results or interpretation in this article. Other authors declare no conflicts of interest.

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Table 1. Hematological and genetic markers in eculizumab-treated PNH patients

	PNH Type	Thrombotic events		Transfusions per year		Hemoglobin (12-16 gr/dL)		% Reticulocytes (0.5-2 %)		LDH (240-480 U/L)		Total Bilirubin (0.2-2 mg/dL)		CR1 Genotype
		Before	During	Before	During	Before	During	Before	During	Before	During	Before	During	
PNH 006	Clasicc	No	No	16	0	8.4	15.7 ± 0.4	4.5	7.9 ± 2.8	2300	304 ± 18	0.80	5.9 ± 0.5	H/L
PNH 004	Classic	No	No	1	0	10.2	9.7 ± 0.2	1.3	7.3 ± 2.3	1050	445 ± 39	0.40	1.6 ± 0.1	H/L
PNH 018	Clasicc	No	No	12	0	2.9	9 ± 0.3	4.4	13.7 ± 0.5	3170	472 ± 11	1.20	3 ± 0.4	H/L
PNH 008	Clasicc	No	No	37	0	4.4	9.5 ± 0.5	4	15.6 ± 1.8	2050	334 ± 20	2.30	8.6 ± 0.9	H/L
PNH 003	Classic	No	No	8	0	8.4	9.6 ± 0.4	3	10.5 ± 1.6	3001	338 ± 9	1.70	1.4 ± 0.1	H/H
PNH 033	Classic	No	No	2	0	7	10.2 ± 0.3	5.9	11.6 ± 0.3	1683	591 ± 46	1.90	3.8 ± 0.7	H/H
PNH 005	Classic	No	No	0	0	9.8	12 ± 0.5	8.6	2.9 ± 0.5	6000	526 ± 22	8.80	1.9 ± 0.4	H/H
PNH 001	Classic	Yes	No	0	0	12.4	12.4± 0.2	2.8	1.1 ± 0.4	3600	525 ± 19	1.00	0.6 ± 0.1	H/H
PNH 002	AA-PNH	No	No	8	2	8.9	9.3 ± 0.2	10.1	4.7 ± 0.6	2800	634 ± 50	3.20	2.1 ± 0.1	H/H
PNH 030	Classic	No	No	2	0	8	10.5 ± 0.1	6.9	5.6 ± 05	8304	489 ± 12	2.50	2.1 ± 0.2	H/H
PNH 014	Classic	No	No	2	0	6.7	12.8 ± 0.3	4.8	3.7 ± 0.6	3728	393 ± 10	3.00	1 ± 0.1	H/H
PNH 026	Classic	No	No	0	0	9	11.5 ±0.5	6.4	0.1 ±0.01	4942	368 ± 27	2.00	1.1 ± 0.1	H/H

* Values are mean ± standard deviation of determinations at all time points.

Table 2. Changes in hemolysis parameters in PNH patients after eculizumab treatment

	All (n=12)		
Variable	**Before treatment**	**After treatment**	**p-value**
Transfusions	7.3 ± 10.7	0.2 ± 0.6	0.03
Hemoglobin	8 ± 2.5	11 ± 2	0.004
% Reticulocytes	5.2 ± 2.5	7 ± 5	0.27
Bilirubin	2.4 ± 2.2	2.8 ± 2.3	0.7
LDH	3552 ± 2025	451 ± 106	<0.001

*Values are mean ± standard deviation of all individuals included in each category. Statistical analysis was performed using Student´s t test.

Table 3. C3 levels in plasma and in erythrocytes of eculizumab treated PNH patients

	FLAER Granulocytes	% E-PNH	% E C3+	Coombs	Plasma C3 (85-135mg/dL)	CR1 Genotype
PNH 006	98.5	94.1 ± 0.3	64.8 ± 2.4	+	59 ± 11.5	H/L
PNH 004	92.4	92.5 ± 0.3	35.7 ± 1.81	+	87 ± 8.1	H/L
PNH 018	61.8	81.2 ± 1.7	36.3 ± 2.2	+	80.5 ± 5.1	H/L
PNH 008	97.6	76.5 ± 2.4	61.7 ± 1	+	53.5 ± 1.8	H/L
PNH 003	88	63.7 ± 0.2	14.6 ± 1.1	+	104.2 ± 15	H/H
PNH 033	92.7	79.1 ± 1.2	47.9 ± 1.7	+	93 ± 5.9	H/H
PNH 005	91.2	70.5 ±1.7	27.3 ± 2.2	+	95.1 ± 6.2	H/H
PNH 001	51	26.9 ± 1.8	1 ± 0.1	-	107.2 ± 9.1	H/H
PNH 002	51.2	43.9 ±1.2	8.8 ± 1.3	-	105.4 ± 11.4	H/H
PNH 030	87.7	61.1 ± 0.7	11.8 ± 2	-	121.2 ± 15.3	H/H
PNH 014	78.3	72.7 ± 0.2	21.3 ± 1.4	-	109.6 ± 8.4	H/H
PNH 026	98.7	20.2 ± 1.6	8.1 ± 1.5	-	89.8 ± 7.5	H/H

*Values are mean ± standard deviation of determinations at all time points.

Table 4. Hemolysis and complement parameters in PNH patients according to free eculizumab in plasma

Variable	Total n= 12	Free Ecu >150µg/mL n= 8	Free Ecu <150µg/mL n=4	p-value
Age (yr)	47.8	48.6 ± 16	46 ± 7	0.8
Gender male (n)	8 (67 %)	5 (63%)	3 (75%)	1
Plasma C3	92.2 ± 20	91 ± 18	94 ± 26	0.8
CR1 H/L	4 (33 %)	3 (38%)	1 (25%)	1
FLAER Granulocytes	82.4 ± 18	77.8 ± 20	91.8 ± 5	0.22
% PNH E	65.3 ± 24	60.6 ± 27	74.5 ± 26	0.36
% C3+ E	28 ± 20	21.3 ± 14	34.8 ± 23	0.26
Coombs +	7 (58%)	4 (50%)	3 (75%)	0.58
Hemoglobin	11.5 ± 2	10.8 ± 2	11.5 ± 3	0.6
% Reticulocytes	7.1 ± 5	6.1 ± 6	8.9 ± 3	0.39
Hemosiderinuria	5 (42%)	3 (43%)	2 (40 %)	1
Bilirubin	2.8 ± 3	2.5 ± 3	3.2 ± 2	0.6
LDH	450 ±107	462 ± 99	430 ± 134	0.7
Free Eculizumab	217 ± 111	268 ± 100	113 ± 24	0.01

* Values are mean ± standard deviation or proportions of all individuals included in each category. For the statistical analysis we used Student´s t-test to compare continuous variables or Fisher´s exact test for the comparison of proportions.

LEGENDS FOR FIGURES

Figure 1. PNH-E cells and C3 opsonized erythrocytes in eculizumab-treated patients

a) Percentage of PNH-E cells determined by flow cytometry as CD59 negative erythrocytes at different time points during two consecutive eculizumab cycles. b) Percentage of C3 opsonized erythrocytes determined by flow cytometry using a goat polyclonal anti human C3 at different time points during two consecutive eculizumab cycles. Samples at day 0 and 14 correspond to those obtained prior and after the eculizumab administration. Each point corresponds to the mean value ± standard deviation of three independent measurements. The correspondence of the symbols with the individual patients is depicted at the bottom of the figure. c) Correlation between percentage of PNH-E cells and percentage of C3 opsonized erythrocytes for the mean of values obtained at all time points. Pearson correlation analysis shows a significant positive correlation ($r^2 = 0.64$; $p<0.002$).

Figure 2. *CR1-L* associates with high % of PNH-E, high % of C3 opsonized cells, decreased C3 plasma levels and elevated hemolysis parameters.

Figures depict the comparison of the mean value ± standard deviation of the individual values (mean of determinations at all time points) obtained for *CR1-H* homozygote (n=8) and *CR1-H/CR1-L* heterozygote (n=4) patients.

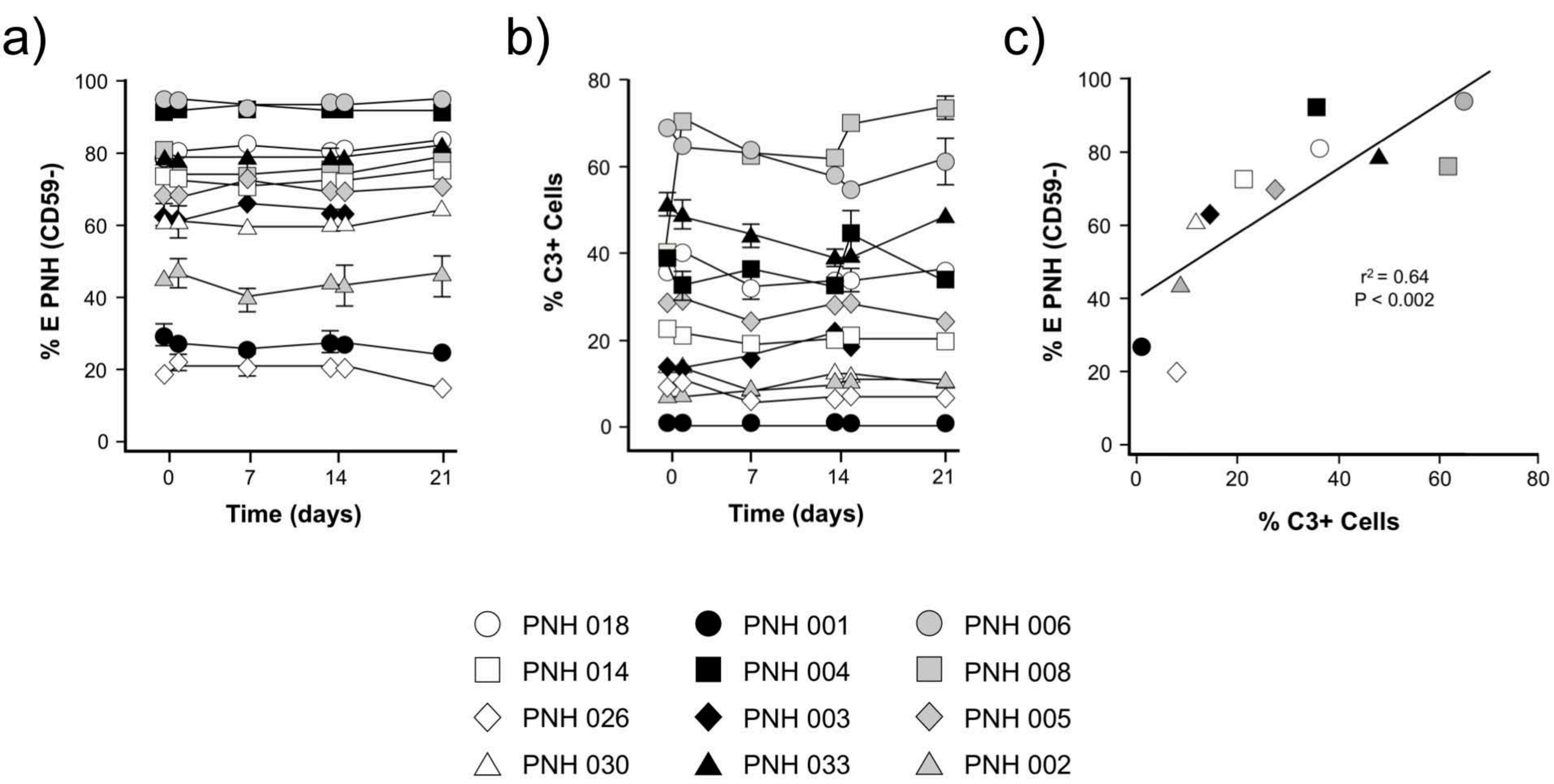
Figure 3. Determination of free-eculizumab levels in the plasma of treated PNH patients

a) Determination of the minimal circulating level of free eculizumab required for blocking completely the activity of the lytic pathway, measured as % of lysis in a CH50 assay (see Materials and Methods).

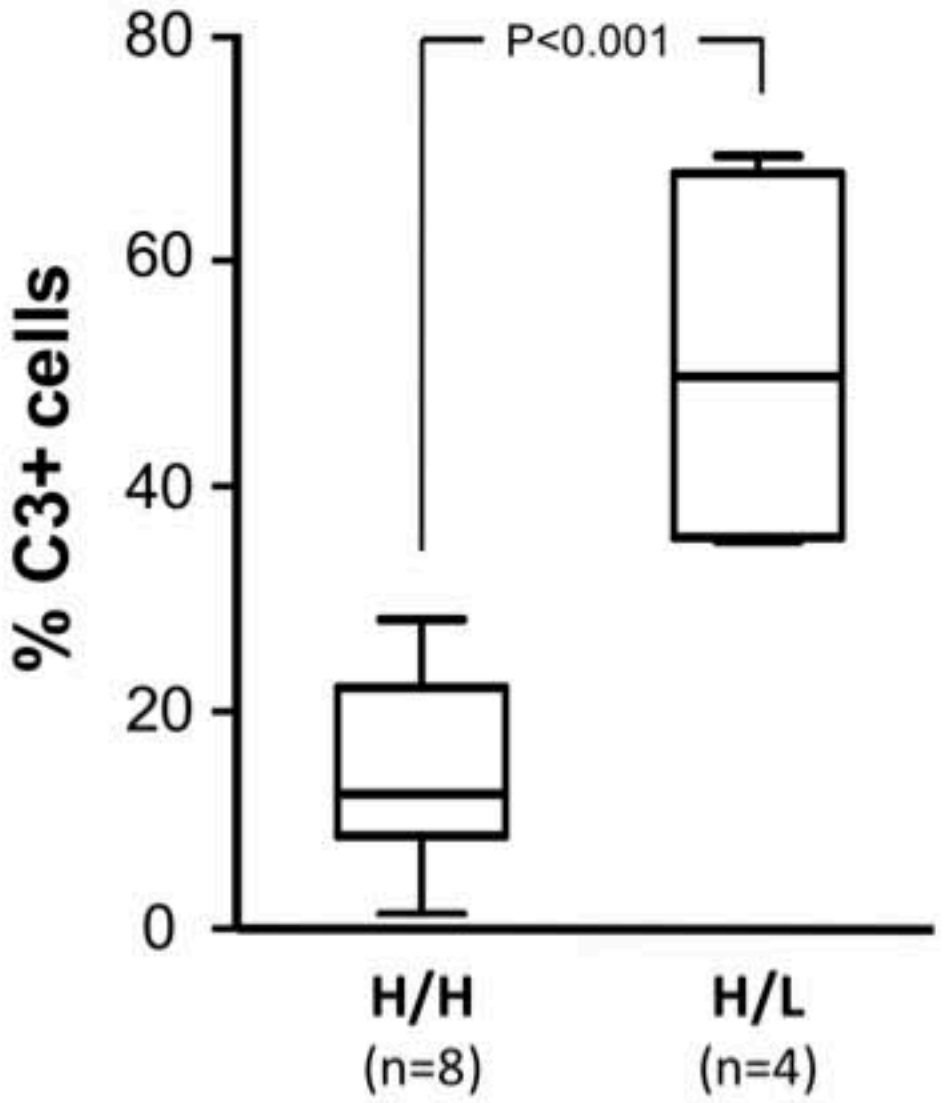
b) Mean value ± standard deviation of circulating free eculizumab in the plasma of patients prior to the next administration of eculizumab. Data from time points 0 and 14 days are included.

Figure 4. Individual variations in free eculizumab levels, sC5b-9 and CH50 during treatment with eculizumab

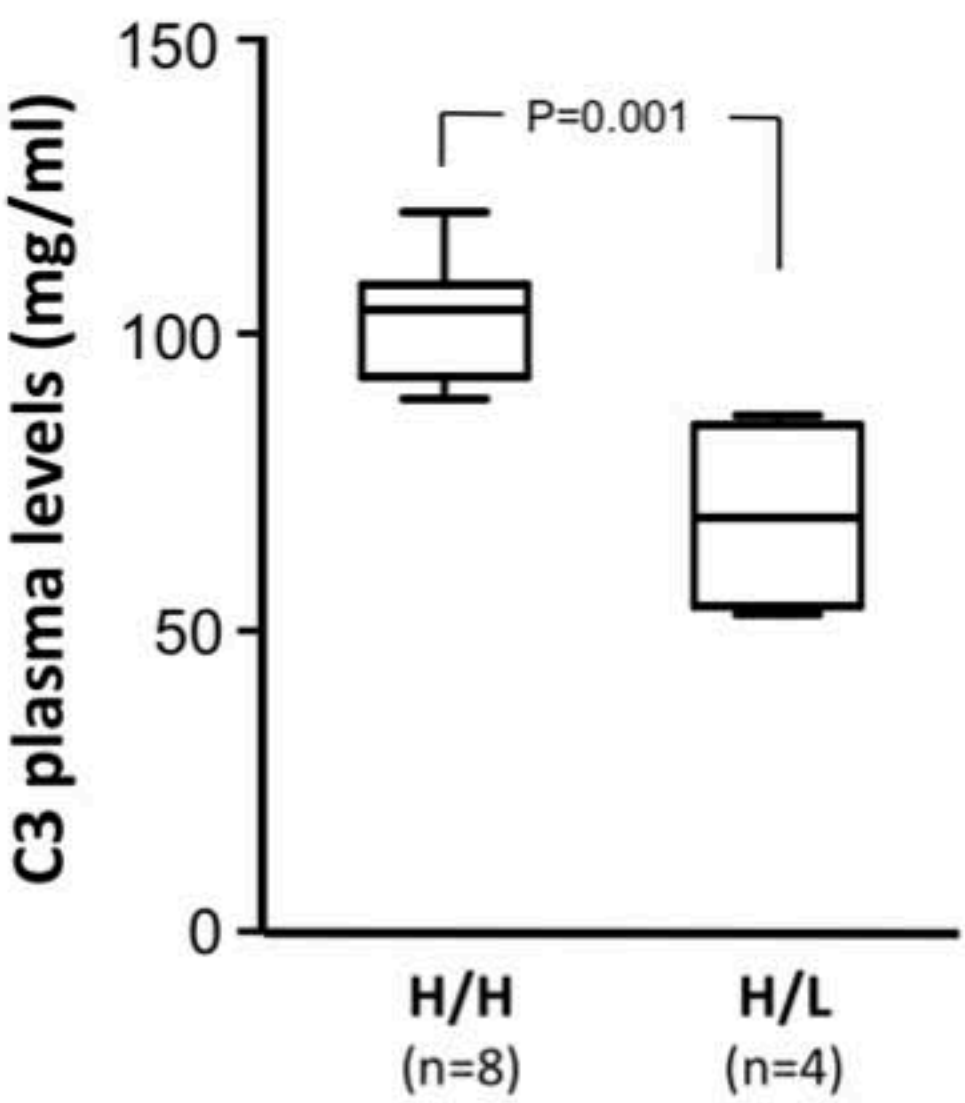
Individual representations of the free eculizumab levels (μg/mL; empty circles) and sC5b-9 levels (ng/mL; solid squares) at all time points during two consecutive cycles of eculizumab treatment. The horizontal line represents the minimal concentration (110μg/mL) of free eculizumab required for blocking completely the lytic pathway. The same line also corresponds to the upper limit of the normal levels of plasma sC5b-9 in this assay, calculated using reference value advisor v2.1 as described by Geffre et al (Geffre, et al., 2011).


a)
% E PNH (CD59-)
Time (days)
b)
% C3+ Cells
Time (days)
c)
% E PNH (CD59-)
% C3+ Cells
r² = 0.64
P < 0.002
PNH 018
PNH 001
PNH 006
PNH 014
PNH 004
PNH 008
PNH 026
PNH 003
PNH 005
PNH 030
PNH 033
PNH 002

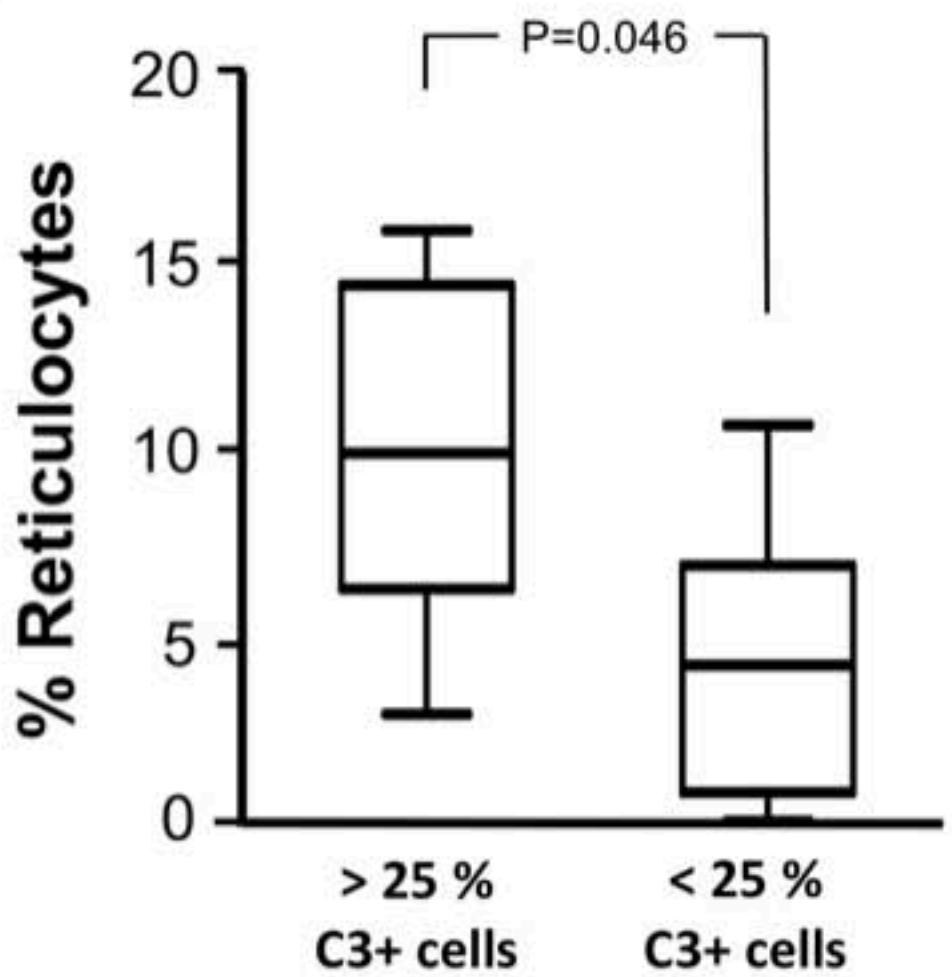
a)


P<0.001
% C3+ cells
80
60
40
20
0
H/H
(n=8)
H/L
(n=4)

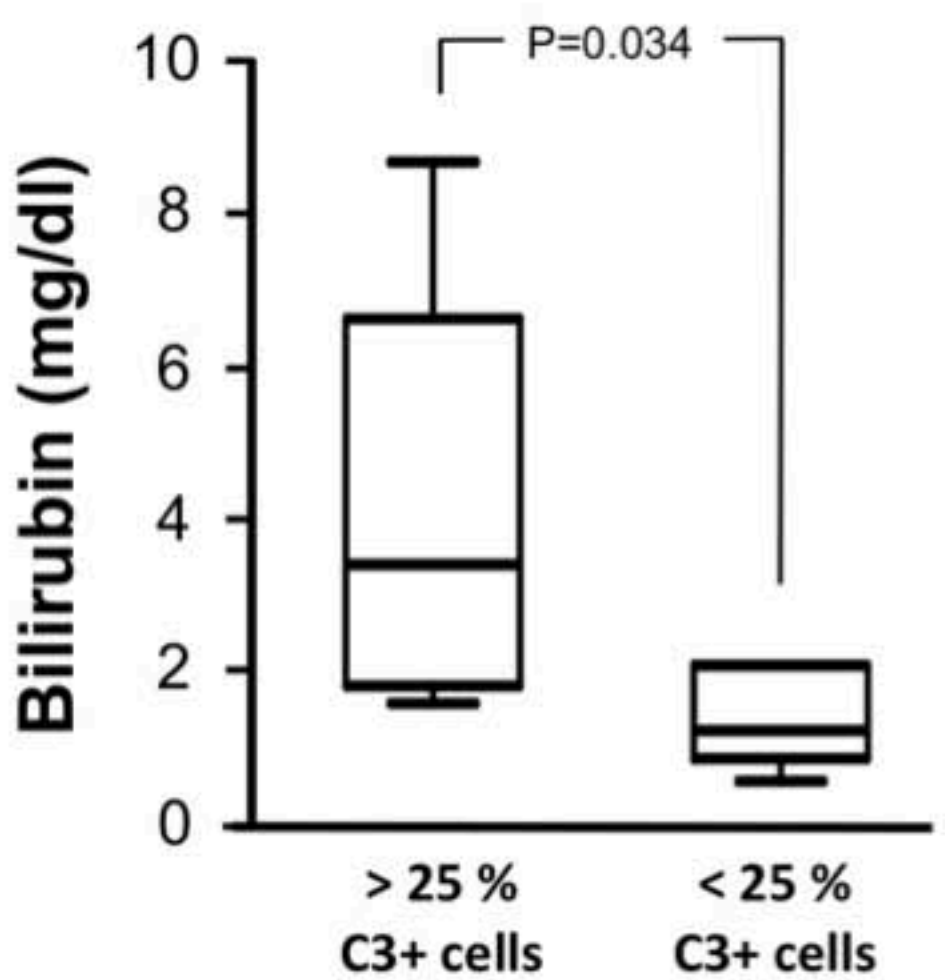
b)


P=0.001
C3 plasma levels (mg/ml)
150
100
50
0
H/H
(n=8)
H/L
(n=4)

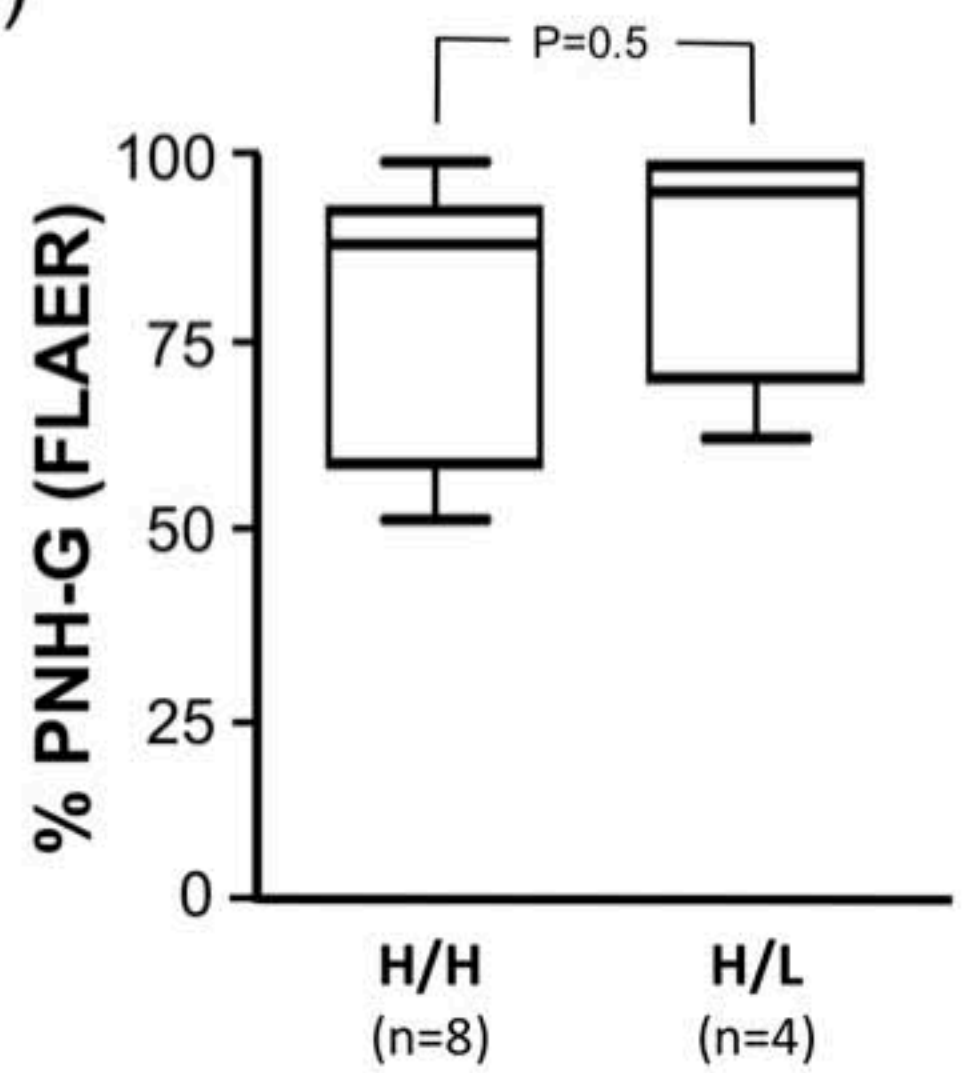
c)


P=0.046
% Reticulocytes
20
15
10
5
0
> 25 %
C3+ cells
< 25 %
C3+ cells

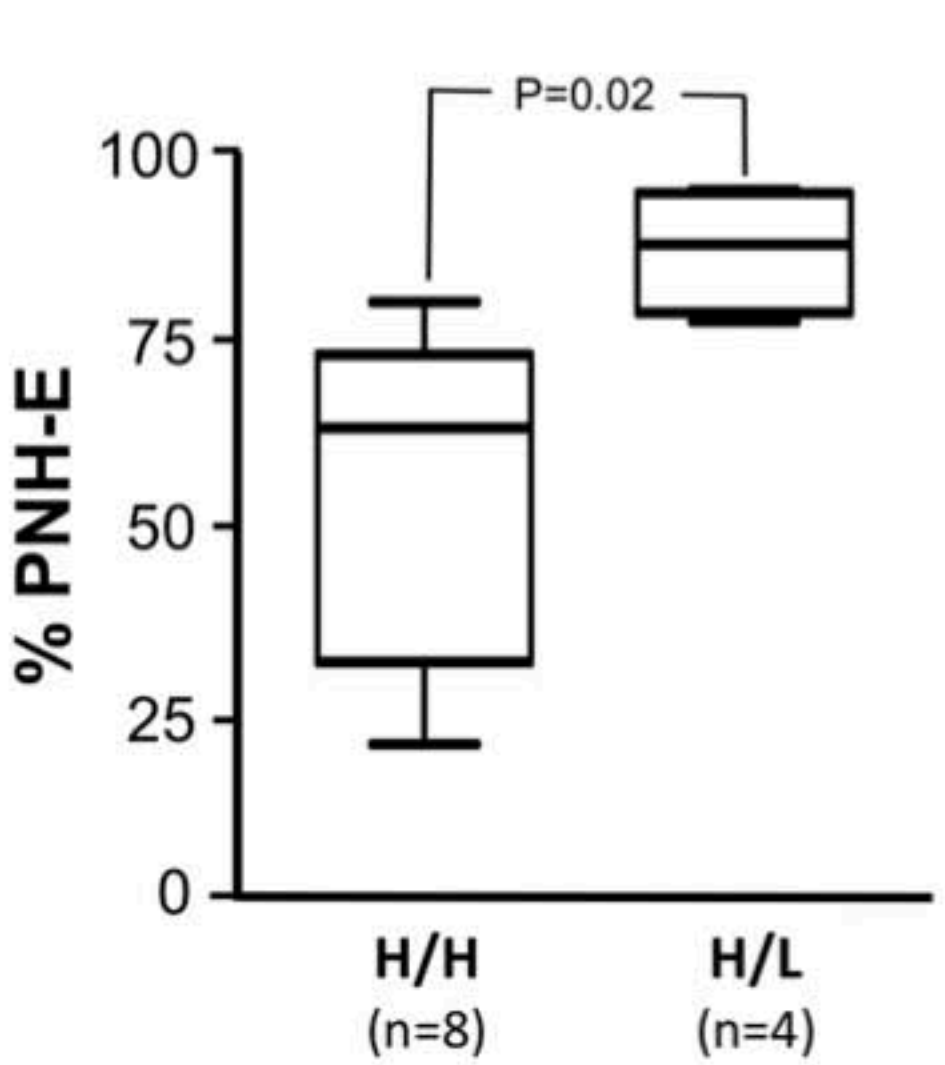
d)


P=0.034
Bilirubin (mg/dl)
10
8
6
4
2
0
> 25 %
C3+ cells
< 25 %
C3+ cells

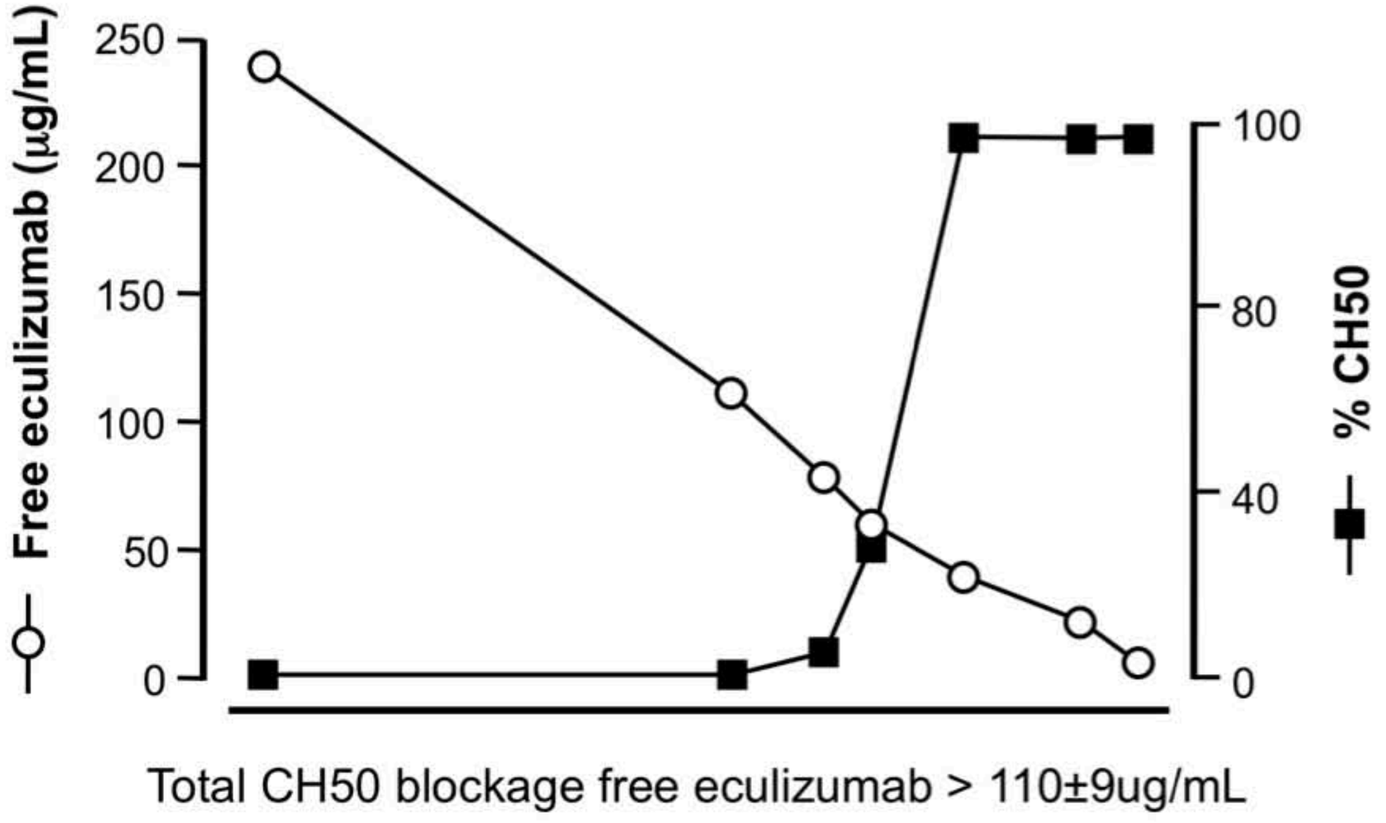
e)


P=0.5
% PNH-G (FLAER)
100
75
50
25
0
H/H
(n=8)
H/L
(n=4)

f)

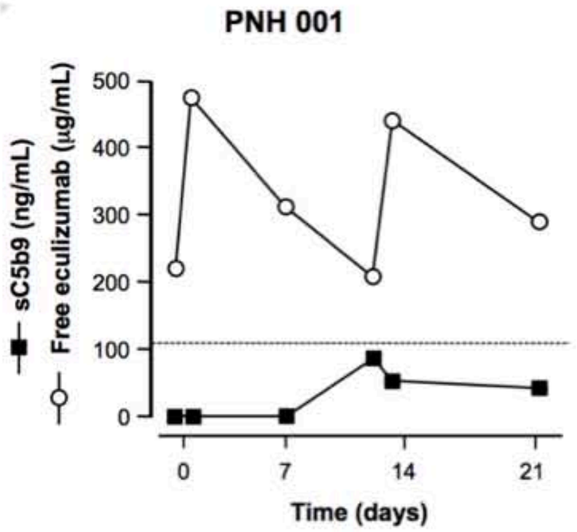

P=0.02
% PNH-E
100
75
50
25
0
H/H
(n=8)
H/L
(n=4)

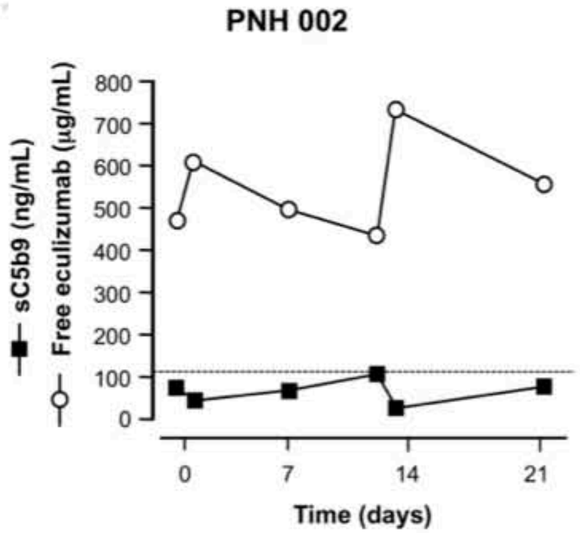
a)

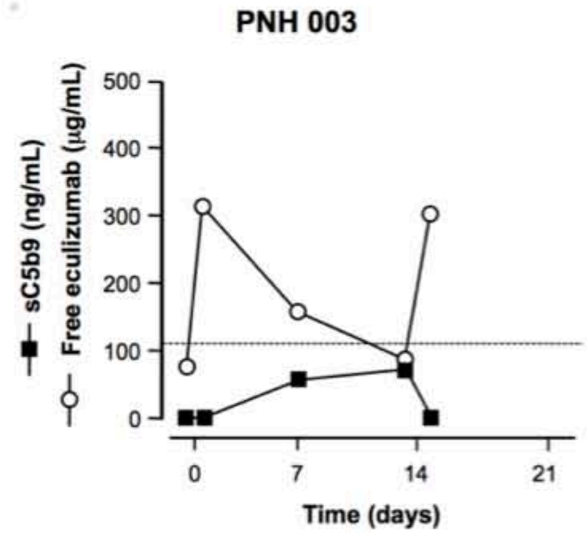


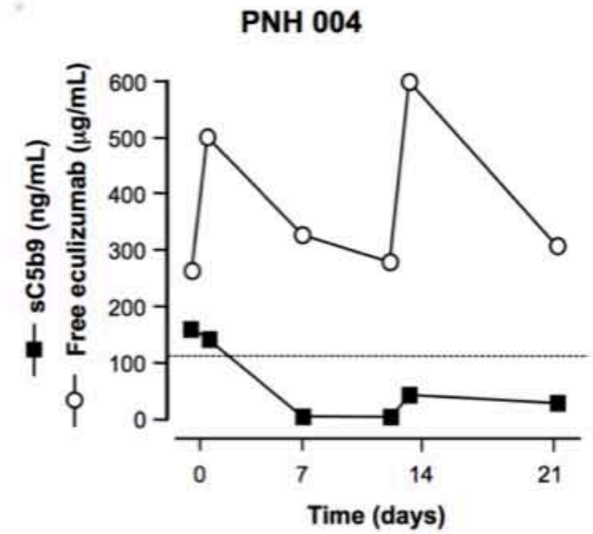
b)

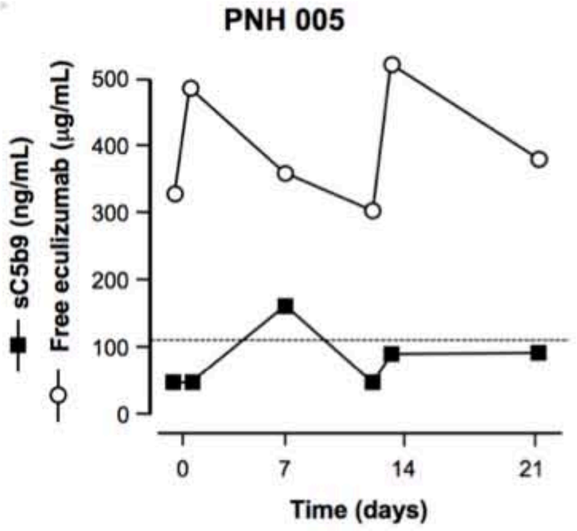
	Free eculizumab (µg/mL)
PNH 001	212 ± 8
PNH 002	499 ± 25
PNH 003	82 ± 8
PNH 004	268 ± 11
PNH 005	235 ± 17
PNH 006	106 ± 4
PNH 008	205 ± 68
PNH 014	242 ± 128
PNH 018	152 ± 3
PNH 026	401 ± 12
PNH 030	133 ± 14
PNH 033	129 ± 13

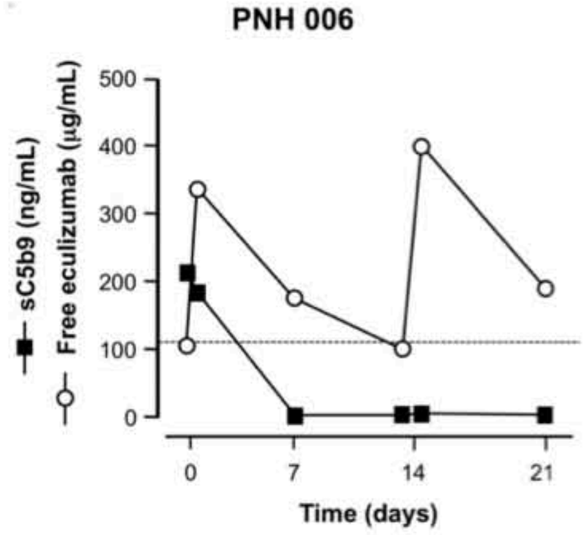

PNH 001
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)

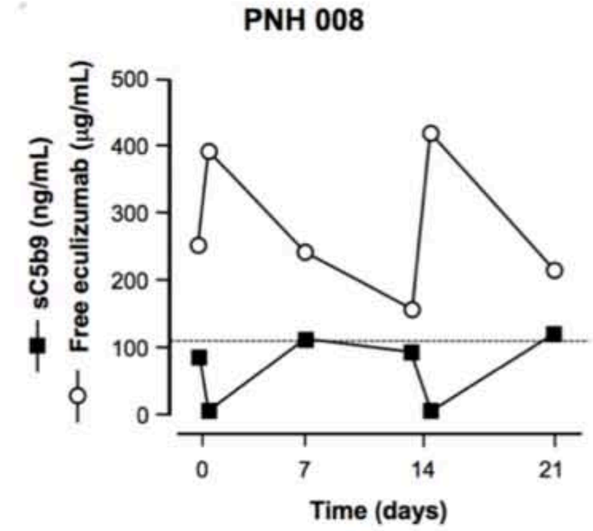

PNH 002
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)

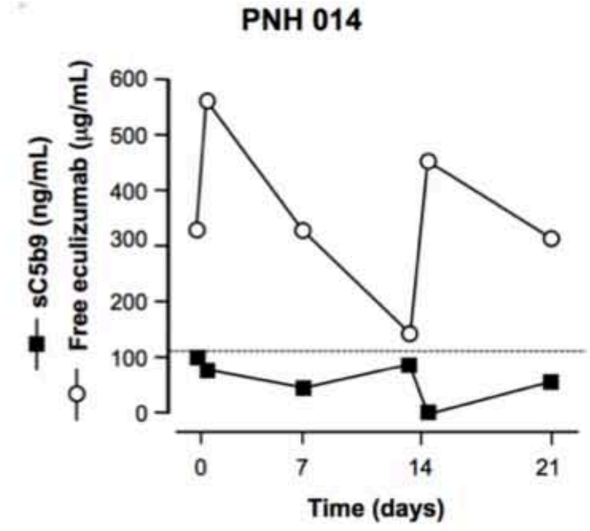

PNH 003
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)

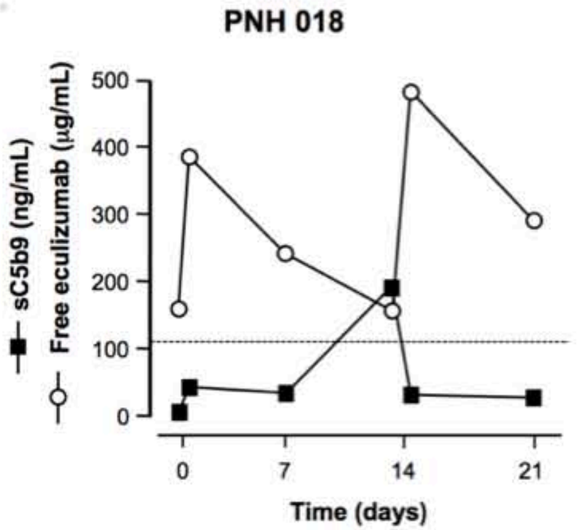

PNH 004
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)

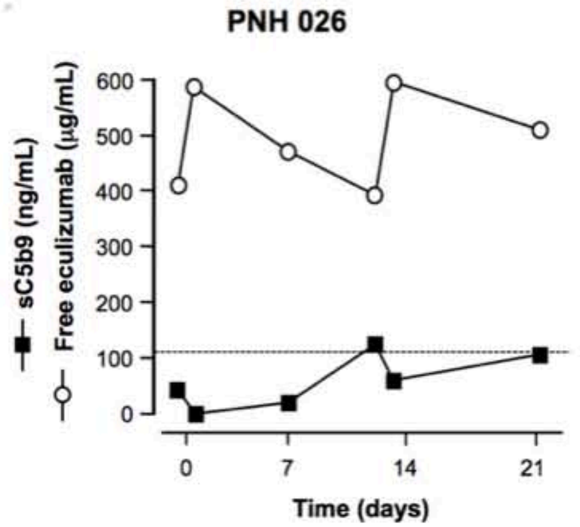

PNH 005
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)

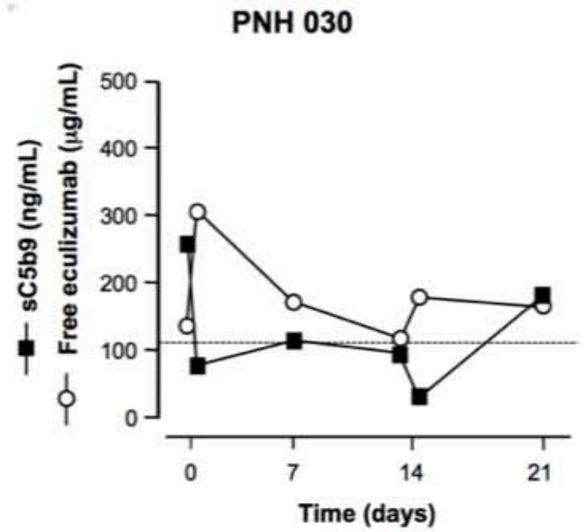

PNH 006
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)

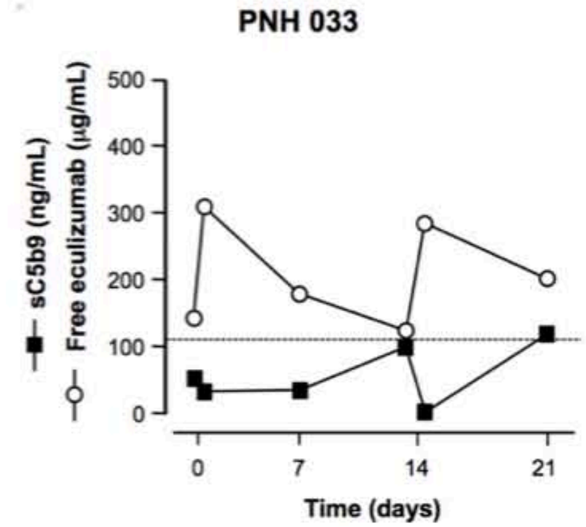

PNH 008
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)


PNH 014
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)


PNH 018
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)


PNH 026
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)


PNH 030
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)


PNH 033
sC5b9 (ng/mL)
Free eculizumab (µg/mL)
Time (days)