



Sensitive, reliable and easy-performed laboratory monitoring of eculizumab therapy in atypical hemolytic uremic syndrome



Elena B. Volokhina^a, Nicole C.A.J. van de Kar^a, Grethe Bergseth^b, Thea J.A.M. van der Velden^a, Dineke Westra^a, Jack F.M. Wetzels^c, Lambertus P. van den Heuvel^{a,d,e,1}, Tom Eirik Mollnes^{b,f,g,h,*}

^a Department of Pediatric Nephrology (774), Radboud University Medical Center, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands

^b Research Laboratory, Nordland Hospital, P. O. Box 1480, 8092 Bodø, Norway

^c Department of Nephrology (464), Radboud University Medical Center, P. O. Box 9101, 6500 HB, Nijmegen, The Netherlands

^d Department of Laboratory Medicine (774), Radboud University Medical Center, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands

^e Department of Pediatrics, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium

^f Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, P. O. Box 6050 Langnes, 9037 Tromsø, Norway

^g Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway

^h Department of Immunology, Oslo University Hospital, and K.G. Jebsen IRC, University of Oslo, P.B. 4950 Nydalen, NO-0424 Oslo, Norway

ARTICLE INFO

Article history:

Received 3 April 2015

accepted with revision 14 May 2015

Available online 23 June 2015

Keywords:

Hemolytic uremic syndrome

Complement

Eculizumab

Personalized therapy

ABSTRACT

Complement C5 inhibitor eculizumab treatment in atypical hemolytic uremic syndrome is effective, but associated with high costs. Complement inhibition monitoring in these patients has not been standardized. In this study we evaluated novel functional assays for application in routine follow-up.

We documented that the Wieslab® complement screen assay showed a sensitivity of 1–2% of C5 activity by adding purified C5 or normal human serum to a C5 deficient serum. All the patient samples obtained during the treatment course, were completely blocked for terminal complement pathway activity for up to four weeks after the eculizumab infusion. Levels of complexes between eculizumab and C5 were inversely correlated to the complement activity ($p = 0.01$). Moreover, titrating serum from eculizumab-treated patients into normal serum revealed that eculizumab was present in excess up to four weeks after infusion.

Thus, we demonstrate sensitive, reliable and easy-performed assays which can be used to design individual eculizumab dosage regimens.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The atypical form of the hemolytic uremic syndrome (aHUS) has a poor prognosis with up to 50% of cases progressing to end stage renal disease and up to 25% of lethal outcomes in the acute phase. Complement dysregulation leading to glomerular endothelial cell damage is considered to be a central element in aHUS etiology [1,2].

The complement system, a part of the innate immune system, can be activated through three pathways: the classical (CP), the lectin (LP), and the alternative (AP) [3,4]. Currently, genetic variants in AP are identified in 50–60% of aHUS patients. Furthermore, the presence of

autoantibodies against factor H or genetic variants of thrombomodulin are associated with aHUS pathogenesis [5–17].

Eculizumab (Soliris®) is a monoclonal antibody that binds to C5 and prevents its cleavage into C5a and C5b, thereby completely blocking the formation of terminal complement complex (C5b-9). It is currently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and aHUS and has opened a new era in the treatment of these diseases [18–23]. However, the optimal or individualized treatment schedule has not yet been established due to lack of reliable and easy routine tests to monitor the treatment.

The current therapy scheme is split into two phases: the initial phase (up to four weeks for patients with ≥ 40 kg body mass), followed by the maintenance phase. For pediatric patients (< 18 years old and < 40 kg), dosage regimen is adjusted to their body mass; the other patients receive the same amount of the drug according to the standardized adult treatment schedule. The present EMA and FDA guidelines recommend a life-long therapy with eculizumab infusions every two weeks (three weeks for the smallest infants) in the maintenance phase to prevent aHUS relapses. This recommendation has been questioned in

Abbreviations: aHUS, hemolytic uremic syndrome; CP, classical pathway; LP, lectin pathway; AP, alternative pathway; FDA, Food and Drug Administration; EMA, European Medicines Agency; PNH, paroxysmal nocturnal hemoglobinuria; C5D, C5 deficient; NHS, normal human serum; EDTA, ethylenediaminetetraacetic acid; MBL, mannose-binding lectin; sC5b-9, soluble C5b-9; E-C5, eculizumab and C5 complexes.

* Corresponding author at: Department of Immunology, Oslo University Hospital, P.B. 4950 Nydalen, NO-0424 Oslo, Norway. Fax: +47 23073510.

E-mail address: t.e.mollnes@medisin.uio.no (T.E. Mollnes).

¹ Contributed equally.

clinical practice, mainly due to the following concerns: a certain, though small, risk of meningococcal infection and sepsis [24,25], possible immunological complications in the long term, including development of neutralizing antibodies, and the extremely high cost of this treatment.

Although approval of eculizumab signified new hope for the aHUS patients, available reports describing pharmacokinetic and pharmacodynamics of this drug remain limited. Thus, in this study we aimed to establish a monitoring regimen for patients treated with eculizumab using novel and reliable complement assays that can be used routinely to follow the complement activity in these patients with high degree of sensitivity and specificity. This approach presents a highly relevant tool to develop individual treatment protocols for patients receiving complement inhibition therapy.

2. Materials and methods

2.1. Study population

All nine patients included in the study received eculizumab treatment at the Departments of Pediatric Nephrology and Nephrology of the Radboud University Medical Center as recommended by the current EMA and FDA guidelines. In several patients the treatment was discontinued or longer intervals between infusions were chosen as indicated.

The genetically C5 deficient (C5D) donor, included in the study as a control, has previously been described [26]. A normal human serum (NHS) pool was made using serum samples from 20 healthy adult volunteers. Purified human C5 was obtained from Quidel (San Diego, CA).

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 before analysis.

2.2. Sample collection

Whole blood was allowed to clot at room temperature for 30–45 min (serum samples) or immediately placed on ice (ethylenediaminetetraacetic acid (EDTA) plasma samples). Serum and EDTA plasma samples were prepared by centrifugation within 1 h after whole blood collection (10 min, 2500 ×g, 4 °C), aliquoted and stored at –80 °C. Urine samples were collected and centrifuged (10 min, 2500 ×g, 4 °C), supernatants were aliquoted and stored at –80 °C.

2.3. Assays to detect complement activity

The Wieslab® complement system screen (Euro Diagnostica AB, Malmö, Sweden) was used according to the manufacturer's protocol. This kit detects serum complement activity through CP, LP and AP with C5b-9 as common detection system for all three pathways. Due to the high degree of mannose-binding lectin (MBL) deficiency in the population, which gives a low LP activity, we included only the CP and AP assays of this kit in the present study. These assays were tested for sensitivity using a C5 deficient serum and were used to analyze serum samples from patients before, during and after treatment with eculizumab. In addition, serum samples from eculizumab-treated patients were diluted in NHS and analyzed in these assays.

The soluble C5b-9 (sC5b-9) levels in urine samples were measured using electroluminescent epitope assay as described before [27].

2.4. Assay to detect eculizumab–C5 complexes

The eculizumab and C5 (E–C5) complexes were detected in serum and urine samples using an enzyme-linked immunosorbent assay as described in detail previously [28]. In several cases serum samples were

not available, then the E–C5 complexes were measured in EDTA plasma, as indicated in footnote of Table 2.

2.5. Statistical analysis

Statistical analyses were performed using one-way ANOVA and unpaired two-tailed t-test. Spearman's rank test was used for correlation testing.

3. Results

3.1. Characterization of the patient group

Nine patients were included in the study, eight with aHUS (P1–8) and one with STEC-HUS (P9) (Table 1). The STEC-HUS patient was diagnosed by detection of antibodies against serotype O157. This patient received a single eculizumab infusion, based on the severe clinical symptoms and non-bloody diarrhea at presentation. Six patients (P1–P5 and P9) were receiving eculizumab therapy during the time of the study, of whom four started during the study period and therefore had a baseline sample included (P1, P3, P4, P9). Therapy of three patients (P6, P7, and P8) was already discontinued and only one single sample from each of these was collected (Table 2). The dosage regimen was in general applied according to the EMA and FDA guidelines (every week initially and then every second week on maintenance), but in patients P1, P4, and P5 the intervals between the eculizumab infusions were extended late in the maintenance phase from three to six weeks. From the patients that discontinued therapy or received eculizumab treatment with extended time intervals, none suffered from aHUS recurrence.

3.2. Sensitivity of the complement activity assay to detect functionally active C5

A useful assay to monitor the complement inhibitory effect of eculizumab requires a high degree of sensitivity for C5 activity at low C5 concentrations in order to find the optimal time-point for the next infusion. Therefore, we first analyzed the sensitivity of the Wieslab® complement system screen test with respect to detection of small amounts of free and functionally active C5. For this purpose we used serum from a C5 deficient (C5D) individual, added purified C5 protein or NHS as C5 source, at increasing concentrations, and measured recovery of complement activity (Fig. 1). C5D serum supplemented with purified C5 at two-fold steps (1 µg/mL–512 µg/mL) showed recovery of complement activity, as measured both by the CP and AP assay, at levels of approximately 10 µg C5/mL serum (Fig. 1A and B). C5D serum was then supplemented with NHS (1%–100% of the total volume) and recovery activity was measured (Fig. 1C and D). Detectable recovery started already at 1–2% of added NHS in both the CP and the AP assay, indicating that both these assays are highly sensitive to detect even trace amounts of C5 present.

Table 1

Patients receiving eculizumab treatment that were included in the study.

Patient number	aHUS genetic change	Gender (F/M)	Age at time of study (years)
P1	Factor H: c.1778T > A, p.Leu593Stop	F	28
P2	Unknown	M	2
P3	Unknown	M	9
P4	Unknown	M	12
P5	Factor H: c.2572T > A, p.Trp858Arg	F	22
P6	Factor H: c.2120delC	F	44
P7	C3: c.481C > T, p.Arg161Trp	F	32
P8	Factor B: c.967A > G, p.Lys323Glu	F	21
P9 ^a	Unknown	F	13

^a STEC-HUS patient.

Table 2
Treatment schedule and complement activity profiles in nine eculizumab-treated patients.

Treatment stage	CP ^a (%)	AP ^b (%)	E–C5 ^c (µg/mL)
P1			
before dose 1 ^u	105	64	0
1 week after dose 1 (900 mg) ^u	4.3	0.2	87
1 week after dose 2 (900 mg) ^u	1.9	0.7	169
1 week after dose 3 (900 mg) ^u	1.4	0.1	187
1 week after dose 4 (900 mg) ^u	0.9	0	137
2 weeks after dose 5 (1200 mg) ^u	0.5	0.1	121
2 weeks after dose 6 (1200 mg)	0.5	0	118
2 weeks after dose 14 (1200 mg)	0.6	0.2	133
3 weeks after dose 15 (1200 mg)	1.0	0.3	133
4 weeks after dose 16 (1200 mg)	1.4	0.2	122
P2			
2 weeks after dose 6 (300 mg)	0.1	0	107
2 weeks after dose 8 (300 mg)	0.2	0	90
2 weeks after dose 9 (300 mg)	n.a.	n.a.	97
2 weeks after dose 11 (300 mg)	0	0	99
2 weeks after dose 13 (300 mg)	0	0	99
2 weeks after dose 15 (300 mg)	0.1	0	91
P3			
before dose 1	87	76	0
1 week after dose 1 (900 mg)	0.6	0.1	67
1 week after dose 3 (900 mg)	0.3	0	107
1 week after dose 4 (900 mg)	n.a.	n.a.	87
2 weeks after dose 5 (1200 mg)	0	0	76
2 weeks after dose 6 (1200 mg)	0.1	0	93
2 weeks after dose 9 (1200 mg)	0	0	146
P4			
before dose 1	n.a.	n.a.	0
1 week after dose 1 (900 mg)	n.a.	n.a.	111
1 week after dose 2 (900 mg)	0.2	0	120
1 week after dose 3 (900 mg)	0	0	183
2 weeks after dose 6 (1200 mg)	0	0	87
2 weeks after dose 8 (1200 mg)	0.1	0	96
2 weeks after dose 11 (1200 mg)	0	0	91
3 weeks after dose 12 (1200 mg)	0	0	63
P5			
2 weeks after dose 6 (1200 mg) ^u	0.9	0	193
2 weeks after dose 7 (1200 mg) ^u	0.7	0	99
2 weeks after dose 8 (1200 mg)	0.8	0.4	101
2 weeks after dose 9 (1200 mg) ^u	0.6	0	89
2 weeks after dose 10 (1200 mg) ^u	0.6	0	110
2 weeks after dose 11 (1200 mg)	1.0	0.1	132
3 weeks after dose 12 (1200 mg)	1.8	0.5	144
4 weeks after dose 13 (1200 mg)	2.2	0.4	127
6 weeks after dose 15 (1200 mg)	73	45	48
P6			
14 weeks after dose 10 (1200 mg)	92	20	0
P7			
8 weeks after dose 10 (1200 mg)	107	81	37
P8			
23 weeks after dose >50 (1200 mg)	65	8.3	0
P9 STEC-HUS			
before dose 1	91	104	0
1 week after dose 1 (600 mg)	4.0	2.1	77
5 weeks after dose 1	77	89	18

n.a.: serum sample not available (EDTA plasma sample was used to measure E–C5).

^a CP = classical complement pathway activity.

^b AP = alternative complement pathway activity.

^c E–C5 = complexes between eculizumab and C5.

^u Urine samples were measured for these treatment points.

3.3. Complement activity and E–C5 complexes during eculizumab therapy

To analyze the effect of eculizumab in the patients, we quantified the complement activity with the Wieslab® assay (Table 2). The complement system was completely blocked by eculizumab in all samples obtained one or two weeks after infusion (AP < 3% in all, CP < 5% in all).

Notably, this was also the case in samples obtained three and four weeks after infusion (P1, P4, and P5). However, in the samples obtained five weeks after infusion (P9) or longer (P5, P6, P7, and P8), recovery of complement activity was observed.

E–C5 complexes in the baseline samples were not detectable, and in samples with complete complement inhibition they were consistently high (median 107 µg/mL, total range of 63–193 µg/mL) (Table 2). However, when complement activity recovered, the amount of E–C5 complexes decreased. Thus, in the samples, taken at five to eight weeks after the last dose (P5, P7, P9) E–C5 complexes were significantly ($p < 0.001$) decreased (range 18–48 µg/mL) compared to those with complete inhibition. After 14 and 23 weeks (P6 and P8) they were undetectable. There was a significant ($r = -0.38$; $p = 0.01$) negative correlation between the complement activity measured by AP and the amount of E–C5 when all samples were included. The complement activity obtained with CP and AP correlated closely ($r = 0.83$; $p < 0.0001$).

3.4. Estimation of eculizumab in excess in serum samples from aHUS patients

To examine the excess of eculizumab in serum samples during treatment, we selected samples from five patients that were obtained four weeks or less after an eculizumab infusion; i.e. they showed complete inhibition in the Wielisa® assays (Fig. 2). We performed careful titration of patient samples with increasing NHS concentrations to observe recovery of the terminal pathway using the CP and AP assays (Fig. 2). Since no effect of NHS was seen when added in an amount less than 80% of the total volume, only the range of 80–95% NHS is shown (i.e. 5–20% patient serum). Serum from the C5D individual (without eculizumab) was used as control. Adding NHS to C5D serum induced a recovery fully consistent with what would be expected if there was no C5 inhibitor present (Fig. 2). CP recovered in the range of 78–91% and AP in the range of 93–112% when added NHS constituted 80–95% of the final volume.

The patient samples showed a substantially delayed recovery compared to the C5D control, consistent with free eculizumab in excess. Thus, when 80% NHS and 20% patient serum was used, complement activity was inhibited in all samples, except for one (P1, one week after the first dose). All samples obtained in the maintenance phase two weeks after the eculizumab infusion required at least 90% NHS to reach a recovery of complement activity above 10%. In two of these samples (the second samples of P3 and P4) complement activity remained below 10% after supplementation with 95% NHS. Samples taken four weeks after the infusion (P1 and P5) required more than 80% NHS for complement recovery.

Thus, our results indicate the presence of excessive eculizumab in aHUS patients, which may bind and inactivate fresh C5 added with NHS. In this case, increase in E–C5 complexes would be expected upon addition of NHS to the patient sera. In order to test this hypothesis, we measured E–C5 complexes in the samples. Indeed, levels of E–C5 complexes consistently increased with addition of NHS to the samples, confirming binding of added C5 to free eculizumab (Fig. 3).

3.5. sC5b-9 and E–C5 complexes measured in urine

We finally investigated whether E–C5 complexes can be found in urine. Normally, large proteins are not secreted via the kidneys due to their size, however, aHUS patients often have proteinuria and therefore may have E–C5 present in urine. The molecular weights of E–C5 complexes are 340 and 530, dependent on monovalent or bivalent binding of C5. Available urine samples from patients P1 and P5, with high E–C5 complexes in plasma were tested and no E–C5 complexes were detected in urine samples. However, in a urine sample obtained from P1 before treatment, sC5b-9 was detected at the level of 63.5 CAU/L, indicating improved effect on renal function after eculizumab treatment was initiated.

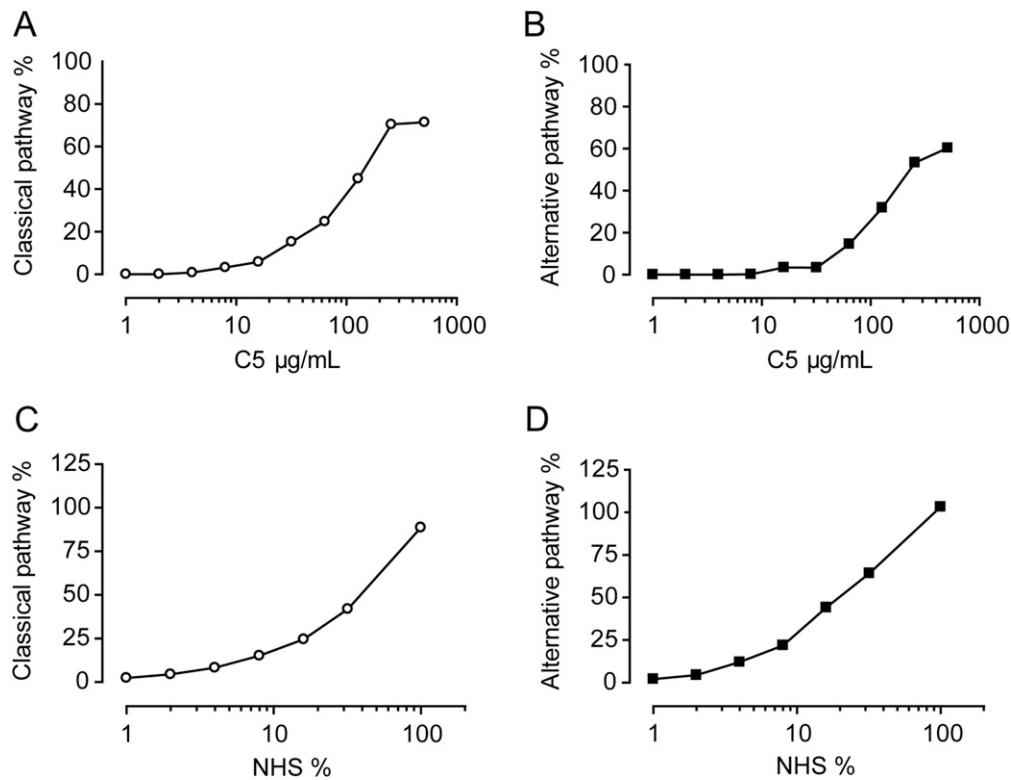


Fig. 1. Recovery of complement activation in serum from a C5 deficient donor. Serum from a C5D donor was supplemented with the commercially available purified C5 at two-fold concentrations steps (1 µg/mL–512 µg/mL) (A and B) or with normal human serum (NHS) at 1%–100% of the total volume (C and D). Activity of classical (A and C) and alternative (B and D) pathways was measured. Data are presented as percentage of activation of the positive control (100%) provided with the assay. The experiments were repeated three times and representative curves are shown.

4. Discussion

In this study, we have documented a high degree of sensitivity of the complement activity screen test. We have used *in vitro* assays to evaluate the efficacy of the treatment on complement activity, and demonstrated an excess of eculizumab in samples obtained up to four weeks after drug infusion. In the samples taken five weeks or longer after an eculizumab infusion, recovery of complement activity was detected, which correlated with decreased levels of the E–C5 complexes. Further studies in larger cohorts should indicate whether profiles that we observed are characteristic for all patients, especially children. Collectively, these data support the current tests to be suitable as a guide for individualized treatment of patients that receive eculizumab.

The sensitivity and specificity of the screen test was documented using C5D serum. Small amounts of purified C5 and NHS increased the activity in C5D from zero. Only 1–2% NHS added increased the activity, indicating a very high sensitivity of the assay. By using purified C5, slightly more protein had to be added to get an increase, which is consistent with the purified protein being less functionally active than native C5 present in serum. This was supported by the fact that 100% recovery was obtained by addition of NHS, whereas 70% was the maximum recovery obtained by purified C5, which had to be added several-folds the normal serum concentration in order to obtain this recovery. Purified C5 obtained from other source (Complement Technology) also was not able to fully reconstitute complement activity (data not shown). Using such C5 preparations may give inaccurate and underestimated results when interpreting eculizumab inhibiting capacity for C5. Nevertheless, in the future in clinical practice commercially available C5 may be used as an internal control to standardize the assay, especially to compare results between the laboratories. Thus, we proceeded with NHS as a C5 source and supplemented serum samples from five patients with various concentrations of NHS and measured complement activity. The results clearly indicate that in the

maintenance phase of the therapy all the samples obtained two weeks after the eculizumab infusion required supplementation with at least 10 volumes of NHS to produce detectable recovery of complement activity. One sample, however, differed from the others (the sample of P1 that was obtained one week after the first eculizumab infusion). This sample showed 4.3% CP activity, though 0.2% AP activity (Table 2), and required less NHS for recovery (Fig. 2) than the others. This may indicate individual differences in requirement for full saturation of circulating C5 at first dose given. After the second dose, samples from this patient were comparable to the others. For the whole material there was, however, a very close correlation between results obtained with CP and AP was seen. Thus, it is possible that in the future only one of these is needed, but more data are needed in order to compare these two and decide which test should be chosen. We have followed a girl on eculizumab treatment over a couple of years with >35 samples tested for CP and AP activity, and all samples in both assays consistently showed <1% activity (personal observation, TEM). The LP assay would not be suitable for this particular purpose, due to the high frequency of MBL deficiency in the population.

So far, it is not clear, whether discontinuation of therapy is a good choice for all recovered aHUS patients. Recent reports indicate that some patients experience relapses after treatment has been discontinued, while others do not [29–31]. Nevertheless, at this point it is crucially important to introduce individualized complement inhibition therapy for aHUS patients, especially in maintenance phase. This should be done in terms of treatment intervals and also in terms of eculizumab dosage. Our data suggest that a majority of patients receive substantially more drug than needed for complete C5 inhibition.

Current guidelines on eculizumab treatment prescribe the same dose regimen for all adults (≥18 years of age), independent of such factors as age, body mass and C5 concentration in blood. Individual approach to dose regimen, based on the novel assay protocol, described here, would optimize quantity of the drug needed and indicate most

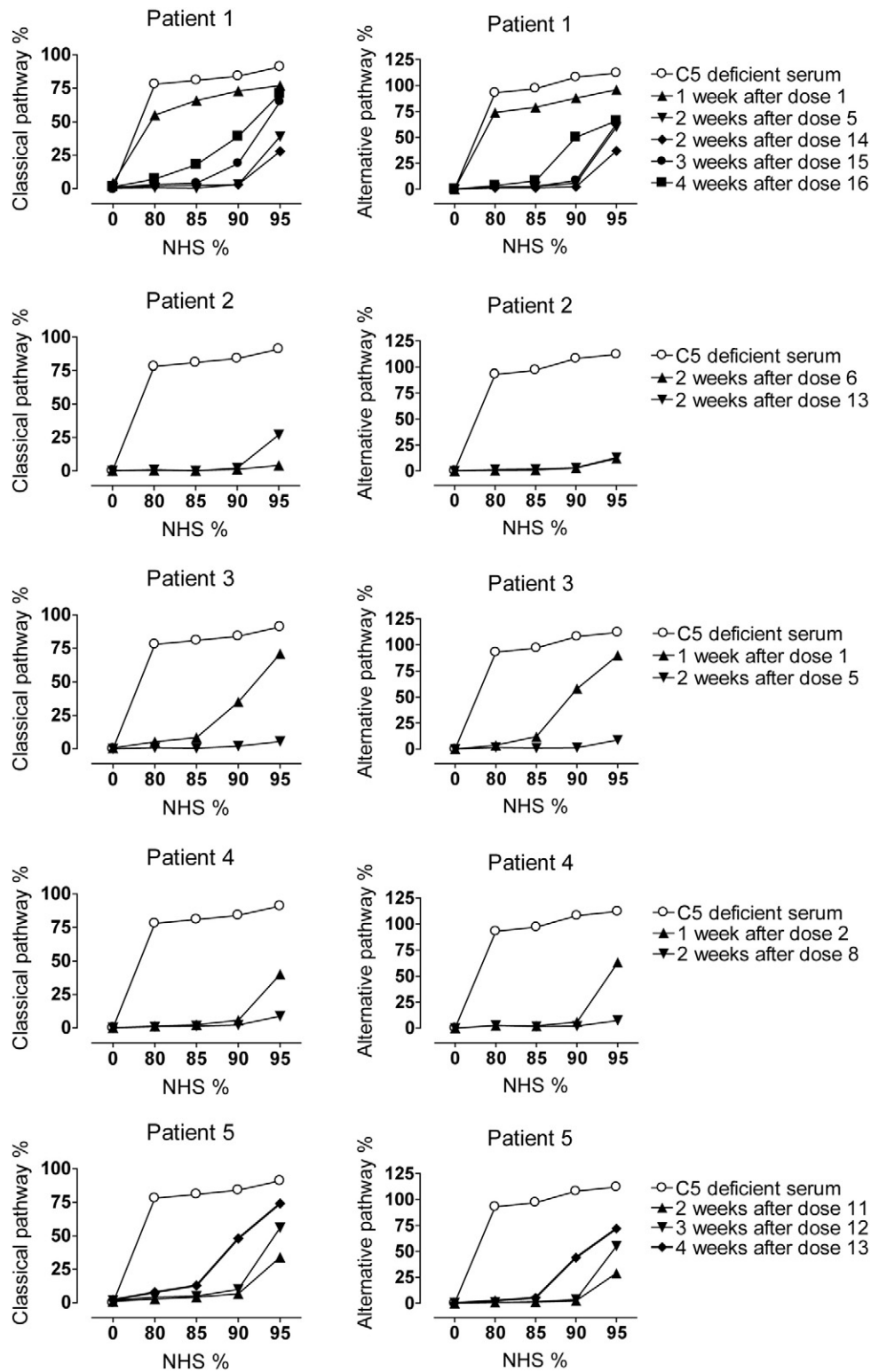


Fig. 2. Recovery of complement activation in eculizumab-treated aHUS serum samples. Samples from five aHUS patients and a C5 deficient donor were supplemented with NHS (range 80–95% of the total volume), after that the recoveries of the classical and the alternative complement pathways were analyzed by the Wieslab® complement system screen. Data are represented as percentage of activation of the positive control provided with the assay by the manufacturer.

favorable intervals between doses. In the future, samples should be analyzed for eculizumab inhibiting capacity using our titration assays and, in case considerable excess will be established, intervals may be extended by one week at a time or dosage can be adjusted in case of absence of signs of active disease. This will relieve the economic pressure on the health care system, making this very expensive—and

very promising—medication more easily available also to other patients who are in need of it.

One previous study has used the Wieslab® kit to monitor aHUS patients [32]. They used doses of eculizumab in the range of 12–33 mg/kg (12 mg/kg corresponds to the normal first dose of 900 mg given to a 75 kg person). They showed a recovery in some patients after three

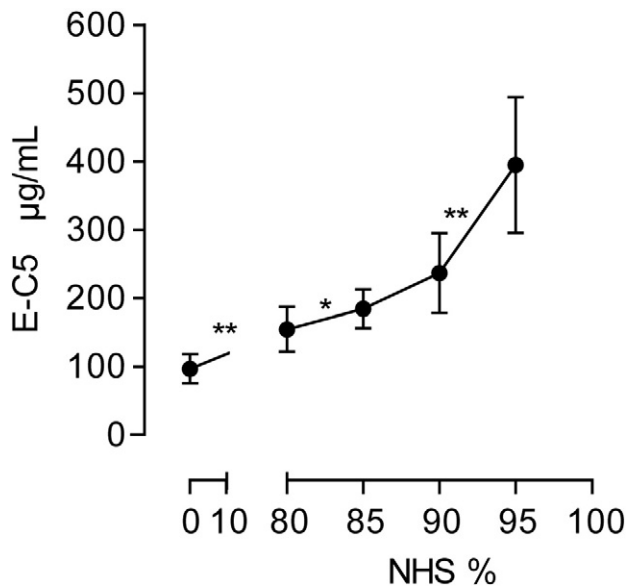


Fig. 3. Increase of the levels of E–C5 complexes with addition of NHS to serum samples from ecuzumab-treated aHUS patients. Samples from patients P1 (after dose 1 and dose 5), P2 (after dose 6 and dose 13), P3 (after dose 1 and dose 5), and P4 (after dose 2 and dose 8) were diluted with various amounts of NHS, and the levels of E–C5 complexes were measured. The data are presented as mean and standard deviation. Statistically significant increases between the dilution steps with $p < 0.05$ (*) and $p < 0.01$ (**) are indicated.

weeks and a significant recovery four weeks after the infusion. In this study the excess of ecuzumab or the E–C5 complexes were not investigated. Despite this, and the difference in dosage and complement activity recovery time, their data support the screen test as a suitable test to monitor these patients. Our test regimen, however, gives more detailed data on the activity and the excess of ecuzumab present at the time of sampling.

In a recent comprehensive study Noris et al. [33] investigated in detail complement activation profile in aHUS patients treated with ecuzumab. They showed that endothelial cell deposition of C5b-9 induced from these patients could be used as a tool to monitor ecuzumab therapy. This assay is interesting from a pathophysiological point of view and documents that if there is sufficient ecuzumab present in serum there is markedly reduced deposition of C5b-9 on the cells. Thus, this is an important assay approach, but it requires a specialized laboratory experienced with endothelial cell work and this assay is far more demanding with respect to personal and economical resources as compared to the simple assays we here describe. Our methods are independent on personal skills, can be performed at every hospital where ELISA equipment is established and the results can be available for the patient's physician the same day as the patient visit the clinic.

In a very recent study PNH patients treated with ecuzumab over a two-year period were followed with serum analysis of CH50 and free ecuzumab [34]. These data are in accordance with ours, but it should be noted that due to batch-to-batch variation in quality of erythrocytes, the traditional hemolytic CH50 assay is prone to variability in lower detection range, which might be clinically significant. Thus, a more accurate detection of low level activity in the 1% range will be obtained with the standardized ELISA-based assay we have used.

Another study suggested the use of novel highly specific antibody against soluble C5b to monitor *in vitro* complement activation blockade by ecuzumab. The authors used hemolytic assay to activate complement in this experiment. In the future, it might be interesting to test whether the use of soluble C5b marker will contribute to better standardization of our assay based on Wieslab® complement system screen test [35].

In conclusion, we have documented sensitive, reliable and easy to perform routine assays to be used as a platform for a therapy regimen that optimizes the use of ecuzumab for individualized treatment.

Disclosures

JFMW and NCAJvdK received speaker's fee from Alexion Pharmaceuticals. All other authors declare that there is no conflict of interest.

Acknowledgments

This work was partially supported by the Dutch Kidney Foundation (1301116, KFB 11.007, IP 10.22, KBSO 11.019, C09.2313), ERA-EDTA (ERA STF 138-2013, ERA LTF 203-2014), The Research Council of Norway (204874/F20), The Norwegian Council on Cardiovascular Disease (NCCD-2014), The Northern Norway Regional Health Authority (SPF914-10), The Southern and Eastern Norway Regional Health Authority, The Odd Fellow Foundation (OFF-2014), and the European Community's Seventh Framework Programme under grant agreement no 602699 (DIREKT). Funding organizations did not have a role in study design, collection, analysis and interpretation of data, writing of the report, decision to submit this article for publication.

References

- Noris, G., Remuzzi, Atypical hemolytic-uremic syndrome, *N. Engl. J. Med.* 361 (2009) 1676–1687.
- Westra, J.F., Wetzels, E.B., Volokhina, L.P., van den Heuvel, N.C., van de Kar, A new era in the diagnosis and treatment of atypical haemolytic uraemic syndrome, *Neth. J. Med.* 70 (2012) 121–129.
- Harboe, E.B., Thorgersen, T.E., Mollnes, Advances in assay of complement function and activation, *Adv. Drug Deliv. Rev.* 63 (2011) 976–987.
- M.J. Walport, Complement. First of two parts, *N. Engl. J. Med.* 344 (2001) 1058–1066.
- A.L. Stahl, F. Vaziri-Sani, S. Heinen, A.C. Kristoffersson, K.H. Gydell, R. Raafat, A. Gutierrez, O. Beringer, P.F. Zipfel, D. Karpman, Factor H dysfunction in patients with atypical hemolytic uremic syndrome contributes to complement deposition on platelets and their activation, *Blood* 111 (2008) 5307–5315.
- M.J. Lehtinen, A.L. Rops, D.E. Isenman, J. van der Vlag, T.S. Jokiranta, Mutations of factor H impair regulation of surface-bound C3b by three mechanisms in atypical hemolytic uremic syndrome, *J. Biol. Chem.* 284 (2009) 15650–15658.
- V. Fremeaux-Bacchi, M.A. Dragon-Durey, J. Blouin, C. Vigneau, D. Kuypers, B. Boudailliez, C. Loirat, E. Rondeau, W.H. Fridman, Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome, *J. Med. Genet.* 41 (2004) e84.
- A. Richards, E.J. Kemp, M.K. Liszewski, J.A. Goodship, A.K. Lampe, R. Decorte, M.H. Muslimanoglu, S. Kavukcu, G. Filler, Y. Pirson, L.S. Wen, J.P. Atkinson, T.H. Goodship, Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 12966–12971.
- M. Noris, S. Brioschi, J. Caprioli, M. Todeschini, E. Bresin, F. Porrati, S. Gamba, G. Remuzzi, Familial haemolytic uraemic syndrome and an MCP mutation, *Lancet* 362 (2003) 1542–1547.
- J. Esparza-Gordillo, E. Goicoechea de Jorge, A. Buil, B.L. Carreras, M. Lopez-Trascasa, P. Sanchez-Corral, S. Rodríguez de Córdoba, Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32, *Hum. Mol. Genet.* 14 (2005) 703–712.
- J. Caprioli, M. Noris, S. Brioschi, G. Pianetti, F. Castelletti, P. Bettinaglio, C. Mele, E. Bresin, L. Cassis, S. Gamba, F. Porrati, S. Bucchioni, G. Monteferrante, C.J. Fang, M.K. Liszewski, D. Kavanagh, J.P. Atkinson, G. Remuzzi, Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome, *Blood* 108 (2006) 1267–1279.
- V. Fremeaux-Bacchi, E.C. Miller, M.K. Liszewski, L. Strain, J. Blouin, A.L. Brown, N. Moghal, B.S. Kaplan, R.A. Weiss, K. Lhotta, G. Kapur, T. Mattoo, H. Nivet, W. Wong, S. Gie, B. Hurault de Ligny, M. Fischbach, R. Gupta, R. Hauhart, V. Meunier, C. Loirat, M.A. Dragon-Durey, W.H. Fridman, B.J. Janssen, T.H. Goodship, J.P. Atkinson, Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome, *Blood* 112 (2008) 4948–4952.
- L.T. Roumenina, M. Frimat, E.C. Miller, F. Provot, M.A. Dragon-Durey, P. Bordereau, S. Bigot, C. Hue, S.C. Satchell, P.W. Mathieson, C. Mousson, C. Noel, C. Sautes-Fridman, L. Halbwachs-Mecarelli, J.P. Atkinson, A. Lionet, V. Fremeaux-Bacchi, A prevalent C3 mutation in aHUS patients causes a direct C3 convertase gain of function, *Blood* 119 (2012) 4182–4191.
- E. Volokhina, D. Westra, X. Xue, P. Gros, N. van de Kar, L. van den Heuvel, Novel C3 mutation p.Lys65Gln in aHUS affects complement factor H binding, *Pediatr. Nephrol.* 27 (2012) 1519–1524.
- E. Goicoechea de Jorge, C.L. Harris, J. Esparza-Gordillo, L. Carreras, E.A. Arranz, C.A. Garrido, M. Lopez-Trascasa, P. Sanchez-Corral, B.P. Morgan, S. Rodríguez de Córdoba,

- Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 240–245.
- [16] M. Delvaeye, M. Noris, A. De Vriese, C.T. Esmon, N.L. Esmon, G. Ferrell, J. Del-Favero, S. Plaisance, B. Claes, D. Lambrechts, C. Zoja, G. Remuzzi, E.M. Conway, Thrombomodulin mutations in atypical hemolytic-uremic syndrome, *N. Engl. J. Med.* 361 (2009) 345–357.
- [17] M. Jozsi, C. Licht, S. Strobel, S.L. Zipfel, H. Richter, S. Heinen, P.F. Zipfel, C. Skerka, Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency, *Blood* 111 (2008) 1512–1514.
- [18] R.P. Rother, S.A. Rollins, C.F. Mojcik, R.A. Brodsky, L. Bell, Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria, *Nat. Biotechnol.* 25 (2007) 1256–1264.
- [19] A. Hill, R.J. Kelly, P. Hillmen, Thrombosis in paroxysmal nocturnal hemoglobinuria, *Blood* 121 (2013) 4985–4996.
- [20] A.M. Waters, C. Licht, aHUS caused by complement dysregulation: new therapies on the horizon, *Pediatr. Nephrol.* 26 (2011) 41–57.
- [21] J. Zuber, F. Fakhouri, L.T. Roumenina, C. Loirat, V. Fremeaux-Bacchi, Use of eculizumab for atypical haemolytic uraemic syndrome and C3 glomerulopathies, *Nat. Rev. Nephrol.* 8 (2012) 643–657.
- [22] E.K. Wong, T.H. Goodship, D. Kavanagh, Complement therapy in atypical haemolytic uraemic syndrome (aHUS), *Mol. Immunol.* 56 (2013) 199–212.
- [23] C.M. Legendre, C. Licht, P. Muus, L.A. Greenbaum, S. Babu, C. Bedrosian, C. Bingham, D.J. Cohen, Y. Delmas, K. Douglas, F. Eitner, T. Feldkamp, D. Fouque, R.R. Furman, O. Gaber, M. Herthelius, M. Hourmant, D. Karpman, Y. Lebranchu, C. Mariat, J. Menne, B. Moulin, J. Nurnberger, M. Ogawa, G. Remuzzi, T. Richard, R. Sberro-Soussan, B. Severino, N.S. Sheerin, A. Trivelli, L.B. Zimmerhack, T. Goodship, C. Loirat, Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome, *N. Engl. J. Med.* 368 (2013) 2169–2181.
- [24] A. Bouts, L. Monnens, J.C. Davin, G. Struijk, L. Spanjaard, Insufficient protection by *Neisseria meningitidis* vaccination alone during eculizumab therapy, *Pediatr. Nephrol.* 26 (2011) 1919–1920.
- [25] G.H. Struijk, A.H. Bouts, G.T. Rijkers, E.A. Kuin, I. ten Berge, F.J. Bemelman, Meningococcal sepsis complicating eculizumab treatment despite prior vaccination, *Am. J. Transplant.* 13 (2013) 819–820.
- [26] G. Grimnes, H. Beckman, K.T. Lappegard, T.E. Mollnes, V. Skogen, Recurrent meningococcal sepsis in a presumptive immunocompetent host shown to be complement C5 deficient—a case report, *APMIS* 119 (2011) 479–484.
- [27] B.J. van Vuuren, G. Bergseth, T.E. Mollnes, A.M. Shaw, Electroluminescent TCC, C3dg and fB/Bb epitope assays for profiling complement cascade activation in vitro using an activated complement serum calibration standard, *J. Immunol. Methods* 402 (2014) 50–56.
- [28] R.F. Hallstensen, G. Bergseth, S. Foss, S. Jaeger, T. Gedde-Dahl, J. Holt, D. Christiansen, C. Lau, O.L. Brekke, E. Armstrong, V. Stefanovic, J.T. Andersen, I. Sandlie, T.E. Mollnes, Eculizumab treatment during pregnancy does not affect the complement system activity of the newborn, *Immunobiology* 220 (2015) 452–459.
- [29] G. Ardissino, S. Testa, I. Possenti, F. Tel, F. Paglialonga, S. Salardi, S. Tedeschi, M. Belingeri, M. Cugno, Discontinuation of eculizumab maintenance treatment for atypical hemolytic uremic syndrome: a report of 10 cases, *Am. J. Kidney Dis.* 64 (2014) 633–637.
- [30] R. Carr, S.R. Cataland, Relapse of aHUS after discontinuation of therapy with eculizumab in a patient with aHUS and factor H mutation, *Ann. Hematol.* 92 (2013) 845–846.
- [31] J.J. Pu, A. Sido, Successful discontinuation of eculizumab therapy in a patient with aHUS, *Ann. Hematol.* 93 (2014) 1423–1425.
- [32] M. Cugno, R. Gualtierotti, I. Possenti, S. Testa, F. Tel, S. Griffini, E. Grovetti, S. Tedeschi, S. Salardi, D. Cresseri, P. Messa, G. Ardissino, Complement functional tests for monitoring eculizumab treatment in patients with atypical hemolytic uremic syndrome, *J. Thromb. Haemost.* 12 (2014) 1440–1448.
- [33] M. Noris, M. Galbusera, S. Gastoldi, P. Macor, F. Banterla, E. Bresin, C. Tripodo, S. Bettoni, R. Donadelli, E. Valoti, F. Tedesco, A. Amore, R. Coppo, P. Ruggerenti, E. Gotti, G. Remuzzi, Dynamics of complement activation in aHUS and how to monitor eculizumab therapy, *Blood* 124 (2014) 1715–1726.
- [34] R. Peffault de Latour, V. Fremeaux-Bacchi, R. Porcher, A. Xhaard, J. Rosain, D. Cadena Castaneda, P. Vieira-Martins, S. Roncelin, P. Rodriguez-Otero, A. Plessier, F. Sicre de Fontbrune, S. Abbes, M. Robin, G. Socie, Assessing complement blockade in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab, *Blood* 125 (2015) 775–783.
- [35] A.M. Blom, A. Österborg, T.E. Mollnes, M. Okroj, Antibodies reactive to cleaved sites in complement proteins enable highly specific measurement of soluble markers of complement activation, *Mol. Immunol.* 66 (2015) 164–170.