Report for ESPN Research Grant 2014.03

Title: Sorting complement dysregulation in renal disorders

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Project summary

A number of rare devastating renal disorders is associated with the dysregulation of complement system, a part of the innate immune system. Important complement-mediated renal disorders include: C3 glomerulopathies (C3G), immune-complex-mediated glomerulonephritis (IgGN) and atypical hemolytic uremic syndrome (aHUS).

In many cases, these disorders have overlapping etiologies. Diagnosis often relies on renal biopsy, which is an invasive procedure for the patient and in case of aHUS should be avoided due to increased chance of bleeding caused by thrombocytopenia. Moreover, current understanding of exact complement pathology in each of these disorders is still limited.

Here we hypothesized that these disorders have different patterns of complement activation biomarkers in fluid phase, which should correspond with biopsy and clinical findings and can be analyzed in serum and/or EDTA plasma samples.

In collaboration with the Department of (Pediatric) Nephrology, University Hospitals Leuven, Belgium (Prof. dr. Kathleen Claes/ Prof. Dr. Ben Sprangers) in this ESPN project we are studying complement activation in renal diseases. This collaboration allows us to combine clinical and laboratory expertise of the departments with strong track record in diagnostics and treatment of complement-mediated renal disorders.

In this highly relevant project, we validated assays to characterize complement activation and performed assays using the samples of patients with various complement-mediated renal diseases. Furthermore, we developed and validated new method for the characterization of C3 convertase stability, which is particularly useful for the characterization of C3G patients. In all disorders activation of the alternative pathway of complement has been established. Furthermore, we established significant differences in complement activation markers between the atypical aHUS and STEC-HUS. Quick and efficient distinction between the two types of HUS is highly important as they demand different approach to treatment. Analysis of samples from 31 C3G patients revealed two distinct groups of patients with stable and highly stable convertase profiles, which may reflect distinct pathophysiological mechanisms in these patients.

Overall, this project has allowed us to develop new techniques, get more insight into the pathophysiology of renal diseases and strengthen the collaboration between the participating research and clinical centers. The project participants are very grateful to the ESPN for the support given to this project.

Results

Sample collection

Currently we have collected samples from 11 aHUS patients, 26 STEC-HUS patients, 31 C3G patients and 7 patients with monoclonal gammopathy of renal significance (MGRS), where complement activation/dysregulation and renal damage are likely to be caused by monoclonal immunoglobulins or their fragments. Moreover, samples from 48 control individuals have been collected. All samples have been collected by using strict protocol for complement studies, in which samples are processed on ice within one hour after blood collection to prevent *in vitro* complement activation. More samples will be collected in the following 1 year of this project.

Validation of ELISA assays to measure complement activation markers C4b/c, C3b/c, C3bBbP and TCC

First, we performed a through validation of the assays to measure the complement activation markers. The assays have been established at the department of Pediatric Nephrology, Radboudumc and validation has been carried out in this project. The current validation results are summarized below (Table 1). Validation of the assay to measure C1rs-C1 inhibitor complex as a marker of complement activation via classical pathway is currently being performed.

Table 1. Validation results of C4b/c, C3b/c, C3bBbP and TCC assays

	Intra-run CV (%)	**Inter-run (CV%)**	**LDL (CAU/ml)[1]**	**Normal range[2]**
C4b/c	5.4	7.2	1.75	<6
C3b/c	4.2	13.2	3.0	<15.0
C3bBbP	10.9	8.5	2.0	<12.0
TCC	1.2	2.2	0.23	<0.5

[1]The values are given in complement activation units per ml (CAU/ml) of the international complement activation standard #2 (Bergseth *et al*., *Mol Immunol*, 2013).

[2]The normal range was defined as mean+ 2 standard deviations of healthy controls (n=48).

Analysis of complement activation markers in pediatric control age groups

Because especially HUS patients are often children, it was important to determine, whether the levels of complement activation markers in various pediatric age groups are different. Samples of the healthy controls (age 2 months-17 years) have been collected (Figure 1). Statistical analysis indicated that there is no difference in levels of complement activation markers between individuals of different ages.

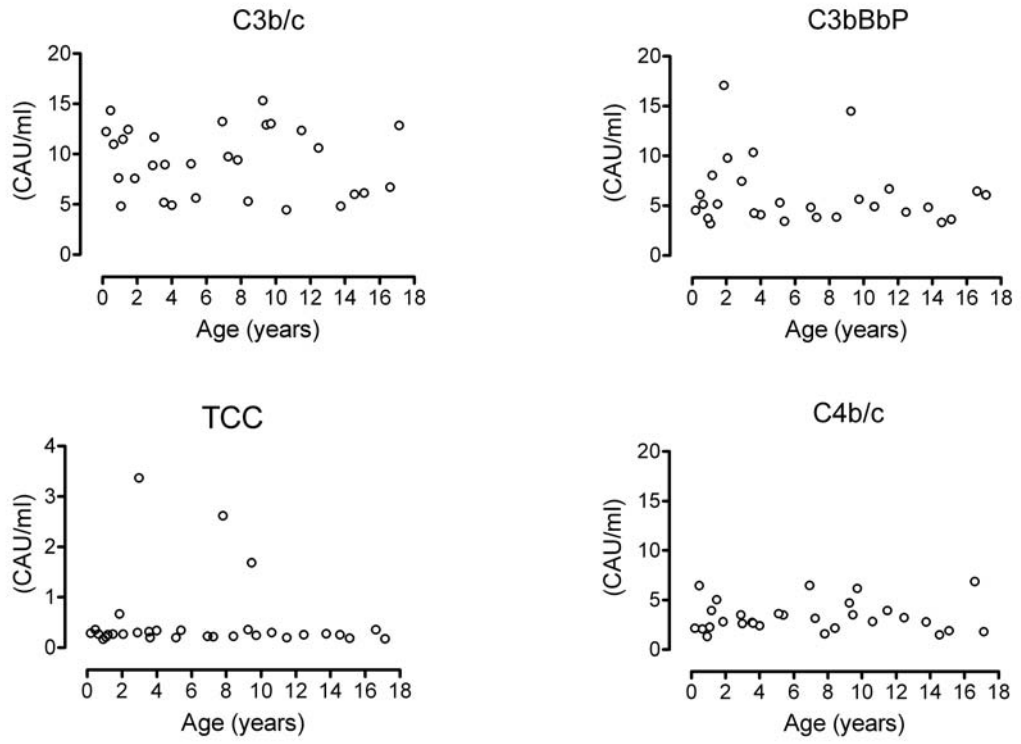


Figure 1. Complement activation markers in samples of healthy pediatric controls. Complement activation markers have been determined in EDTA plasma samples from 30 pediatric controls (age 2 months- 17 years). Data were quantified using international complement standard#2 (ICS#2) in complement activation units per ml (CAU/ml) (Bergseth et al., Mol Immunol, 56:232-239 2013).

Analysis of complement activation in patient samples

Analysis of complement activation in patient samples is currently ongoing. In the first experiments, alternative complement pathway activation was analyzed by measuring C3bc, C3bBbP and TCC in a number of patients from three patient groups (Figure 2). All patient groups demonstrate increased alternative complement pathway activation profiles, compared to healthy individuals, however, no significant differences between the patient groups were found at this point.

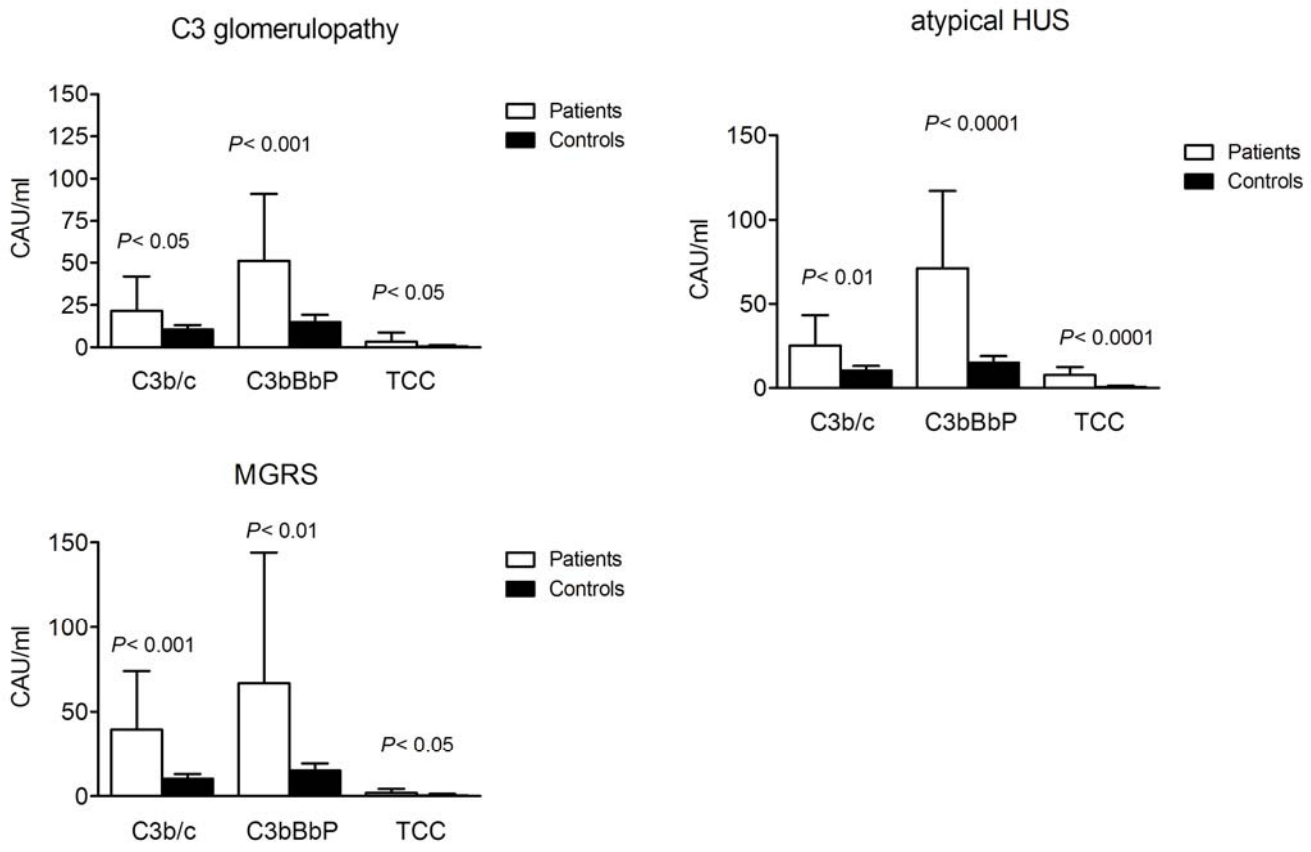


Figure 2. Complement activation products measured in patient samples. EDTA plasma samples of patient with C3 glomerulopathy (n=8), monoclonal gammopathy of renal significance (MGRS, n=5) and aHUS (n=6) were analyzed for the levels of C3b/c, C3bBbP and TCC. The values were compared to those of 19 healthy controls. Data were quantified using international complement standard#2 (ICS#2) in complement activation units per ml (CAU/ml) and are presented as mean+ standard deviation. Values that were statistically different from those of healthy controls (P<0.05) are indicated.

Next, we analyzed whether complement activation patterns are different in the aHUS and more common and less severe STEC-HUS (Figure 3). Our data indicate that the levels of C3b/c and C3bBbP are higher in aHUS than in STEC-HUS in acute phase of the disease. Such difference is in line with the genetic background of aHUS, where 60-70% of patients are reported to have genetic aberrations affecting complement regulation at the level of the C3 convertase. STEC-HUS patients demonstrated higher TCC levels than the aHUS patients. Our findings indicate that there are significant differences in patterns of complement activation in STEC-HUS and aHUS and may provide useful tools for the distinguishing between the two forms of HUS, which require different approach to treatment.

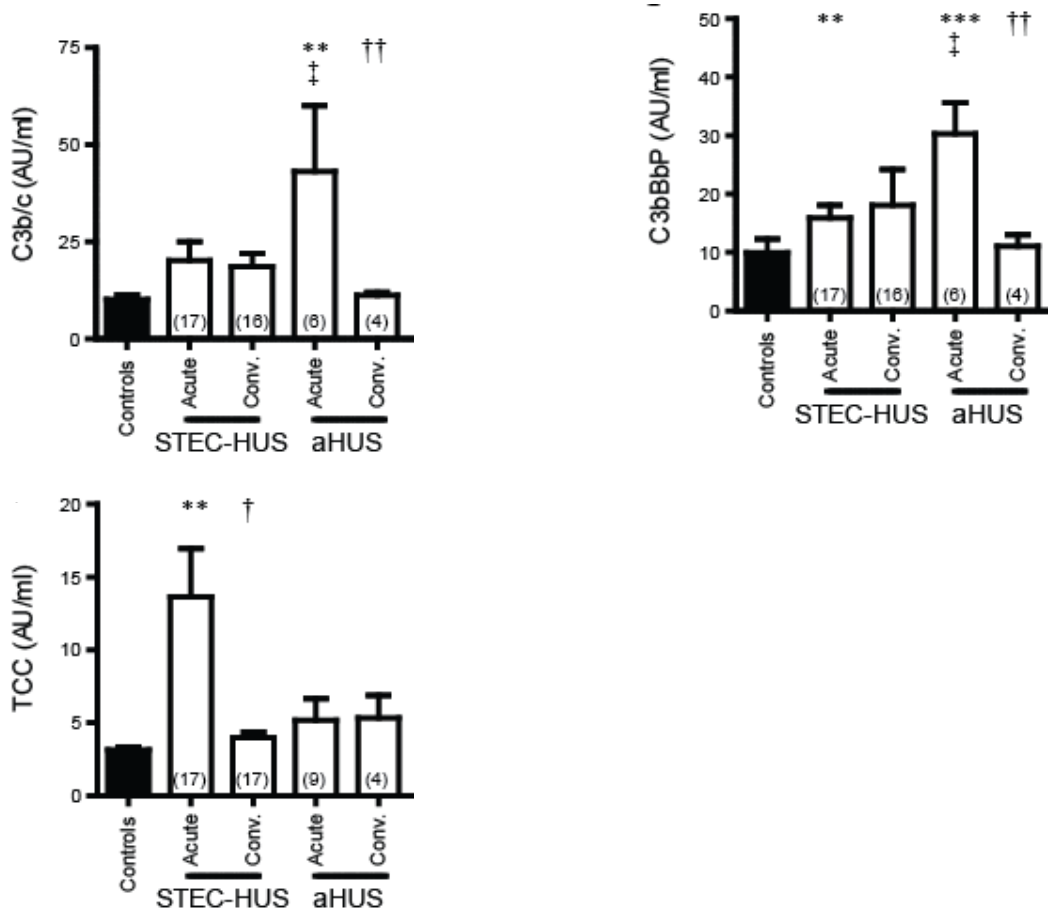


Figure 3 Complement activation products of alternative pathway activation measured in samples obtained from patients with STEC-HUS and aHUS in the acute and convalescent phases of disease. Serum and plasma samples of HUS patients in the acute and convalescent phase were analyzed for the levels of C3bBbP, C3b/c, and TCC. The number of screened patients are indicated within parentheses. The statistically significant differences in patients vs controls are indicated: ** ($P<0.01$), *** ($P<0.001$); convalescent phase vs. acute phase: † ($P<0.05$), †† ($P<0.01$); and STEC-HUS vs aHUS: ‡ ($P<0.05$).

Analysis of C3 convertase stability in C3 glomerulopathy patients

One of the cohorts of this study consists of C3 glomerulopathy patients. To characterize this group properly, it is extremely important to establish the C3NeF status of each patient. In collaboration with the group of Prof. dr. Anna Blom (Section of Protein Chemistry, Department of Laboratory Medicine, Lund University, Malmö, Sweden) a very promising method was developed to measure alternative pathway convertase stability, which is a highly reliable marker for C3NeF activity in samples (Blom *et al*., CEI, 2014). Explanation of this method can be found in the Appendix I of this report. In October-November 2014, Dr. E. Volokhina visited the lab of Prof. dr. Anna Blom to learn this novel technique and to analyze samples from our patients. Currently, this promising method is being implemented at the Pediatric Nephrology Department of Radboudumc, Nijmegen, The Netherlands. In this project we performed validation of this method and tested convertase stability in a large cohort of C3G patients that were included in this project.

First, we tested the 15 control samples (Figure 4). Serum of C3G patients is often depleted of C3 and other complement factors due to continuous complement activation and consumption. To compensate for this depletion, the samples are diluted in 1:1 with normal human serum (NHS). Therefore, the controls were also diluted 1:1 with NHS, to match the conditions used for the patient samples.

Based on the data from NHS-diluted controls, cut-off values were defined for the normal convertase stability profile. A sample is considered positive for prolonged convertase activity if two criteria are met:

1. The measured lysis is higher than the mean+2 standard deviations of healthy controls at t=30, 40 and 50 min of incubation in Step 1 (please see Appendix I for method explanation). Thus in the range, where convertase activity in control samples is sharply decreased (Figure 4).
2. The area under the curve of convertase activity profile is higher than that of mean+2 standard deviations of healthy controls. This criterion was introduced, because relevance of prolonged convertase activity as defined in criterion 1 without overall higher activity is not clear.

Then the samples of 31 C3G patients were analyzed for the alternative complement pathway convertase activity profiles. In our cohort, 11 samples (36%) were positive for prolonged convertase activity and thus are likely to carry C3NeF (Figure 4). Visually, the positive samples could be divided into strongly positive (n=4, shown in green) and positive (n=7 shown in blue). It is possible that the strongly positive and positive samples may carry different types of C3NeF, such as properdin-dependent and properdin- independent (Tanuma *et al*., Clin Immunol Immunopathol, 1990) and/or bind to different epitopes on C3 convertases, which will be investigated in further studies. Moreover, questionable (n=2, pink), and negative (n=18, grey) patient profiles were observed. The two questionable samples did not meet all the criteria of the positive samples, although their results were at the top of the normal range, new samples of these patients will be analyzed again in the future.

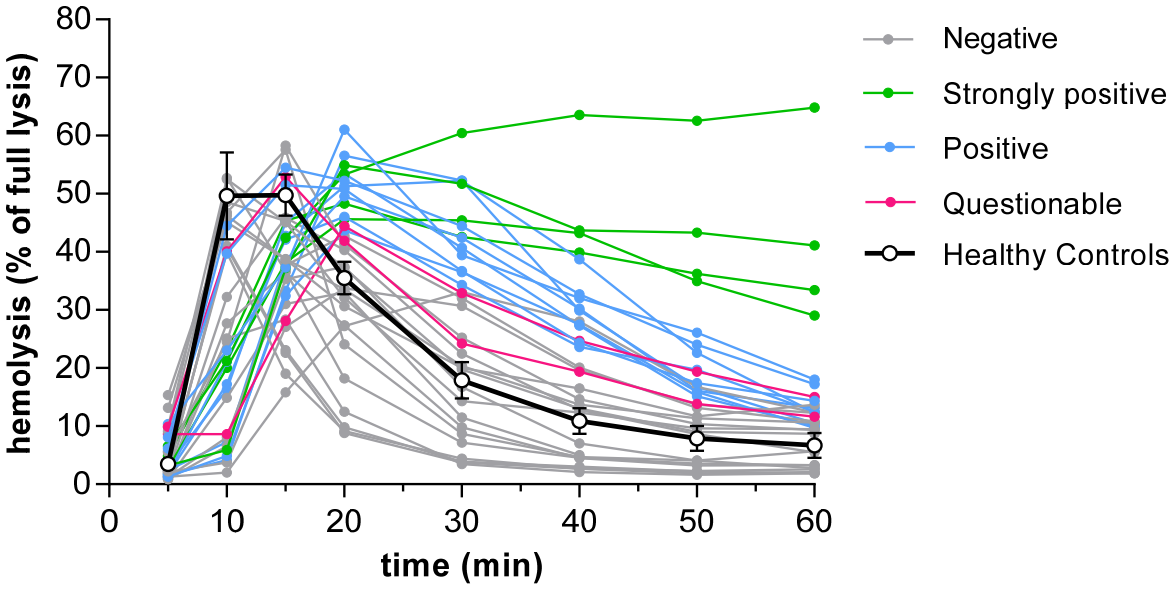


Figure 4. Alternative complement pathway convertase stability in C3G patients. Samples of C3G patients (n=31) and of healthy controls (n=15) were analyzed. Data of controls are presented as mean ± standard deviation, convertase activity profiles of patients are presented individually.

Conclusion

In this project we have established protocol for collection of samples, that would ensure sufficient sample size for the three patient groups at the end of the project. Furthermore, we have validated important assays for the measurement of complement activation biomarkers (C4b/c, C3b/c, C3bBbP and TCC). We have characterized levels of these markers in healthy control groups (pediatric and adult). Analysis of pediatric control samples is particularly important for aHUS, which often affects children.

Measurements of complement activation markers C3b/c C3bBbP and TCC in patients from the three groups (C3G, MGRS and aHUS and STEC-HUS) indicated alternative pathway complement activation in all patient groups. The aHUS and STEC-HUS samples demonstrated significantly different profiles of the complement activation markers. I the next part of the project, more patient samples will be measured for all five complement activation markers (C1rs-C1inh complex, C4b/c, C3b/c, C3bBbP and TCC) that would allow to determine complement activation of all three complement pathways and perform statistical analysis.

Furthermore, in collaboration with the group of Prof. dr. Anna Blom (Malmö, Sweden) we have established a novel method to analyze C3NeF activity, which is highly important for the characterization of C3G samples. We established cut-off values for the C3NeF-positive samples and analyzed a large cohort of 31 C3G patients in this assay.

Future plans

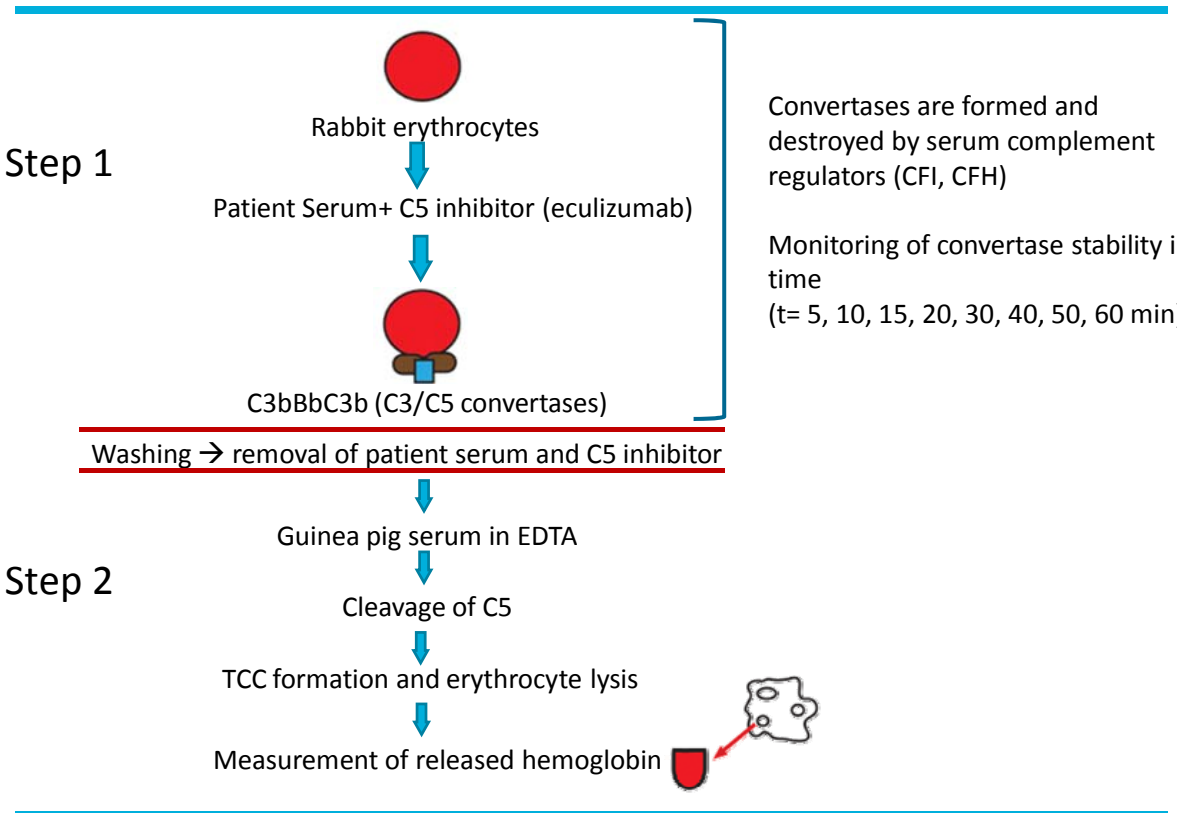
In the remaining period of the project more samples will be collected to reach target sample size for the three patient groups. To asses complement activation in these disorders, complement activation markers (C1rs-C1 inhibitor complex, C4b/c, C3b/c, C3bBbP and TCC) will be analyzed together with complement activation kinetics. The results of complement analyses will be correlated with the clinical and biopsy data and with the findings of complement genetic testing in order to obtain more insight into pathophysiology of the complement-mediated renal disorders. In the future, results of this project may contribute to developing of diagnostic techniques based on EDTA plasma/serum biomarkers.

Appendix I. Assay to measure alternative pathway convertase stability.

We designed a new assay, in which C5-blocking compounds (eculizumab or OmCI) enable separation of the complement cascade into two steps as illustrated in figure below. The assay is performed in Mg-EGTA buffer on rabbit erythrocytes, where only alternative complement pathway can be activated.

The Step 1 (upper part) terminates with the formation of the alternative pathway C5 convertase (C3bBbC3b) on rabbit erythrocytes. In this step we incubate erythrocytes with patient/control serum for various time periods to obtain convertase stability profiles.

The Step 2 (bottom part) begins after removal of excessive serum, C5 blockers and complement inhibitors by washing. In the Step 2 guinea pig serum diluted in EDTA buffer is added as a source of MAC components These conditions allow initiation of MAC formation only by convertases formed in Step 1 and disable *de novo* convertase formation. Guinea pig serum is fully compatible with human components in the hemolysis of rabbit erythrocytes.



Blom *et al*., Clin Exp Immunol. 2014 178:142-53

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