

Progress report for ESPN Research Grant 2014.03

Title: Sorting complement dysregulation in renal disorders

Duration: October 1st 2014-September 30th 2016 (two years)

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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 hypothesize 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.

This is a highly relevant project, which will allow getting more insight into pathology of various complement-mediated glomerular diseases. In the future, it may contribute to the development of the diagnostic techniques based on EDTA plasma/serum biomarkers. These tests will distinguish disorders with alternative complement pathway activation in the fluid phase (such as C3G) from disorders caused by complement activation at the cell surface (aHUS) and from disorders that involve activation of the classical pathway (IgGN). Furthermore, it would be possible to monitor patients for early signs of the disease relapse and guide the development of individual treatment schemes for complement inhibition therapy.

Current progress

Sample collection

Currently we have collected samples from 30 aHUS 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.

Characterization of the control group

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

First, we performed a thorough 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 range²
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

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

²The 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 aHUS 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 (Spearman's rank correlation test).

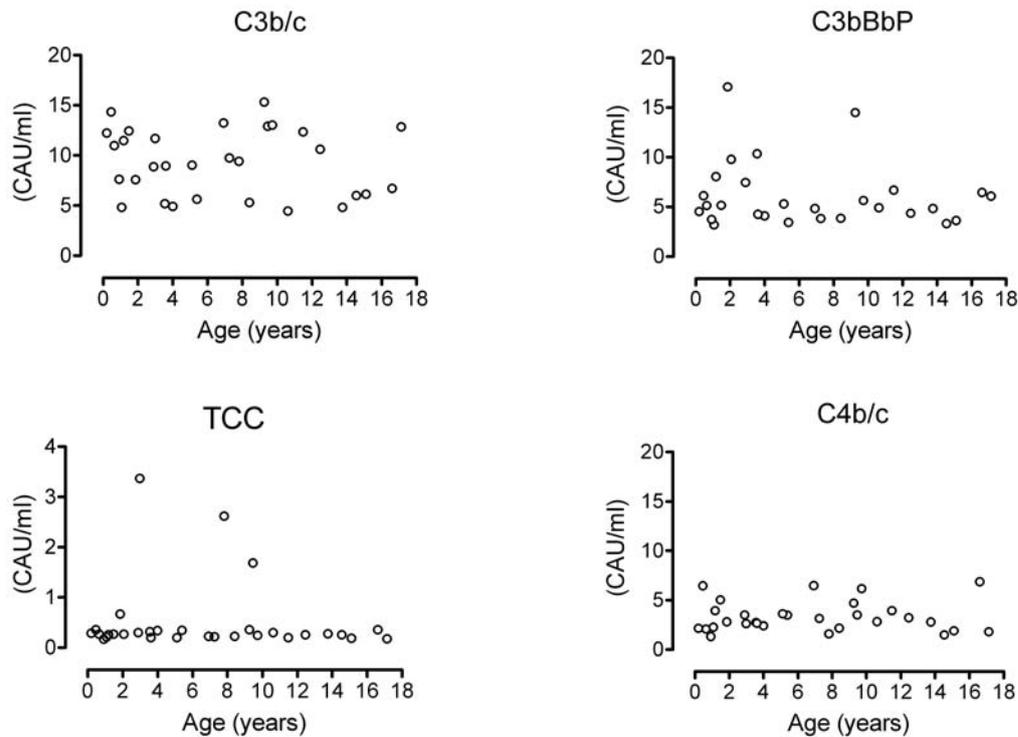


Figure 1. Complement activation markers in samples of healthy pediatric controls. Complement activation markers have been determined in EDTA plasma samples from 30 pediatric controls (age 2 months- 17 years). Data were quantified using international complement standard#2 (ICS#2) in complement activation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

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. Future experiments on more samples for these activation markers and C4b/c and C1rs-C1 inhibitor complex that will be performed in the following (final) year of the project will indicate whether significant differences between the patient groups exist.

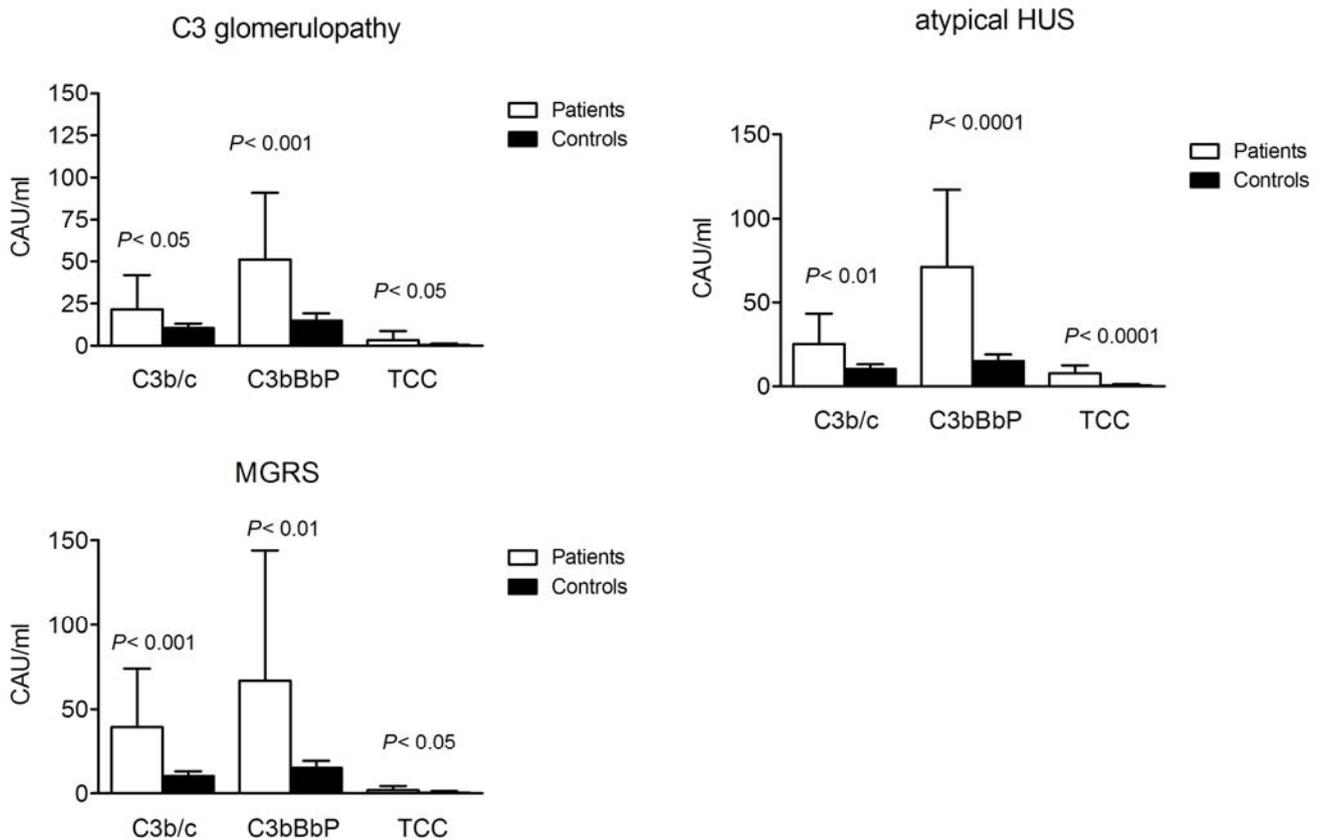


Figure 2. Complement activation products measured in patient samples. EDTA plasma samples of patient with C3 glomerulopathy (n=8), monoclonal gammopathy of renal significance (MGRS, n=5) and aHUS (n=6) were analyzed for the levels of C3b/c, C3bBbP and TCC. The values were compared to those of 19 healthy 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. Statistical analysis has been performed by using unpaired two-tailed t-test.

Convertase stability assay

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 3). 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 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 3).
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 3). 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.

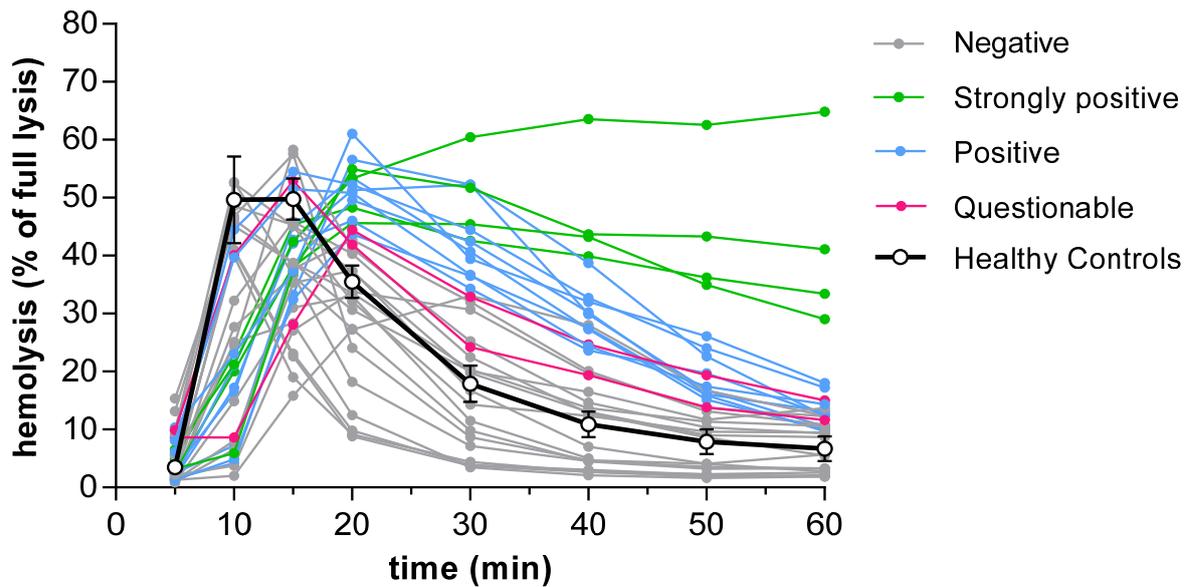


Figure 3. Alternative complement pathway convertase stability in C3G patients. Samples of C3G patients (n=31) and of healthy controls (n=15) were analyzed. Data of controls are presented as mean \pm standard deviation, convertase activity profiles of patients are presented individually.

Conclusion from current results

In the first part of the 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) indicated alternative pathway complement activation in all three groups. In 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 (until September 30th 2016) more samples will be collected to reach target sample size for the three patient groups. To assess 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, as proposed in the original grant application. 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.

