OTKA PD116119

Exploring the role of complement factor H-related protein 5

in complement-mediated kidney disorders

Final closing report, 2015-2018

This collaborative study on the role of complement factor H-related protein 5 was conducted by two research teams of two universities:

Semmelweis University, 3rd Department of Internal Medicine, Research Laboratory: Dorottya Csuka (PI), Zoltán Prohászka, Nóra Garam, Ágnes Szilágyi

Eötvös Loránd University, Department of Immunology: Mihály Józsi, Marcell Cserhalmi

The aim of our project was to determine the role of the complement factor H-related protein 5 in the development of kidney disorders, such as atypical hemolytic uremic syndrome (aHUS) and C3-glomerulopathy (C3GP).

The study was conducted and finished according to the plans, as detailed below.

I. Expanding our biobank

Our previously established biobank (including samples of patients with aHUS, dense deposit disease or C3-glomerulonephritis, as well as from healthy subjects) was enlarged by collecting serum and EDTA-plasma samples from further subjects, in an international collaboration. Thus, we accumulated finally a huge number of samples from patients with aHUS (n=135) or with C3-glomerulopathy (n=151) and also from healthy individuals (n=200); this sample number is larger then originally planned.

We also managed to collect several follow-up samples from patients with aHUS (n=6) or with C3-glomerulopathies (n=6) that enabled us to monitor the changes of the complement parameters in response to treatment.

DNA was isolated using a salting-out method from the blood samples of all patients and healthy subjects.

II. Analyzing the complotype of the patients with aHUS or C3GP

The entire coding region of the well-known disease-associated genes (*CFH*, *CFI*, *CD46*, *THBD*, *C3*, *CFB, DGKE*) as well as of the *CFHR5* gene was analyzed by bidirectional Sanger sequencing, following the PCR amplification of coding exons and flanking regions, in order to detect possible mutations, risk variations and risk haplotypes. Sequencing chromatograms were evaluated with the CLC DNA Workbench 6.5 (CLC Bio, Aarhus, Denmark).

By the end of the grant, we have identified altogether 22/133 aHUS (16.5%) and 20/151 C3GP (13.2%) patients, who carry at least one (rare) variation or mutation in the gene encoding CFHR5. Altogether 17 different (rare) missense variations or mutations were identified in the patient groups, all in heterozygous form: **Val16Phe** (c.329T>C, in 1 aHUS patient), **Pro46Ser** (c.136C>T, in 4 aHUS and 3 C3GP patients), **c.254-5C>T** (in 1 aHUS patient), **Val110Ala** (in 3 aHUS and 5 C3GP patients), **Lys144Asn** (c.432A>T, in 2 aHUS and 2 C3GP patients), **Cys208Arg** (in 1 aHUS and 1 C3GP patients), **Asn244Asn** (in 1 aHUS patient), **Gly278Ser**

(c.832G>A, in 1 aHUS and 3 C3GP patients), **Lys343Lys** (in 1 C3GP patient), **Arg356His** (c.1067G>A, in 6 aHUS and 7 C3GP patients), **Glu378Lys** (in 1 aHUS patient), **Gly471Glu** (in 1 C3GP patient), **Thr491Thr** (c.1473A>G, in 1 C3GP patient), **Leu504Gln** (in 1 C3GP patient), **Asp505Asn** (1 C3GP), **Met514Arg** (c.1541T>G, in 1 aHUS patient), **Arg555Pro** (in 1 C3GP patient) (Figure 1).

Furthermore, the following 5 frameshift mutations were identified, all in heterozygous form: **c.254-2_266dup** (in 1 aHUS patient), **Glu163Lysfs*10** (c.479_480insAA, in 3 aHUS patients), **Glu163Argfs*34** (c.479_480insA, in 2 aHUS and 1 C3GP patients), **Leu504Glnfs*11** (c.1511_1511delT, in 1 C3GP patient), **Asp505Ilefs*10** (c.1513_1513delG, in 1 C3GP patient) (Figure 1).

Eight of the above patients carried even 2, or 3 different *CFHR5* mutations (one patient suffering from aHUS carried the following three *CFHR5* mutations: c.479_480insA, c.254-2_266dup and Cys208Arg).

If available, the family members of the patients were also screened for the variations or mutations mentioned above.

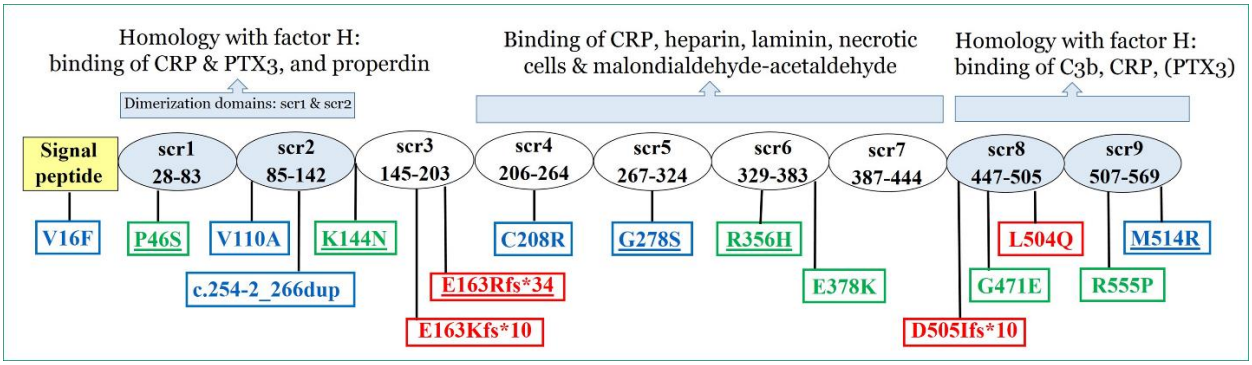


Figure 1. Schematic structure of CFHR5 including those mutations that were detected in our patient cohort.

In order to study copy-number alterations and to detect deletions or duplications in the chromosomal region of complement factor H gene (*CFH*) and related genes (*CFHR1, CFHR2, CFHR3, CFHR5*) of the subjects enrolled, multiplex ligation-dependent probe amplification (MLPA) was performed with SALSA MLPA probemix P236-A3 (MRC-Holland, Amsterdam, The Netherlands). We managed to identify a CFHR2-CFHR5 hybrid gene in two siblings suffering from C3GP, the functional characterization of this extremely rare hybrid gene will be described in a further section of the closing report.

The following 5 manuscripts were/are prepared based on the sequencing results of the included aHUS or C3GP patients:

1. Marcell Cserhalmi, Barbara Uzonyi, Lubka T. Roumenina, Dorottya Csuka, Edgar Meusburger, Karl Lhotta, Zoltán Prohászka, Mihály Józsi.
   Functional characterization of the disease-associated N-terminal complement factor H mutation W198R.
   Published in Frontiers in Immunology, 2017 Dec 13;8:1800. IF: 5.511
   The described patient, initially diagnosed with chronic glomerulonephritis, 6 years later had an episode of aHUS, and a heterozygous *CFH* W198R mutation was identified that affects the complement control protein (CCP) 3 domain of Factor H. Our functional

studies showed that the W198R mutant confers a decreased C3b binding, as well as reduced cofactor and decay accelerating activities compared with the wild-type protein.

2. Martina Gaggl, Christof Aigner, Dorottya Csuka, Ágnes Szilágyi, Zoltán Prohászka, Renate Kain, Natalja Haninger, Maarten Knechtelsdorfer, Raute Sunder-Plassmann, Gere Sunder-Plassmann, Alice Schmidt.
   Maternal and Fetal Outcomes of Pregnancies in Women with Atypical Hemolytic Uremic Syndrome.
   Published in Journal of the American Society of Nephrology, 2018 Mar;29(3):1020-1029. IF: 8.655
   In this study, we detected mutations in genes that encode the complement alternative pathway proteins or the molecules that regulate this pathway in 71% of the women suffering from aHUS, with no relationship to pregnancy outcome.

3. Nóra Garam, Zoltán Prohászka, Ágnes Szilágyi, Christof Aigner, Alice Schmidt, Martina Gaggl, Gere Sunder-Plassmann, Dóra Bajcsi, Jürgen Brunner, Alexandra Dumfarth, Daniel Cejka, Stefan Flaschberger, Hana Flögelova, Ágnes Haris, Ágnes Hartmann, Andreas Heilos, Thomas Mueller, Krisztina Rusai, Klaus Arbeiter, Johannes Hofer, Dániel Jakab, Mária Sinkó, Erika Szigeti, Csaba Bereczki, Viktor Janko, Kata Kelen, György S. Reusz, Attila J. Szabó, Nóra Klenk, Krisztina Kóbor, Nika Kojc, Maarten Knechtelsdorfer, Mario Laganovic, Adrian Catalin Lungu, Anamarija Meglic, Rina Ru, Tanja Kersnik-Levart, Ernesta Macioniene, Marius Miglina, Anna Pawłowska, Tomasz Stompór, Ludmila Podracka, Michael Rudnicki, Gert Mayer, Romana Rysava, Jana Reiterova, Marijan Saraga, Tomáš Seeman, Jakub Zieg, Eva Sládková, Tamás Szabó, Andrei Capitanescu, Simona Stancu, Miroslav Tisljar, Kresimir Galesic, András Tislér, Inga Vainumäe, Martin Windpessl, Tomas Zaoral, Galia Zlatanova, Dorottya Csuka.
   Validation of distinct pathogenic patterns in a cohort of membranoproliferative glomerulonephritis patients by cluster analysis.
   Under review at the Clinical Journal of the American Society of Nephrology

   Our results in brief: A novel data-driven cluster analysis identified distinct pathogenic patterns in C3-glomerulopathies and immune-complex mediated membranoproliferative glomerulonephritis. Here, we replicated these observations in an independent cohort and elucidated disease pathophysiology with detailed analysis of functional complement markers. Ninety-two patients with clinical, histological, complement and genetic data were involved in the study, and hierarchical cluster analysis was done by Ward method, where 4 clusters were generated. High levels of sC5b-9, low serum C3 levels and young age at onset (13 years) were characteristic for cluster 1 with a high prevalence of likely pathogenic variations (LPVs) and C3NeF, whereas for cluster 2 strong IgG staining, low C3 levels and high prevalence of nephritic syndrome at disease onset were observed. Low plasma sC5b-9 levels, decreased C3 levels and high prevalence of LPV and sclerotic glomeruli were present in cluster 3, and patients with late onset of the disease (median: 39.5 years), near-normal C3 levels and low prevalence of LPV and/or C3NeF were in cluster 4. A significant difference was observed in the incidence of end-stage renal disease during follow-up between the different clusters. Patients in cluster 3-4 had worse renal survival than patients in clusters 1- 2. Our results confirm the main findings of the original cluster analysis and indicate that the observed, distinct pathogenic patterns are replicated in our cohort.

4. Nóra Garam, Zoltán Prohászka, Ágnes Szilágyi, Christof Aigner, Alice Schmidt, Martina Gaggl, Gere Sunder-Plassmann, Dóra Bajcsi, Jürgen Brunner, Alexandra Dumfarth, Daniel Cejka, Stefan Flaschberger, Hana Flögelova, Ágnes Haris, Ágnes Hartmann, Andreas Heilos, Thomas Mueller, Krisztina Rusai, Klaus Arbeiter, Johannes Hofer, Dániel Jakab, Mária Sinkó, Erika Szigeti, Csaba Bereczki, Viktor Janko, Kata Kelen, György S. Reusz, Attila J. Szabó, Nóra Klenk, Krisztina Kóbor, Nika Kojc, Maarten Knechtelsdorfer, Mario Laganovic, Adrian Catalin Lungu, Anamarija Meglic, Rina Rus, Tanja Kersnik-Levart, Ernesta Macioniene, Marius Miglinas, Anna Pawłowska, Tomasz Stompór, Ludmila Podracka, Michael Rudnicki, Gert Mayer, Romana Rysava, Jana Reiterova, Marijan Saraga, Tomáš Seeman, Jakub Zieg, Eva Sládková, Tamás Szabó, Andrei Capitanescu, Simona Stancu, Miroslav Tisljar, Kresimir Galesic, András Tislér, Inga Vainumäe, Martin Windpessl, Tomas Zaoral, Galia Zlatanova, Dorottya Csuka.
   C4 nephritic factor in patients with Immune-complex-mediated membranoproliferative glomerulonephritis and C3-glomerulopathy.
   Under review at the Frontiers in Immunology
   Our results in brief: please find the short description of this study at section VIII./2.

5. Nóra Veszeli, Nóra Garam, Adrian Catalin Lungu, Zoltán Prohászka, Dorottya Csuka. **Identification of rare hybrid genes of the CFHR gene family in patients suffering from C3-glomerulopathy**. – manuscript under preparation

   Our results in brief: In one of our C3GP patients, the data obtained by MLPA showed an unusual heterozygous deletion in *CFHR2* exon 3-4 as well as in *CFHR5* exon 1 besides the common copy number polymorphism that results in the heterozygous deletion of both *CFHR3* and *CFHR1* genes. We hypothesized that the complex rearrangement resulted in a *CFHR2-CFHR5* hybrid gene in addition to one normal copy of both *CFHR2* and *CFHR5* genes. It raised the possibility that a pathological hybrid protein expression/production occurs in the patient. We obtained blood samples from our patient and from his healthy mother as well as his bother who was diagnosed with ankylosing spondylitis. We designed a PCR targeting the breakpoint in the CFHR2-CFHR5 region using a primer localized in *CFHR2* intron 2 as a forward primer and one in *CFHR5* exon 2 as a reverse primer. The presence of the *CFHR2-5* hybrid gene in our patient as well as in his brother was confirmed by the detection of the presumed amplicon at cc. 3 kb. No amplicon was generated in individuals with two copies of the *CFHR2* and *CFHR5* genes used as controls. Based on the amplicon sequencing, we identified the breakpoint in *CFHR2* intron 3. The deletion spanned as far as intron 1 of *CFHR5* (1889 base pairs before exon 2), leading to a heterozygous deletion of 18,556 base pairs, which comprised *CFHR2* exons 4 and 5, as well as exon 1 of *CFHR5*.
   Western blot analysis by using polyclonal and monoclonal antibodies against CFHR5 showed two extra bands (at 74 and 70 kDa position) besides the normal CFHR5 protein in both the patient and his brother, whereas the mother of our patient showed only normal CFHR5 protein. The two bands possibly represent differently glycosilated forms of the CFHR2-CFHR5 hybrid protein. Based on the genetic and western blot analysis the hybrid protein consists of the CFHR2 SCR1-2 domains as well as all nine SCRs of CFHR5. Our results suggest that the identified mutation may have a disease-causing role in the development of C3GP.

Further parts of this project were presented as a poster presentation:
27th International Complement Workshop, Santa Fe, USA, 16-21 September 2018:
Nora Veszeli, Barbara Uzonyi, Nora Garam, Adrian Lungu, Mihály Józsi, Zoltán Prohászka, Dorottya Csuka.
Identification of a CFHR3 hybrid protein in a patient with familial form of C3-glomerulonephritis.
Molecular Immunology, Volume 102, October 2018, Page 224

6-7. Two other manuscripts focusing on the occurrence of *CFHR5* mutations in C3GP or aHUS patients (as detailed above) are in progress and will be submitted in the first half of 2019. These results were presented at the following scientific conferences:

- International Complement Workshop in Kanazawa, Japan, 4-8 September 2016: Dorottya Csuka, Nóra Garam, Ágnes Szilágyi, Mihály Józsi, Michael Rudnicki, Gere Sunder-Plassmann, Alice Schmidt, George S Reusz, Zoltán Prohászka. **Identification of CFHR5 variations in patients with atypical hemolytic uremic syndrome or with C3-glomerulopathies.**
  Immunobiology 221 (2016) 1131–1225

- 16th European Meeting on Complement in Human Disease, Copenhagen, Denmark, 8-12 September 2017: Dorottya Csuka, Nóra Garam, Ágnes Szilágyi, Mihály Józsi, Michael Rudnicki, Gere Sunder-Plassmann, Alice Schmidt, George S Reusz, Zoltán Prohászka. **Relationship between CFHR5 and complement parameters in patients suffering from complement-mediated kidney disorders, with or without CFHR5 mutations.**
  Molecular Immunology, Volume 89, September 2017, Page 177

III**. Introduction of an ELISA method for the determination of the plasma CFHR5 level**

We have introduced a sensitive and specific sandwich ELISA method that is suitable for measuring the plasma CFHR5 concentration. For this purpose, commercially available monoclonal and polyclonal anti-human CFHR5 antibodies (R&D Systems) were used, and the CFHR5 concentration of the samples was determined based on the commercially available recombinant CFHR5 (R&D Systems) that was applied as a standard. This newly developed ELISA method was used to analyze the plasma level of CFHR5 in the samples of 135 patients suffering from aHUS, in 151 C3GP patients, as well as in 130 healthy subjects. We have shown that the concentration of CFHR5 is significantly decreased in C3GP (mean±SD: 1.86±0.71 μg/ml, $p<0.0001$), and also in aHUS (1.87±0.73 μg/ml, $p=0.0216$), compared to the healthy subjects (2.31±0.79 μg/ml). Furthermore, CFHR5 levels were significantly higher in male C3GP patients ($p=0.032$) compared to the female C3GP patients, which difference did not occur in the control group.
The two manuscripts summarizing these determinations in aHUS or C3GP patients are currently prepared and will be submitted in the first half of 2019.

These results were presented as poster presentations at the following congresses:

- 16th European Meeting on Complement in Human Disease, Copenhagen, Denmark, 8-12 September 2017:
  Dorottya Csuka, Nóra Garam, Ágnes Szilágyi, Mihály Józsi, Michael Rudnicki, Gere Sunder-Plassmann, Alice Schmidt, George S Reusz, Zoltán Prohászka. **Relationship**

between CFHR5 and complement parameters in patients suffering from complement-mediated kidney disorders, with or without CFHR5 mutations.
Molecular Immunology, Volume 89, September 2017, Page 177

- Congress of the Hungarian Society of Immunology, Velence, Hungary, 18-20 October 2017

IV. Analyzing the complement activation profile of the patients with aHUS or C3GP (with or without a *CFHR5* mutation) and of healthy subjects

To determine whether and to which extent the detection of complement components, activation products or further complement activation markers in the blood samples of the involved patients shows a relationship with the CFHR5 level or the disease characteristics, detailed complement biomarker measurements were done. We have determined the level of several complement activation products (C4d, C3a, Bb and sC5b-9) using commercial ELISA kits (Quidel), the activity of the classical and alternative complement pathways (Eurodiagnostica), as well as the concentration of the complement components such as C3, C4, C1q and factor B (using immunoturbidimetry, Beckman-Coulter), factor H (in-house ELISA test using polyclonal sheep anti-human factor H antibody, BindingSite) and factor I (radial immunodiffusion using polyclonal sheep anti-human factor I antibody, BindingSite) in all the three study groups (including follow-up samples).

V. Establishing a database on the clinical and laboratory parameters of C3GP and aHUS patients

Parallel with the above mentioned laboratory measurements, Dr. Nóra Garam (my PhD student working on this project) has established a C3GP database that contains the laboratory (including eGFR) and clinical (age, gender, previous medical history, baseline disease, biopsy results) data of the C3GP patients originating from numerous European patient centers.

Our results in brief: By analyzing this comprehensive database, we have examined whether there is any connection with the C3GP patients' clinical characteristics and the CFHR5 levels. CFHR5 concentration showed a moderate tendentiously negative correlation with patients' eGFR ($p=0.06$, $r=-0.18$); patients with normal renal function had lower CFHR5 levels than patients with renal impairment (eGFR: 15-60 mL/min/1.73m2). There was no difference as regards the CFHR5 levels between patients with no proteinuria, non-nephrotic or nephrotic-range proteinuria. CFHR5 titers showed a positive correlation with the presence and extent of sclerotic glomeruli ($p=0.018$; $r=0.22$) and crescent formation ($p=0.028$; $r=0.2$) on light microscopy which indicates more severe disease.
We have collected the data of C3GP patients' progression to end-stage renal disease (ESRD) during follow-up period and followed 103 subjects successfully for a median follow-up of 1.52 years (range: 0.05-18.18 years). During the follow-up period 17 patients progressed to ESRD. Low and high CFHR5 levels were determined by ROC-curve analysis (cut-point: 1.565 ng/ml) and C3GP patients with higher CFHR5 levels had tendentiously worse renal survival by Kaplan-Meier analysis ($p=0.043$) (Figure 2).

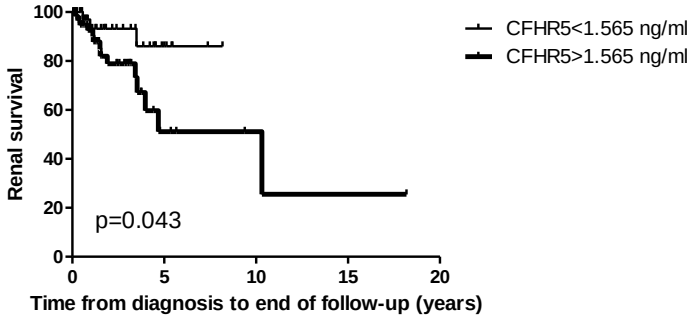
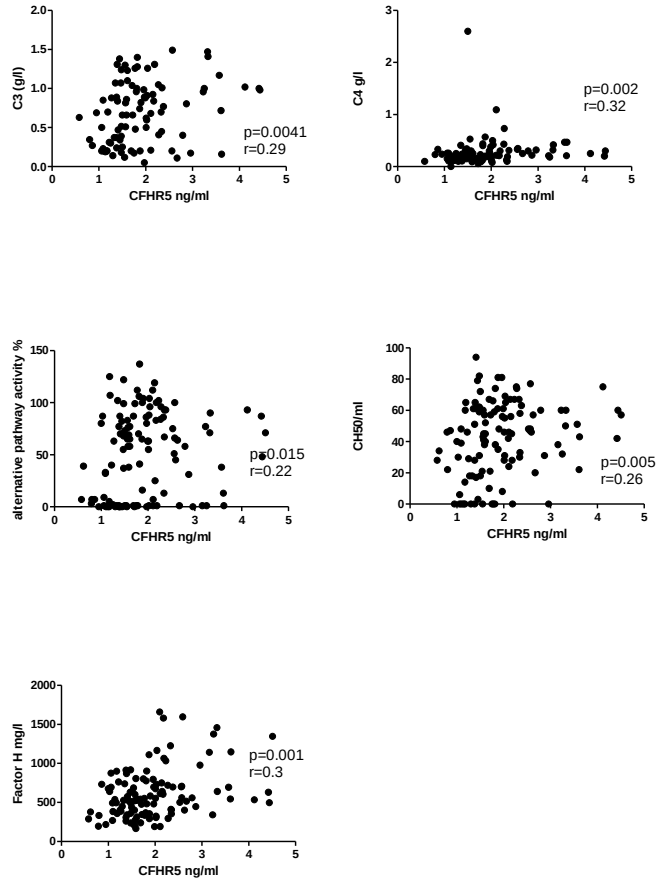


Figure 2.

C3GP patients with higher CFHR5 levels had tendentiously worse renal survival by Kaplan-Meier analysis.

We also investigated whether there is any relevant connection between the C3GP patients' complement profile and CFHR5 levels. Several complement components showed significant correlation with serum CFHR5 concentrations such as C3 (p=0.004; r=0.29), C4 (p=0.002; r=0.32), alternative pathway activity (p=0.015; r=0.22), classical pathway activity (p=0.005; r=0.26) and Factor H antigenic level (p=0.001; r=0.3) (Figure 3). Similar correlations were observed in patients suffering from aHUS but not in healthy individuals. C3GP patients with complement dysregulation (decreased serum C3 level) had significantly lower CFHR5 levels (p=0.029). In case of decreased alternative pathway activity only a trend could be seen as regards the different CFHR5 levels (p=0.59).

Figure 3. Correlations between the C3GP patients' complement profile and CFHR5 levels.



Interestingly, C3GP patients with at least one *CFHR5* mutation showed a trend to have lower CFHR5 serum levels compared to the patients with no *CFHR5* mutation (p=0.055).
We also compared the CFHR5 levels in the different hypothesis-free data-driven clusters which were previously described in our submitted manuscript (as mentioned above in manuscript 3). CFHR5 concentrations were significantly decreased in cluster 1 (p=0.0004) (Figure 4), which cluster was characterized by higher complement activation, early disease onset and the higher prevalence of autoantibodies against complement components. *CFHR5* mutations were more prevalent in cluster 1 compared to the other clusters (p=0.033).

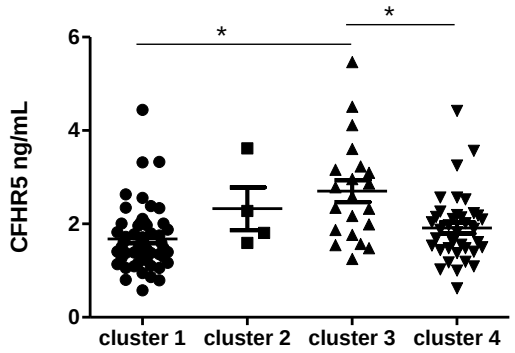


Figure 4. CFHR5 levels in the generated hypothesis-free data-driven clusters.

Currently, we are working on this manuscript focusing on the occurrence of *CFHR5* and further complement mutations in C3GP patients, in relation to their clinical and laboratory data. As mentioned in Section II/6-7, this manuscript on C3GP, along with a second manuscript focusing on the results of aHUS patients, will be submitted in the first half of 2019.

VI. Introduction of a new method for screening the *CFHR5* R356H variation

We have observed the frequent occurrence of the *CFHR5* R356H polymorphism both in patients with aHUS or C3GP. In order to study its frequency in a cheap and quick manner, we have introduced an RFLP (restriction fragment length polymorphism) method using the HpyCH4IV restriction enzyme (New England Biolabs). This assay was validated using the sequencing data of previously determined samples. No significant difference was observed as regards the frequency of the rare allele of the R356H polymorphism between C3GP patients and healthy subjects (0.9% vs. 0.6%, respectively).

VII. Functional characterization of the identified *CFHR5* variations

In order to functionally characterize the identified *CFHR5* variations, we have applied the following approaches:
VII./1. We applied 5 different *in silico* prediction tools (PolyPhen, PROVEAN, SIFT, MutationTaster, Human Splicing Finder), and cathegorized the *CFHR5* variations based on these predictions: 7 damaging mutations, 8 probably damaging mutations, and 6 rare variations with an ambiguous effect.

VII./2. Analyzing the frequency data of the identified *CFHR5* variations in comparison with data of international databases including healthy subjects (1000Genomes Project, Exome Variant Server).

VII./3. Measuring the CFHR5 plasma concentrations associated with the identified *CFHR5* mutations (using the newly developed assay as described above in Section III).

VII./4. Investigating the binding ability of CFHR5 protein variants to immobilized C3b or C-reactive protein: up to date, the G278S and R356H CFHR5 variants were recombinantly expressed (the M514R is in progress) by our collaborating partners, Dr. Mihály Józsi, Dr. Barbara Uzonyi and Marcell Cserhalmi (Eötvös Loránd University, Department of Immunology) from all the CFHR5 variants identified in this study. Surface plasmon resonance tests were applied to analyze the ligand binding ability of these CFHR5 variants to C3b. The interaction of wild-tpye CFHR5 and its mutant variants, G278S and R356H with the C3b fragment was analyzed in real time using a ProteOn XPR36 surface plasmon resonance system (Bio-Rad). The CFHR5 proteins were immobilized by a standard amine coupling technology to a GLC biosensor chip. Different concentrations of C3b were injected with 30 µl/min flow rate and allowed to interact with each of the three immobilized proteins for 300s. The dissociation was also followed for 300s. The experiment was repeated three times on three different chips. SPR analysis showed that the G278S and R356H variants are characterized with a decreased association to the C3b fragment, whereas the R356H variant had a slower dissociation and the G278S mutant had a faster dissociation compared to the wild-type CFHR5 protein. Similar results were obtained by ELISA, indicating weaker C3b binding by the CFHR5 G278S variant. Epitope mapping revealed that C3b binding to the CFHR5 peptide with K144N was increased and binding of CRP to the R356H and M514R mutant peptides was decreased compared to wild type peptides.

These results were presented as a poster presentation at the following congress:
27th International Complement Workshop, Santa Fe, USA, 16-21 September 2018:
Marcell Cserhalmi, Barbara Uzonyi, Dorottya Csuka, Katalin Uray, Attila Iliás, Zoltán Prohászka, Mihály Józsi. **Functional characterization of disease associated variants of human complement factor H-related protein 5.**
Molecular Immunology, Volume 102, October 2018, Pages 170-171

The manuscript summarizing these results will be submitted in the second half of 2019. Therefore, we will kindly ask the evaluation panel for a second re-assessment of our project, upon publication of this manuscript, after 1 year of the closing date.

VII./5. We have introduced a novel ELISA method that is suitable for measuring the C3b- or iC3b binding ability of the identified CFHR5 variants, using the patients' serum samples. In brief, the microtiter plates were coated with either purified C3b or iC3b, and after incubating the diluted serum samples, the bound CFHR5 from the samples was detected using monoclonal mouse anti-human CFHR5 antibody (R&DSystems). Samples of 31 aHUS patients and 34 C3GP patients (with a known genetic background), as well as of 20 healthy subjects were analyzed with this assay. We have shown that the CFHR5 G278S and R356H variants along with further damaging frameshift mutations had a significantly decreased C3b- or iC3b-binding capacity, whereas the identified CFHR2-CFHR5 hybrid gene showed an increased ligand-binding abilty, compared to the wild-type protein (Figure 5).

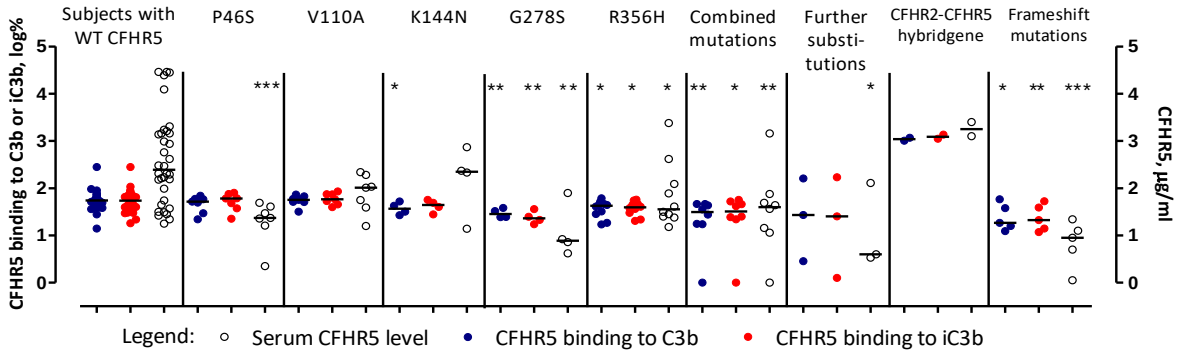


Figure 5. The extent of the C3b- or iC3b-binding obsereved in the individual samples was expressed as the percent of the same calibrator sample, that contained 2.89 µg/ml CFHR5 and carried no mutation in CFHR5. Asterisks show significant difference of each mutation group, compared to the subjects with wild-type CFHR5.

Furthermore, we have found significant correlations between the C3b-/iC3b-binding capacity of CFHR5 and the concentrations of Factor H, C3 or CFHR5, both in aHUS and in C3GP patients.

These results were presented as a poster presentation at the following congress:
27th International Complement Workshop, Santa Fe, USA, 16-21 September 2018:
Dorottya Csuka, Nóra Garam, Mihály Józsi, Marcell Cserhalmi, Barbara Uzonyi, Michael Rudnicki, Gere Sunder-Plassmann, Alice Schmidt, George S Reusz, Zoltán Prohászka.
Assessment of the C3b- and iC3b-binding ability of CFHR5 variants.
Molecular Immunology, Volume 102, October 2018, Page 141

The manuscript summarizing these results will be submitted in the second half of 2019 along with the results mentioned in Section VII./4. Therefore, we will kindly ask the evaluation panel for a second re-assessment of our project, upon publication of this manuscript, after 1 year of the closing date.

VIII. Further tasks that were completed but not included in the original research plan (although they are additional substantial parts of the project):

VIII./1. Development of ELISA tests for the measurement of autoantibodies against human C3 or factor B, in all the included C3GP patients (performed by Nóra Garam, whose PhD project overlaps with fundamental parts of this grant).

These results are described in the following manuscript:
Nóra Garam, Zoltán Prohászka…..Dorottya Csuka.
Validation of distinct pathogenic patterns in a cohort of membranoproliferative glomerulonephritis patients by cluster analysis.
Under review at the Clinical Journal of the American Society of Nephrology

VIII./2. Development of a hemolytic assay for detecting C4-nephritic factor in all the included C3GP patients (performed by Nóra Garam).

These results are described in the following manuscript:
Nóra Garam, Zoltán Prohászka, Ágnes Szilágyi…..Dorottya Csuka.
C4 nephritic factor in patients with Immune-complex-mediated membranoproliferative glomerulonephritis and C3-glomerulopathy.
Under review at the Frontiers in Immunology

Our results in brief: Less is known about the presence and role of C4 nephritic factor (C4NeF) which may stabilize the classical pathway C3 convertase. Our aim was to examine the presence of C4NeF and its connection with clinical features and with other pathogenic factors in IC-MPGN and C3GP patients. Clinical and histological data were collected whereas genetic and complement parameters were determined in 119 IC-MPGN/C3GP patients. 17 patients (14.3%) were positive for C4NeF with lower prevalence of renal impairment and lower C4d level, and tendentiously higher C3 nephritic factor (C3NeF) prevalence at time of diagnosis compared to the C4NeF negative patients. Patients positive for both C3NeF and C4Nef had the lowest C3 levels and highest terminal pathway activation. End stage renal disease did not develop in any of the C4NeF positive patients during the follow-up period. Positivity to other complement autoantibodies, such as anti-C1q and anti-C3, was also linked to the presence of nephritic factors. An unsupervised, data-driven cluster analysis identified a group of patients (cluster 1) with high prevalence of multiple autoantibodies to complement proteins, including C4NeF. In conclusion, C4NeF may be a possible cause of complement dysregulation in approximately 10-15% of IC-MPGN/C3GP patients.

VIII./3. Determining the systemic level of pentraxin-3 and C-reactive protein in acute phase TMA (including patients suffering from aHUS, STEC-HUS, secondary TMA and thrombotic thrombocytopenic purpura) and analyzing their relation to the complement profile, laboratory parameters and clinical outcome of patients. Our manuscript describing these results has just been accepted:
Eszter Trojnar, Mihály Józsi, Zsóka Szabó, Marienn Réti, Péter Farkas, Kata Kelen, George S Reusz, Attila J Szabó, Nóra Garam, Bálint Mikes, György Sinkovits, Blanka Mező, Dorottya Csuka, Zoltan Prohászka.
Elevated systemic pentraxin-3 is associated with complement consumption in the acute phase of thrombotic microangiopathies.
Frontiers in Immunology, 2019 (accepted for publication 28 January 2019) IF: 5.511

VIII./4. Introducing next-generation sequencing and the analysis, validation along with the quality control of the resulting raw data, in order to explore novel mutations in those patients, in whom no mutations or pathogenic antibodies were identified in the traditional disease-associated genes. These results were presented as poster presentations at the following congresses:

- 16th European Meeting on Complement in Human Disease, Copenhagen, Denmark, 8-12 September 2017: Aino Koskinen, Eszter Trojnár, Dorottya Csuka, Zoltán Prohászka, Sakari Jokiranta. **Whole exome sequencing in diagnostics of atypical hemolytic uremic syndrome.**
  Molecular Immunology, Volume 89, September 2017, Pages 148-149

- 16th European Meeting on Complement in Human Disease, Copenhagen, Denmark, 8-12 September 2017: Susan A. Lagerstedt, Dorottya Csuka, Zoltan Prohaszka, Roshini S. Abraham.
  Analysis of Complement Gene Variants In The Clinical Laboratory: Comparison of Next-Generation Sequencing (NGS) and Sanger Methods.
  Molecular Immunology, Volume 89, September 2017, Page 149

In total, this grant provided support to the PI to publish 3 grant-related articles as a co-author; 2 manuscripts are currently under review where the PI is the last author, and 3+1 manuscripts are currently under preparation where the PI is the first, or the last author, respectively. Therefore, we will kindly ask the evaluation panel for a second re-assessment of our project, upon publication of the mentioned manuscripts, after 1 year of the closing date.