„Role of the human factor H-related serum proteins CFHR1 and CFHR3 in physiology and pathophysiology” (2014-2018)

## 1. Introduction

The complement system is a major humoral component of innate immunity that plays important roles in the protection against infections, in the clearance of cellular debris and in modulation of immune responses. Complement can be activated via three major pathways, the alternative, the lectin and the classical pathways. In addition, the alternative pathway amplifies complement activation initiated by any of the three pathways. Complement activation, however, is strictly regulated to prevent damage to the host. Excessive or insufficient activation or defective regulation of this system may lead to various diseases, such as atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathies and age-related macular degeneration (AMD).[1]

*Factor H (FH) is a major regulatory protein of the complement system, which plays an important role in the protection of the host from complement-mediated damage. The function of the factor H-related (FHR) proteins, however, is poorly characterized to date.* The deficiency of the FHR1 and FHR3 proteins in particular is associated with certain diseases (e.g., aHUS), whereas it protects against e.g. age-related macular degeneration.[2] Our major aim was the functional characterization of these proteins, in order to elucidate their role in physiology as well as in pathological processes.

The FHR proteins are encoded by separate genes and their domains show high degree of sequence similarity to those of FH. All FHRs lack the complement regulatory domains (CCPs) 1-4 of FH. The conserved domains in these proteins are homologous to domains 6-9 and 18-20 of factor H (**Fig. 1.**), which mediate important interactions of the complement regulator with C3b, the pentraxins C-reactive protein (CRP) and pentraxin-3 (PTX3), malondyaldehide (MDA) epitopes generated upon oxidative stress, host cells and basement membranes; several microbial proteins also bind within these FH domains. Our central hypothesis was that the FHR proteins appeared during evolution to inhibit binding of FH to pathogens.[2,3] Thus, these proteins would lack any significant complement regulatory activity, but would compete with FH for binding to microbial proteins. Due to the conserved homologous domains, however, this would also result in competition for certain host ligands. *The project in particular focussed on the ligand binding and functional characterization of FHR1 and FHR3 as competitors of FH in AMD, kidney diseases and infectious diseases, and the characterization of their potential complement activation/opsonization enhancing ability.*

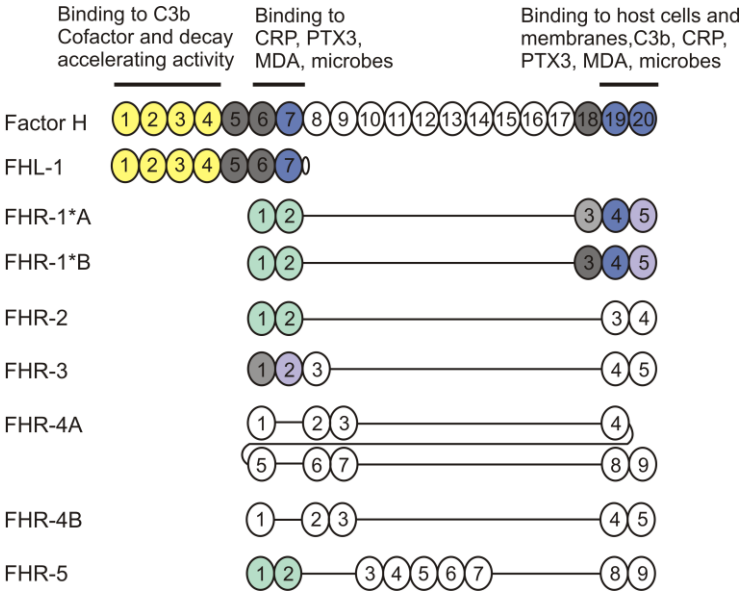


**Fig. 1. The human factor H protein family.** The five human FHR proteins retained domains homologous to domains 6-9 and 18-20 of factor H (showed by vertical alignment). Colors indicate domains identical between factor H and FHRs; light shades indicate high sequence similarity but not complete identity. The domains marked green are closely related to each other but only distantly to factor H and mediate dimerization of FHR1, FHR2 and FHR5. Functional sites in factor H are shown by horizontal lines. FHL1 is a splice variant of factor H.

## II. Results

### *Specific Aim 1: What are the functions of CFHR1 and CFHR3 related to AMD and aHUS?*

The FHR proteins were expressed in the Baculovirus-insect cell system and used for the studies. First, we investigated the interaction of FHR1 and FHR3 with disease-relevant ligands, such as pentraxins, extracellular matrix (ECM) and MDA-epitopes, which are implicated in the pathology of both diseases.

Previously, we showed that FHR1 binds to the pentraxin PTX3. In the current project, we investigated the interaction of FHR1 with the native, pentameric C-reactive protein (pCRP) and that of the modified, monomeric CRP form (mCRP) by ELISA (**Fig. 2**). We found that pCRP did not bind to recombinant FHR1; there was weak binding of pCRP to FHR3 at higher concentrations, and strong binding to FHR4A (as described before). However, there was a strong binding of the mCRP form to FHR1. Using a recombinant C-terminal fragment of FHR1 (comprising CCP domains 4-5), we could localize the mCRP binding site to these FHR1 domains (that are homologous to the C-terminal mCRP binding site in FH). The two FHR1 isoforms, FHR1*A and FHR1*B bound equally well to mCRP. Mutational analyses identified partly overlapping binding sites in FH and FHR1 for PTX3 and mCRP; this was confirmed by demonstrating partial inhibition of PTX3 binding to FHR1 by mCRP.[4]

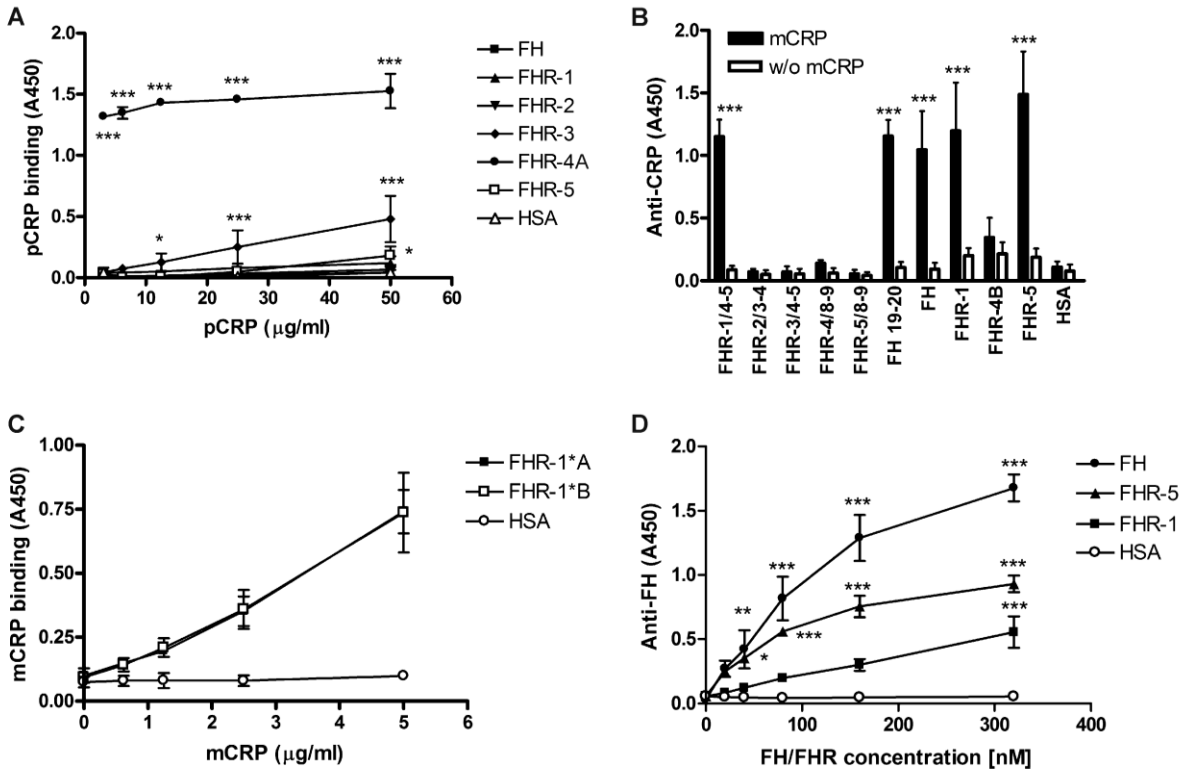


**Fig. 2. Comparison of the binding of pCRP and mCRP to the FHR proteins.** (A) Binding of pCRP to recombinant FHR proteins and FH (200 nM) by ELISA. *p < 0.05, ***p < 0.001, two-way ANOVA. (B) Binding of 10 μg/ml mCRP to FHR proteins, FH and the homologous C-terminal two domains of all five FHR proteins. ***p < 0.001, one-way ANOVA. (C) Binding of increasing concentrations of mCRP to immobilized FHR-1*A and FHR-1*B. (D) Binding of FH, FHR-5 and FHR-1 to immobilized CRP detected with polyclonal anti-FH antibody. *p < 0.05, **p < 0.01, and ***p < 0.001, two-way ANOVA. *(Published in: Csincsi et al., J Immunol. 2017;199:292-303.)*[4]

We confirmed these results using serum of sepsis patients as a source of CRP. There was no binding of CRP under normal pH 7.4; however, at slightly acidic pH, which is characteristic to inflammatory conditions, there was evident binding, indicating that CRP when undergoes structural change due to pH shift („mCRP") can interact with FHR1.[4]

To investigate the functional relevance of the FHR1/mCRP interaction, we first studied whether FHR1 can inhibit FH binding to mCRP. These experiments indicated only a partial inhibition, and when investigating complement activation on mCRP in normal serum or in FH-depleted serum, FHR1 did not significantly increase C3 deposition.[4] Thus, under the experimental conditions used, FHR1 did not significantly compete with FH for mCRP binding in serum, which could be due to the weaker binding of FHR1 to mCRP than that of FH, explained by the multiple CRP binding sites in FH compared with the single mCRP binding site in FHR1.

We further analyzed the interaction of FHR3 with the pentraxins. Both CRP forms and also PTX3 bound to FHR3 (**Fig. 2 and 3**). The binding of PTX3 to FHR3 was stronger than to other members of the FH family (**Fig. 3**). Since the C-terminal FHR3 fragment did not bind mCRP and PTX3 (**Fig. 2** and ref. 5),[5] we concluded that the FH CCP7-homologue domain harbors the pentraxin binding site in FHR3.

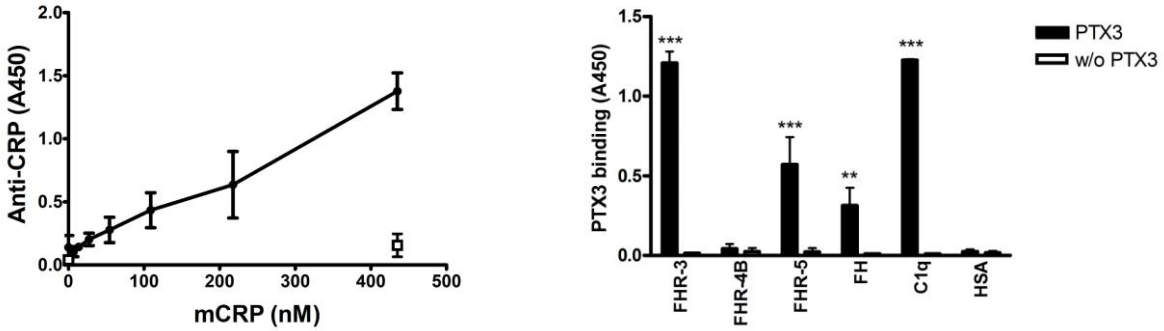


**Fig. 3. FHR3 interacts with pentraxins.** Left panel: Binding of the monomeric CRP form (mCRP) to recombinant FHR3 and human serum albumin (HSA, empty square), immobilized in microplate wells at 5 µg/ml. Right panel: Binding of the long pentraxin PTX3 to FH family proteins (immobilized in equimolar amounts); C1q was used as a positive control, and HSA as a negative control. ***$p < 0.001$, **$p<0.01$, one-way ANOVA.

FH was shown to bind to certain ECM components, and also to the ECM extract and *in vitro* ECM model MaxGel. Because laminin is a major constituent of MaxGel, we first analyzed the interaction between FHR1 and laminin, in comparison to FH. The major laminin binding site was identified in the C-terminal domains of FH, using recombinant deletion mutants, and similar laminin binding was detected to FHR1, indicating that the homologous FHR1 C-terminus mediated laminin binding (**Fig. 4**). We also used a multiplex approach, when selected ECM components and controls were spotted on nitrocellulose-covered slides and incubated with recombinant FHR1. Binding of FHR1 to certain ECM components could be detected, including osteoadherin, PRELP, fibromodulin, laminin, vitronectin and collagen IV. In parallel experiments, the slides were incubated with FH together with FHR1 or without FHR1. In these experiments, FHR1 dose-dependently inhibited FH binding to MaxGel, osteoadherin, PRELP, fibromodulin, laminin, vitronectin and collagen IV (**Fig. 4**). FHR3 did not significantly bind to ECM under these conditions.

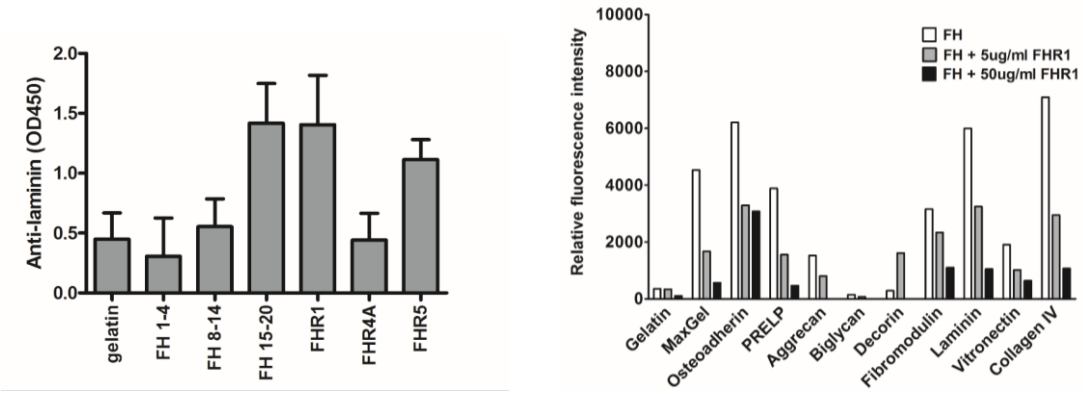


**Fig. 4. Binding of FHR1 to ECM proteins and competition with FH.** Left panel: Laminin binding to immobilized FH fragments and FHRs was analyzed in ELISA. Right panel: MaxGel, gelatin and ECM components were printed onto nitrocellulose-covered slides in triplicates. After drying and blocking, slides were incubated with 25 µg/ml FH or FH-FHR1 mixture as indicated. FH binding was detected with a mAb not recognizing FHR1.

Because malondialdehyde (MDA) adducts of lipids and proteins are implicated in the pathogenesis of AMD, and FH was shown to bind to MDA epitopes via domains CCP7 (a homologous domain is contained in FHR3) and CCPs 19-20 (homologous domains are contained in FHR1), we set out to characterize the interaction of FHR1 and FHR3 with MDA-epitopes. To this end, the binding of the proteins to MDA-BSA adduct was measured.

In collaboration with Dr. Agustín Tortajada and Prof. Santiago Rodríguez de Córdoba (Madrid), first the binding of purified FH variants (Y402 and H402) and the two FHR1 isoforms FHR1*A and FHR1*B was analyzed. FHR1 bound stronger in these experiments to MDA-BSA, compared to FH, but both isoforms bound equally well (**Fig. 5**). Both FHR1 and FHR3 reduced the binding of FH to MDA-BSA in a dose-dependent manner (**Fig. 6**), but the inhibitory effect of FHR3 was more pronounced compared to that of FHR1, which is likely explained by the stronger binding of FHR3 to MDA-epitopes (saturation of binding reached for 10 nM FHR3; **Fig. 5**).

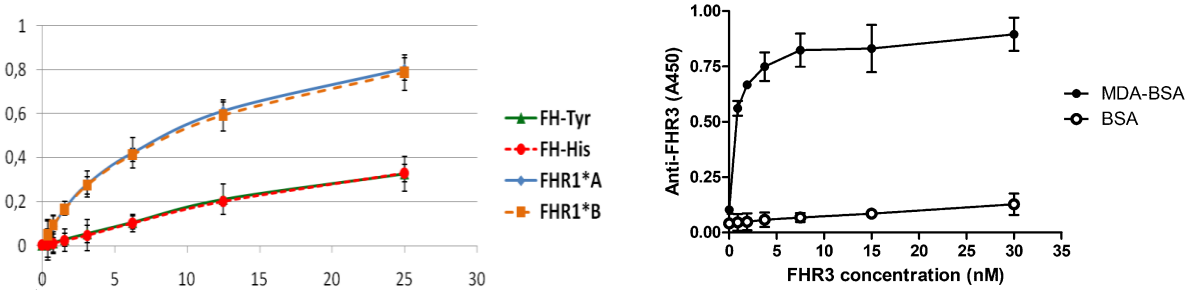


**Fig. 5. Binding of FHR1 and FHR3 to MDA-epitopes.** Left panel: In ELISA, both variants FH-402Tyr and FH-402His, bound equally well to immobilized MDA-BSA adducts. Similarly, the binding of FHR1*A and FHR1*B was comparable. Right panel: Dose-dependent binding of FHR3 to MDA-BSA.

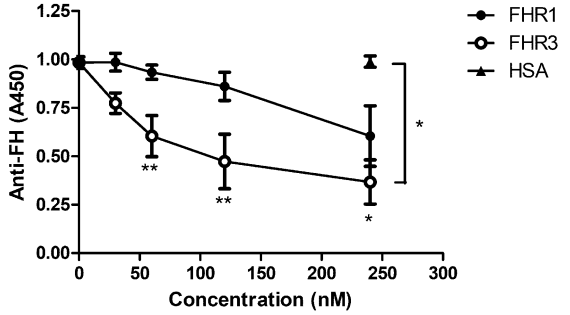


**Fig. 6. FHR1 and FHR3 inhibit FH binding to MDA-epitopes.** Binding of constant amounts of FH to MDA-BSA in the presence of increasing concentrations of FHR1 and FHR3 was measured using a mAb recognizing only FH. HSA did not inhibit FH binding. **p<0.01, *p<0.05, two-way ANOVA.

*Altogether, these results suggest that FHR1 and FHR3 can hamper the binding and, consequently, the complement inhibitory and anti-inflammatory role of FH* on ligands in the context of AMD and aHUS, such as pentraxins upregulated during inflammation, ECM components exposed in the fenestrated basal membranes in the eye and kidney and when overlying cells become activated, and MDA modifications during oxidative stress. Thus, FHR1 and FHR3 may promote complement activation and complement-mediated inflammation. This could also explain in part the described protective effect of the *CFHR3/CFHR1* double gene deletion in the case of AMD, since this common polymorphism removes the FH antagonists FHR1 and FHR3.

***Specific Aim 2: Are CFHR1 and CFHR3 able to facilitate complement activation?***

Because of the contradiction in the literature regarding the complement regulatory roles of the FHRs, we tested whether these proteins can inhibit complement activation. First, we analyzed the cofactor activity of both FHR1 and FHR3. For this, we incubated both proteins with C3b and the enzyme factor I; however, even at supraphysiological 2 μM concentrations neither FHR1 nor FHR3 could act as a cofactor for factor I to cleave (and thus inactivate) C3b. When measuring the capacity of these proteins to facilitate the decay of the solid phase C3bBb alternative pathway C3 convertase enzyme, we again found no significant decay accelerating activity of FHR1 and FHR3. Furthermore, they did not affect the convertase decay accelerating activity of FH.

To analyze the influence on the activity of the terminal pathway, we measured the amounts of soluble terminal complement complexes (sC5b-9) in serum activated by zymosan in the presence of exogenously added recombinant FHR protein. In contrast to FH, no inhibitory effect on the terminal pathway was found.[4] We also tested the potential capacity of FHR1 to inhibit the terminal pathway in an *in vitro* model of nanomedicine-induced complement activation. Certain liposomes and Cremophor EL micelles when added to human serum can cause complement activation, which is in part responsible for the infusion reaction (a phenomenon termed complement activation-related pseudoallergy). In these experiments, FHR1 did not inhibit the formation of sC5b-9.[6]

Next, we studied if FHR1-bound mCRP can still bind C1q and allow for activation of the classical complement pathway. We found that mCRP when bound to FHR1 could bind C1q and also the C1 complex (containing the serine proteases C1s and C1r in addition to C1q).

These interactions, in turn, resulted in enhanced classical pathway activation when exposed to human serum, as measured by the deposition of C4 fragments (**Fig. 7C**). In other experiments, ARPE-19 retinal pigmented epithelial cells were cultured in 96-well plates for 7 days, then the cells were removed and the cell-free ECM was incubated sequentially with FHR1 and mCRP. Enhanced C4-fragment deposition was observed when exposed to human serum on ECM-bound FHR1+mCRP compared with FHR1 alone (**Fig. 7D**).[4] Similarly, we found that FHR1 bound to late apoptotic and necrotic cells and recruited mCRP and PTX3 to the surface of these dying cells. The FHR1/mCRP and FHR1/PTX3 interactions resulted in enhanced C4 deposition on necrotic cells when exposed to human serum, as deteced by flow cytometry.

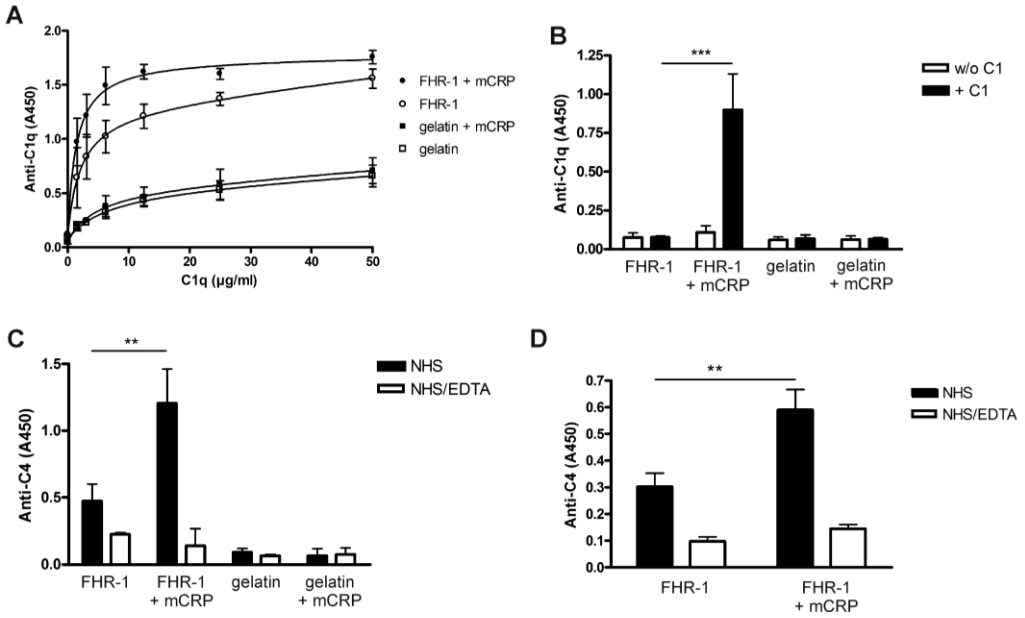


**Fig. 7. FHR1 bound mCRP binds C1q and allows for classical pathway activation.** (A) FHR1 was immobilized in microplate wells and incubated with or without 5 μg/ml mCRP. After washing, serial dilutions of purified C1q were added and the C1q binding was measured. Gelatin was used as a negative control. The binding of C1q to FHR1 was significantly different in the presence of mCRP compared with the binding in the absence of mCRP ($p < 0.0001$, two-way ANOVA). (B) Binding of 10 μg/ml purified C1 complex to wells coated with 4 μg/ml recombinant FHR1 and preincubated with 5 μg/ml mCRP as in (A). ***$p < 0.001$, one-way ANOVA. (C) FHR1 and gelatin were immobilized in microplate wells and preincubated or not with 5 μg/ml mCRP. 1% normal human serum in DPBS containing $Ca^{2+}/Mg^{2+}$ (NHS) or NHS in DPBS containing 20 mM EDTA was added for 30 min at 37ºC, then C4-fragment deposition was detected. **$p < 0.01$, one-way ANOVA. (D) ARPE-19 cells were cultured in 96-well plates for 7 days. After removal of the cells, the cell-derived ECM was incubated sequentially with FHR1 and mCRP, then exposed to 1% normal human serum (NHS) or NHS/EDTA for 30 min at 37ºC. C4-fragment deposition was detected. **$p < 0.01$, one-way ANOVA. *(Published in: Csincsi et al., J Immunol. 2017;199:292-303.)*[4]

When analyzing FHR3, we found that FHR3-bound PTX3 but not mCRP could still bind C1q (**Fig. 8**). In line with this, and in contrast to the case of FHR1, we could not measure increased C4 deposition when FHR3 was preincubated with mCRP. This is likely due to the different nature of the interaction of mCRP with the two FHR proteins. However, on immobilized CRP and PTX3, but not on MaxGel (to which FHR3 did not significantly bind), enhanced C3 deposition was detected when exposed to human serum in the presence of FHR3 (**Fig. 8**).

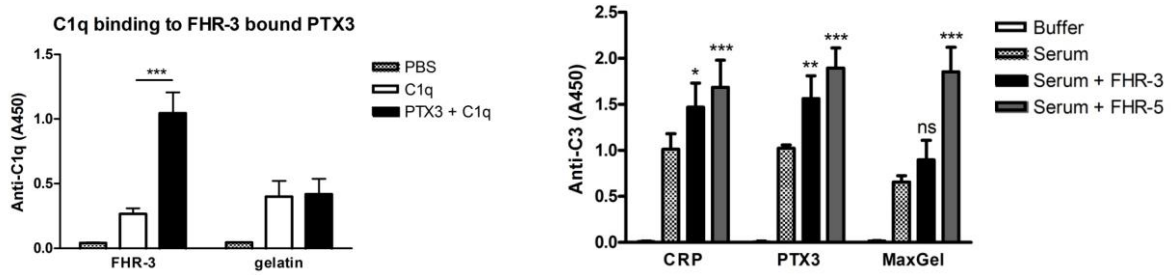


**Fig. 8. C1q binds to FHR3-bound PTX3, and modulation of complement activation by FHR3.** Left panel: Recombinant FHR3 and gelatin were immobilized in microplate wells, and 5 μg/ml recombinant PTX3 was added, followed by the addition of 25 μg/ml C1q. C1q binding was detected. Right panel: CRP, PTX3 and MaxGel were immobilized in microplate wells, then human serum was added in the absence or presence of FHR-3 (300 nM for CRP, 600 nM for PTX3 and ECM) and FHR-5 (300 nM). C3 fragment deposition was detected. The data are mean absorbance values + SD derived from three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, one-way ANOVA.

Because FHRs, in addition to competing with FH and interacting with pentraxins, can also bind C3b and directly enhance alternative pathway activation, we also analyzed whether the C3b binding to FHR1 and FHR3 allows the formation of an active C3 convertase enzyme, like it does for FHR4 and FHR5.[5] To this end, first FHR1 was preincubated with C3b, followed by the addition of factors B, D and P. We found that using purified proteins the C3bBb convertase can assemble on FHR1 (detected with anti-factor B) (**Fig. 9A**). This convertase was functional: when C3 was added for 1 hr, its cleavage was detected by the generation of the soluble C3a fragment (**Fig. 9B**). When using normal human serum, convertase formation on immobilized FHR1 could similarly be observed by detecting C3 fragment deposition, as well as the deposition of factors B and P (**Fig. 9C**). Thus, FHR1 can support alternative pathway activation by serving as a platform for the assembly of a fully active C3bBb alternative pathway C3 convertase.[4]

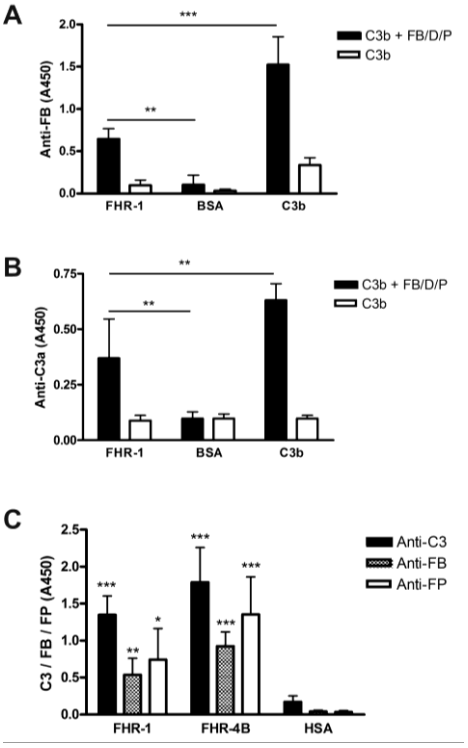


**Fig. 9. FHR1 supports rather than inhibits complement activation.** (A) Assembly of the C3bBb convertase on FHR1. Recombinant FHR1, BSA as negative control and C3b as positive control were immobilized in microplate wells. After incubation with C3b, the alternative pathway C3 convertase was built up by adding purified factors B, D and P. **p < 0.01, ***p < 0.001, one-way ANOVA. (B) Activity of the FHR1-bound convertase was measured by adding C3 to the wells for 1 h at 37°C; C3a generation was measured by an ELISA kit. **p < 0.01, one-way ANOVA. (C) FHR1 was immobilized on microplate wells and incubated with 10% normal human serum in 5 mM $Mg^{2+}$-EGTA buffer to allow only alternative pathway activation. Deposition of C3b, factors B and P was detected using the corresponding antibodies. FHR-4B was used as positive control and human serum albumin (HSA) was used as negative control. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA. *(Published in: Csincsi et al., J Immunol. 2017;199:292-303.[4]*

We also found that such a convertase can form on mCRP-bound FHR1, even when mCRP recruits FHR1 to ECM and the surface of necrotic cells (**Fig. 10**).

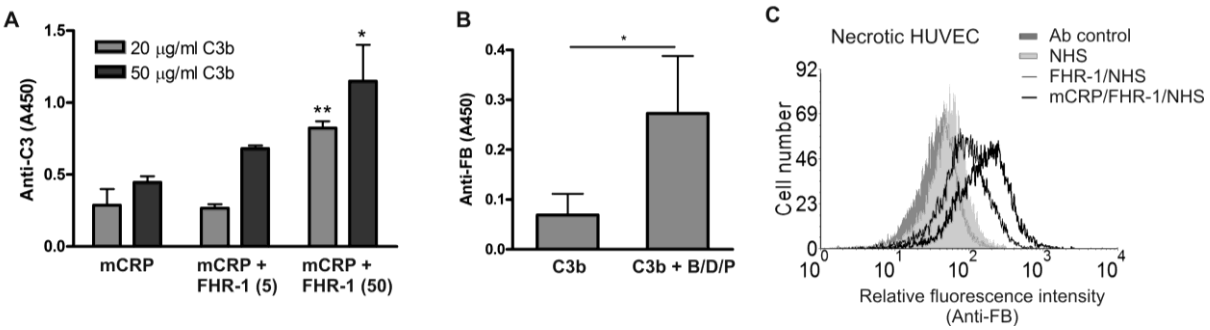


**Fig. 10. FHR1 activates the alternative pathway when bound to mCRP on surfaces.** (A) C3b can bind to mCRP-bound FHR-1 on microplate wells (*p < 0.05, **p < 0.01, one-way ANOVA). (B) Assembly of the alternative pathway C3 convertase C3bBb was measured on ECM produced by ARPE-19 cells *in vitro*, by incubating the washed, cell-free ECM with mCRP followed by FHR1. After preincubation with C3b, the convertase was built up by adding purified factors B, D and P; the convertase was detected with anti-FB antibody. *p < 0.05, one-way ANOVA. (C) Necrotic HUVEC were generated by heat treatment and preincubated or not with 2.5 μg/ml mCRP, followed by 25 μg/ml FHR1, then exposed to 5% normal human serum (NHS) for 30 min at 37°C. Activation of the alternative pathway was detected by flow cytometry using polyclonal anti-FB antibody. *(Published in: Csincsi et al., J Immunol. 2017;199:292-303.)*[4]

Similarly, C3 convertase can assemble on FHR3 due to its C3b binding ability and this FHR3-bound C3bBb enzyme can cleave C3 into C3a and C3b. FHR3 could also activate complement when bound to mCRP and PTX3 (**Fig. 11**).

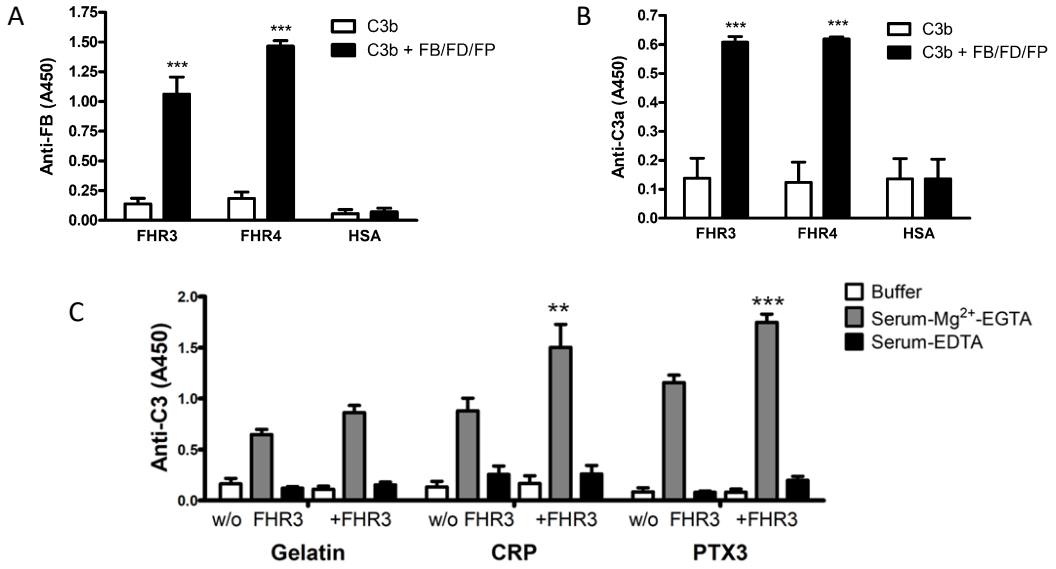


**Fig. 11. FHR3 can activate the alternative pathway.** (A) Assembly of the C3bBb convertase on FHR3 and (B) its activity were measured as in Fig. 9. *** $p < 0.001$, one-way ANOVA. (C) Complement activation by pentraxin-bound FHR3 was measured by incubating pentraxins or gelatin (as negative control) immobilized on microplate wells at 5 μg/ml with or without 5 μg/ml FHR3. 10% serum was added in 5 mM $Mg^{2+}$-EGTA-containing buffer to allow only alternative pathway activation or in 5 mM EDTA-containing buffer to inhibit complement activation. C3 deposition was detected with polyclonal anti-C3. ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.

*Altogether, these results show that FHR1 and FHR3, while lacking complement inhibiting activity, can activate complement directly via C3b binding and supporting the formation of the C3bBb convertase, and also via their interactions with pentraxins.*

### *Specific Aim 3: What is the role of FHR1 and FHR3 in infectious diseases?*

To test our hypothesis that FHRs can compete with FH binding to bacterial ligands, we first analyzed the binding of FHR1 to bacterial ligands that are known to bind FH, such as OspE from *Borrelia burgdorferi*, Lig proteins and LcpA from *Leptospira interrogans*, GBS enolase from *Streptococcus pyogenes*. Recombinant FHR1 bound to these bacterial FH binding proteins (**Fig. 12A**) and the binding was mediated by the FH CCP19-20 homologue C-terminal domains of FHR1, which was shown by inhibition of FHR1 binding by the mAb C18 that binds to the most C-terminal domain of FHR1. Binding of FHR1 to these bacterial proteins could also be detected from serum.

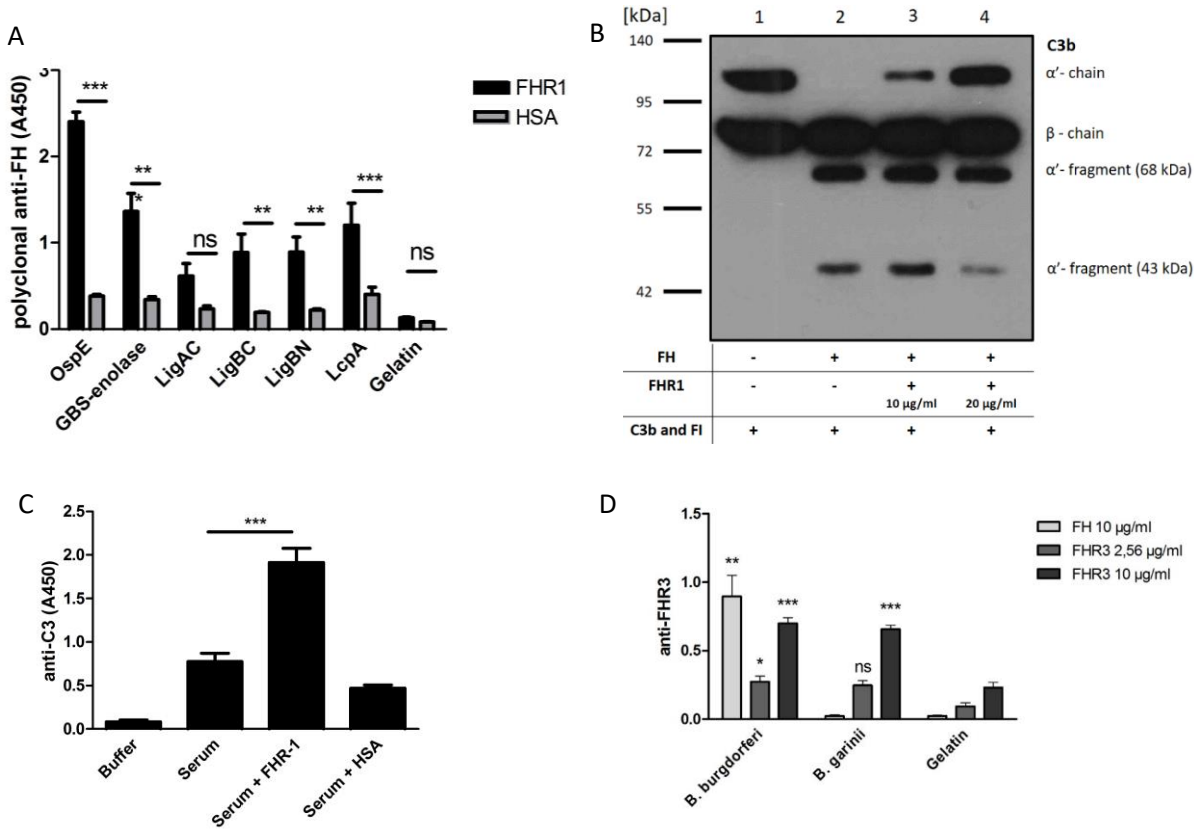


**Fig. 12.** (A) OspE, GBS-enolase, Lig-AC, Lig-BC, Lig-BN, Lcp-A and gelatin were immobilized on microtiter plate (10 µg/ml). After blocking, 67 nM human FHR1 or HSA were added and FHR1 binding was detected. (B) Competition between FH and FHR1 causes reduced cofactor activity of FH. OspE was coated in 10 µg/ml in microplate wells. 30 µg/ml FH was added in the absence or presence of 10 or 20 µg/ml FHR1. After washing, 140 nM C3b and 300 nM FI were added. Samples were subjected to 7.5% SDS-PAGE and analyzed by Western blotting. C3 fragments were detected with a HRP-conjugated anti-human C3 Ab. (C) FHR1 increases C3 deposition and alternative pathway activation on OspE. The immobilized (5 µg/ml) OspE was incubated with 10% serum with or without 10 µg/ml FHR1, diluted in buffer containing $Mg^{2+}$/EGTA. Binding of C3 fragments was detected. (D) Binding of recombinant FHR3 to borrelial proteins. 10 µg/ml bacterial surface protein OspE from *B. burgdorferi* and *B. garinii* was immobilized on microplate wells. After blocking, FHR3 and FH were added in equimolar concentrations (67 nM) and FHR3 also in a higher concentration (262 nM). The bound proteins were detected with polyclonal anti-human FHR3 Ab.

FHR1 inhibited FH binding to OspE in a dose-dependent manner. This translated to a functional effect, i.e. the competitive inhibition of FH binding resulted in reduced cofactor activtiy of FH on OspE (**Fig. 12B**). FHR1 could also activate complement on OspE when added to human serum in $Mg^{2+}$/EGTA containing buffer, which allows only activation of the alternative pathway. When OspE was exposed to human serum containing exogenously added FHR1, increased C3 deposition was detected compared to serum alone or when HSA was added (**Fig. 12C**). We got similar results with FHR5 when applied in these experiments in parallel (although e.g. FHR5 bound less strongly to OspE), indicating overlapping functions among these proteins.

When analyzing FHR3, we made the interesting observation that it bound not only to OspE of *B. burgdorferi*, but also to a homologue protein from *B. garinii* that is known for not binding FH (and it also did not bind FHR1) (**Fig. 12D**). We are currently further analyzing the functional relevance of these interactions.

In addition, several microbes express complement inhibitors to evade the host complement system. These include C3b binding proteins that can block convertase enzymes and thus further complement activation. We found that while the staphylococcal protein Efb (extracellular fibrinogen binding protein) enhanced C3b binding to FHR1, via domains 4-5 of FHR1, and inhibited C3b binding to FHR3 (domains 4-5 of FHR3), the Efb homologous protein (Ehp) enhanced C3b binding to both FHR1 and FHR3. Complement activation on FHRs, however, was inhibited by both Efb and Ehp. Thus, our data suggest that these staphylococcal secreted proteins can inhibit the FHR-bound form of the alternative pathway C3 convertase and thus reveal a novel point of interference of this bacterial pathogen with the human complement system. A manuscript is in preparation describing these results.

Because in our institute it is not possible to work with human-pathogenic microbes, we set up cooperation with researchers at the Dept. Bacteriology and Immunology in Helsinki to measure opsonization and survival of microbes (particularly *Borrelia*) and analyze the effect of the FHRs. These studies are ongoing.

*These data support a role for FHR1 and FHR3 (and likely other FHRs) in counteracting the complement evasion strategy of microbes, and indicate that the different FHRs may bind to similar or disctinct microbes (microbial proteins) and enhance opsonization.*

***FHRs and cells:***

We have previously shown that FHR1 can bind to human neutrophil granulocytes. Therefore, we analyzed cellular (monocytes, neutrophils) responses to factor H family proteins (**Figs. 13 and 14**). First, we measured binding of FHR1, FHR3 and FHR5 to neutrophil granulocytes and monocytes isolated from blood. All these FHRs bound to the cells, with different intensity, FHR3 binding being the weakest. However, their effect was different. While FHR1 immobilized in cell culture plates supported the spreading of neutrophils, FHR3 did not; on the contrary, FHR3 supported the spreading of monocytes. FHR5 did not show any effect in these experiments. However, these proteins did not significantly alter cytokine production of the cells (IL-1β, TNF-α, IL-8, IL-10), even after stimulation with LPS. An invited review article on this non-canonical cellular role (i.e., unrelated to complement regulation) of the factor H family proteins has recently been published by our group (Józsi et al., Seminars in Cell & Developmental Biology, 2019).[7]

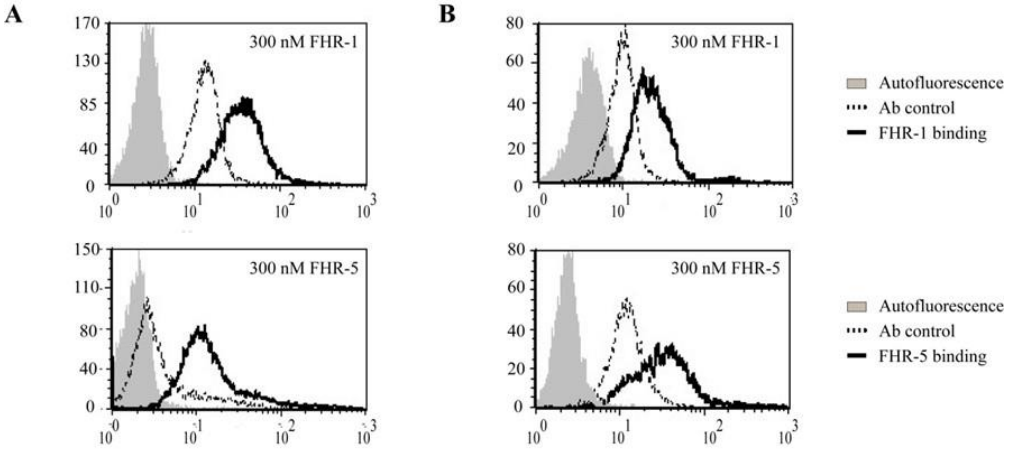


**Fig. 13.** Binding of recombinant FHR1 and FHR5 to primary (A) neutrophils and (B) monocytes was measured by flow cytometry using specific antibodies. The x-axes show relative fluorescence intensities and the y-axes the cell number.

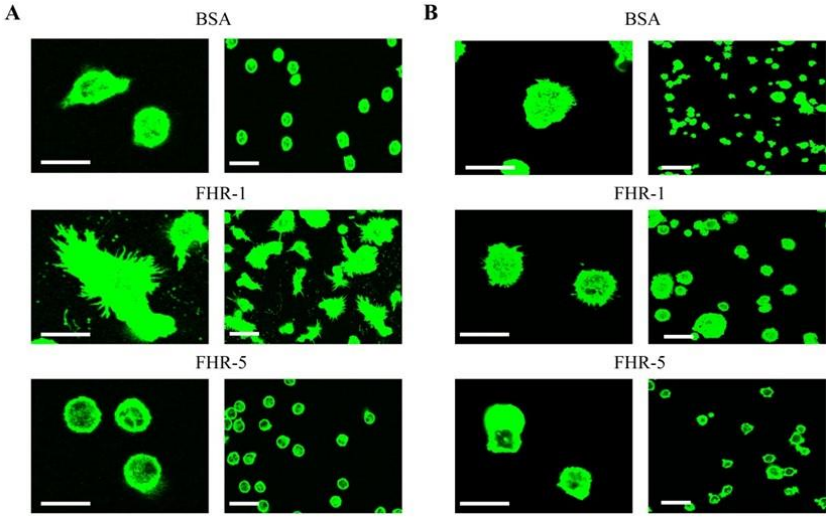


**Fig. 14.** FHR1 when surface bound could promote spreading of primary neutrophils (A) but not that of monocytes (B). FHR5 had no such effect. (Analyzed by CLSM using fluorescently labeled cells.)

***FHR1 and autoimmunity:***

Because we previously found that FHR1 deficiency was linked to the generation of autoantibodies against FH in aHUS,[8] we further invertigated the role of FHR1 in this context. In collaboration with the group of Prof. Zoltán Prohászka (Semmelweis University, Budapest) and the groups of Dr. Pilar Sánchez-Corral (La Paz University Hospital, Madrid) and Dr. T. Sakari Jokiranta (University of Helsinki) we analyzed the FH autoantibodies of several aHUS patients. We found that the isotype, the avidity and the binding site of the FH autoantibodies did not significantly change during disease course and follow up (only the titer of the autoantibodies); however, in patients with homozygous FHR1 deficiency the autoantibodies mainly bound to the FH residues 1183-1189 in CCP20, whereas in patients without FHR1 deficiency to residues1203-1215.[9] We mapped the major autoantibody binding sites in the FH/FHR1 C-terminus using single amino acid mutants and identified a major autoantibody binding site. We found that the conformation of the FH and FHR1 most C-terminal domains

are slightly different in exactly the identified autoantigenic loop and proposed a model of infection-related autoimmunity that is also supported by the observations of gastrointestinal or upper respiratory tract infections as prodromal symptoms in most patients.[10] Thus, binding of FH to microbial ligands may induce a conformational change making the autoantigenic loop in FH similar to that in FHR1; however, due to lack of tolerance to this alternate conformation in the absence of FHR1, this could lead to the generation of autoantibodies.[10]

We have also screened the sera of patients with neuromyelitis optica spectrum disorders (NMO-SD) and identified FH autoantibodies in 4 out of 45 patients (~9%). In contrast to aHUS patients, these patients did not have FHR1 deficiency. Furthermore, the autoantibodies recognized different epitopes: in three patients the main autoantibody binding site was in FH CCP19, and only one patient had autoantibodies recognizing similar epitope in CCP20 as in aHUS. All four NMO-SD associated autoantibodies also recognized FHR1. Importantly, the autoantibodies caused impaired C3b binding, suggesting a functional effect. The manuscript describing these results is in revision.

**III. Dissemination**

Our results revealed that FHR1 and FHR3 interact with several ligands also bound by FH and may inrease complement activation by competing with FH for ligand binding and also directly by supporting the formation of the alternative pathway C3 convertase enzyme. This may cause complement dysregulation on ligands implicated in the pathology of eye and kidney diseases and, favourably, on the surface of microbes. Thus, our data provide important insights into the function of the factor H family proteins and their role in disease.

The results described in the report have been published in part,[4,9,10] from the results concerning FHR3 and interaction of FHR1 and FHR3 with bacterial ligands three manuscripts are in preparation. A further mansucript regarding autoantibodies in patients with neuromyelitis optica spectrum disorders is in revision. We have summarized the role of the FH family and the FHRs in the regulation/modulation of complement activation in two review articles,[2,11] and their role in the complement evasion of microbes[3] and the role of complement and the FH family in C3 glomerulopathy[12] in additional review articles. Two further manuscripts are in preparation that describe the role of FHR1-pentraxin interactions in the modulation of opsonization of apoptotic cells and the direct effect of FHR1 on neutrophil granulocytes and monocytes. We have summarized our current understanding of the non-canonical functions of the FH family proteins in the modulation of cellular functions in a recent review article.[7]

Ádám Csincsi defended his PhD thesis from this topic in November 2018 (https://doktori.hu/index.php?menuid=193&lang=HU&vid=19324). The PhD studies of Éva Kárpáti includes the opsonic effect of FHR/pentraxin interaction on dead cells and the influence of FH family proteins on the activation of immune cells; her thesis is in preparation. One MSc student's (Bianca Brandus') TDK work was prepared from the characterization of FHR1-microbial proteins interactions; she won first prize at the university TDK conference (2018) and is going to present her data at the national TDK conference in April 2019.

The results have been presented at several national and international conferences (see published abstracts in the online report).

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