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The balance between complement factor H and its related proteins in health and disease

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Publication date
2018
Document Version
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Citation for published version (APA):
Pouw, R. B. (2018). *Setting the scale: The balance between complement factor H and its related proteins in health and disease*. [Thesis, externally prepared, Universiteit van Amsterdam].

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9

Summarizing discussion

The complement system in balance

The complement system is a central part of the human immune system, being important for the defense against invading micro-organisms but also involved in the removal of damaged and dying human cells. There is a delicate balance between complement activation and inhibition[1,2]. On the one side, a constant activation state of the complement system is required to detect invading pathogens and cells that have to be cleared. On the other side, activation has to be tightly controlled to keep the system from attacking healthy host cells and prevent excessive inflammation. Too much of either, activation or inhibition, will lead to disease. Too much complement activation results in excessive inflammation and damage while too little complement activation (or too much inhibition) predisposes to infections. Complement regulator factor H (FH) is a key player in maintaining this balance. FH inhibits complement activation on human cells, while leaving the complement system unchecked when it targets invading pathogens. With the discovery of FH-related (FHR) proteins, it is now hypothesized that FH-mediated complement inhibition itself is also tightly regulated. The balance between the inhibiting FH on the one side and the FHR proteins, that might allow or even increase complement activation, on the other side, is thought to dictate when and where complement activation via the alternative pathway takes place[3]. However, while the function of the various FHR proteins has been extensively studied *in vitro*, only little is known about the concentration and function of these proteins *in vivo*, especially in relation to FH.

In this thesis the balance between FH and FHR proteins 3, 4A and 4B was investigated. To this end, novel antibodies were developed to specifically distinguish the highly similar FH and FHR proteins. These antibodies were used to study the function of FH, as well as to set up novel assays to quantify FH, FHR-3 and FHR-4A. We applied these assays to establish normal levels in healthy donors and compared these with levels found during disease. Furthermore, we investigated the genotype-phenotype relationships for FHR-3 in clinical settings, most prominently in meningococcal disease, and revealed a new genetic link between FH and FHR-3 that influences the balance in the complement system.

FH in health

FH has two major regulatory functions in the complement system. First, fluid phase regulation of C3 activation by FH prevents complete consumption of C3 in blood plasma, allowing C3 to activate on surfaces. Second, when activated C3, in the form of C3b, binds to human surfaces, FH binds as well and inhibits deposited C3b to prevent further activation of the complement system. Human cells do express several additional regulators to inhibit complement activation and at first sight, there seems to be a level of redundancy within the system: multiple inhibitors

are present on the human cell surfaces that inhibit the complement cascade. The membrane-bound complement regulators complement receptor 1 (CR1), CD46 and CD55 inhibit C3b deposition on healthy human cells (reviewed in [4]). CR1 functions very similar as FH, as it has decay accelerating activity, competes with factor B (FB) and is a cofactor for factor I (FI), resulting in degradation of C3b into C3dg. CD46 is also known as membrane cofactor protein (MCP), and like FH is a cofactor for FI for the degradation of C3b. CD55 is also known as decay accelerating factor, and like FH, increases the decay of formed C3bB and C3bBb complexes. In addition, human cells express CD59, which blocks formation of the lytic C5b-9 complex. Despite the presence of CR1, CD55 and CD59 on human erythrocytes, we demonstrate in **Chapter 2** that normal FH function is required in protecting human erythrocytes from complement. CR1, CD55 and CD59 on human erythrocytes do prevent complement-mediated lysis, but C3b deposition is greatly increased when blocking FH with inhibiting antibodies. As C3b-opsonized erythrocytes are prone to extravascular hemolysis[5–7], these results indicate a crucial role for FH in protecting healthy human erythrocytes. On the other hand, when cells lack CD55 and CD59, which is the case for a subpopulation of erythrocytes in a disease called paroxysmal nocturnal hemoglobinuria (PNH), complement-mediated clearance also occurs, despite the presence of FH[5,8]. PNH erythrocytes are more dependent on FH, and variations in FH levels might reflect variations in disease severity[9]. Due to the lack of CD55 and especially CD59, C3b deposition on PNH erythrocytes results in intravascular complement-mediated hemolysis. PNH occurs in flares, indicating sufficient complement inhibition can be maintained in general, but changes in complement activating or regulating factors can trigger the disease. This highlights the tight balance of the complement system and while there seems to be redundancy in the complement regulators, all are needed to prevent unwarranted complement activation to occur on healthy human cells. When lacking functional FH on the cell surface, C3 deposition is likely to accumulate on the cell surface resulting in inflammatory responses and opsonisation-mediated clearance, while surface regulators do prevent complement-mediated lysis. When lacking surface regulators, any C3 activation on the surface that is not blocked by FH can easily trigger inflammation and ultimately result in complement-mediated lysis.

Binding of FH

The function of FH has been extensively studied, mainly by using FH fragments[10–17]. The complement inhibiting activity resides in domains 1-4, while the binding activity of FH has been mapped to domains 6-8 and 19-20. Current models suggest that initial FH binding is mediated by domain 19 and 20 to C3b and polyanionic residues on a human cell, followed by binding of domain 6-8 to polyanionic residues to stabilize the binding[17]. It is therefore thought that the binding region in domain 19-20 is the primary binding region, with a minor, secondary role for domains 6-8. However, the splice variant of FH, FHL-1, which has no additional binding sites besides domains 6-7, does inhibit complement efficiently on

cell surfaces[18]. Likewise, mini-FH's, which are rationally designed FH constructs in which domains 1-4 are directly linked to domains 19-20, are also highly effective in protecting cells from complement[19,20]. Thus, while FHL-1 and mini-FH's function with only one additional binding site (6-7 or 19-20) next to CCPs 1-4, FH seems to require both 6-8 and 19-20 for efficient binding. When binding via domain 6-8 does not occur, FH will not inhibit C3b due to either too weak interaction of FH with the surface, or through inappropriate binding of the FH molecule to C3b. Likewise, when binding via domains 19-20 is impaired, which is often the case in atypical hemolytic uremic syndrome (aHUS) due to mutations or auto-antibodies[21], FH is less efficient in protecting human surfaces from complement. Indeed, as we demonstrated in **Chapter 2**, blocking either domain 6 or 20 with monoclonal antibodies inhibited FH and resulted in increased C3b deposition on the cell surface. The reason for requiring both binding sites by FH is unclear, but may reside in the necessity to distinguish between foreign and human surfaces.

The structure of FH is involved in its function

With functions described for domains 1-4, 6-8 and 19-20, it is unclear what the role is of the remaining domains. As demonstrated with the mini-FH constructs[19,20], FH would be a more effective complement inhibitor without domains 5-18, raising the question why evolution did not drive the development of a natural 'mini-FH' or why full-length FH even exists next to the smaller FHL-1. Structural studies on FH have elucidated that parts of 5-18 are bent, folding full length FH back onto itself and bringing the two binding regions of FH closely together[22–26]. Possibly, the conformation of FH is an additional feature, next to its two binding sites conferring polyanionic specificity, involved in the discrimination of human cell surfaces from non-human surfaces[27]. Indeed, mini-FH was also found to confer complement regulation to bacterial surfaces to some extent, although direct comparison with FH was not performed[28]. In addition, FHL-1 has been reported to be impaired in distinguishing human from foreign cells[29]. In our experiments, we did not observe protection of bacteria by plasma-derived FH (**Chapter 3**). Thus, while the bent back conformation (domain 8-18) and the presence of two binding sites (domains 6-7 and 19-20) seems to make FH less effective as a complement regulator, its trade-off might be a better selectivity towards human cells over bacteria and thus allowing clearance of pathogens. It appears that having two regions (6-8 and 19-20) that are required to bind to the surface and/or C3b, confers specificity for human surfaces (FH), while having only one binding region results in protection of foreign, possibly pathogenic surfaces as well (FHL-1 and mini-FH's).

The bent back conformation of FH is thought to be flexible, and FH might undergo conformational changes when it binds to C3b on human cells. It has been hypothesized that such a conformational change increases the regulatory function of FH on cell surfaces[27]. In **Chapter 3**, we investigated a monoclonal antibody of which we hypothesize it induces or

stabilizes such a conformational change in FH, making it more active. Of the antibodies described in **Chapter 2**, one antibody, named anti-FH.07, was found to bind to domain 18 of FH. When performing complement activation experiments in the presence of this antibody, a decrease in C3 deposition was observed. Further experiments showed this effect was dependent on FH. Addition of anti-FH.07 to aHUS patient sera could even overcome the complement-mediated cell lysis caused by impaired FH function. These results imply that

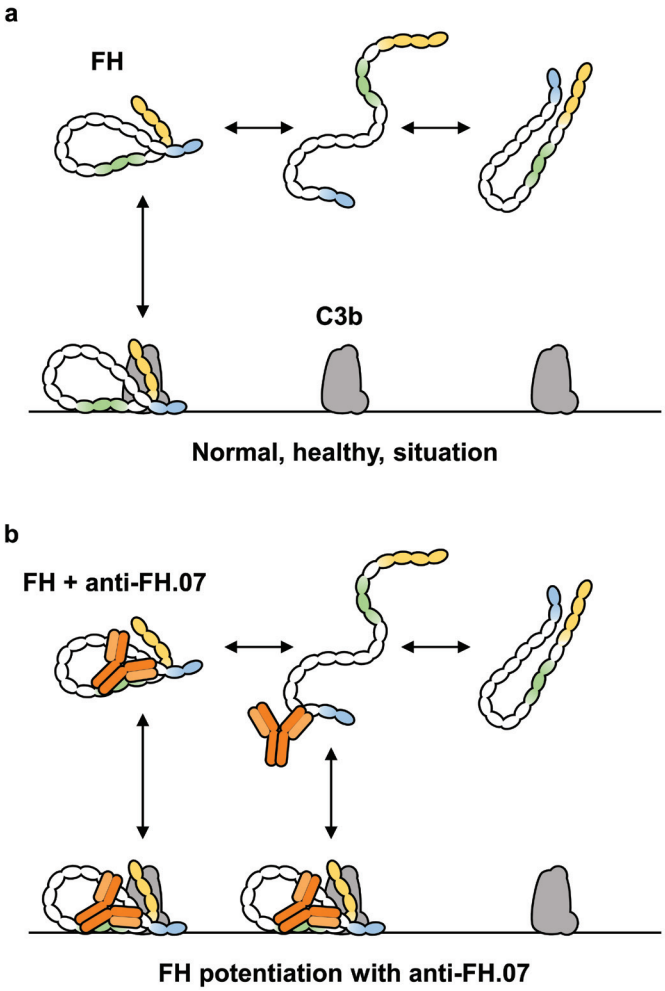

Figure 1 Proposed mechanism of action of anti-FH.07-mediated FH potentiation. (**a**) In a normal healthy situation, FH circulates in multiple, interchangeable conformations, of which a few are in the correct conformation to bind deposited C3b on human cells, controlling complement activation. (**b**) Anti-FH.07 binds to multiple conformational isoforms of FH, resulting in a conformational change into the form that binds deposited C3b on human cells, thus enhancing complement regulation.

there is indeed a less active form of FH in the circulation, which can become more active. Our antibody increased the amount of plasma-derived FH binding to C3b, suggesting that 1) not all FH bound to C3b in the first place, and 2) this can be improved by manipulating FH (**Fig. 1**). This is indirect evidence that there are indeed multiple FH conformations present, that these differ in functionality, and that these conformations are flexible and can change to improve functionality. Formal proof using crystal structures of FH in different conformations, in the presence of our antibody, is required to confirm these assumptions. Furthermore, we only were able to test the binding of FH to C3b, and did not test binding to other ligands. It is unknown whether anti-FH.07 would also improve binding of FH to other ligands, such as heparin or other polyanionic residues. This is to be expected, as only the increase in C3b binding seems unlikely to fully explain the effect of anti-FH.07. If only C3b binding would increase, increased FH regulation would occur on any surface with deposited C3b. However, our antibody did not improve FH function on bacteria or rabbit erythrocytes, which both are surfaces that, under normal conditions, are not protected by FH. Thus, the specificity of FH for human polyanionic residues remains intact when FH function is enhanced by anti-FH.07. This opens a new opportunity for the development of a therapeutic that targets and enhances FH to increase complement regulation specifically on human cells.

FH as a therapeutic target

Antibody-mediated potentiation of FH with anti-FH.07 seems to have an advantage over mini-FH's, as no protection of bacteria following FH potentiation was found *in vitro* (**Chapter 3**). Of note, this was only tested with two Gram-negative bacteria, *Escherichia coli* and *Neisseria meningitidis*. The latter is infamous for its dependence on inhibiting complement for its survival, underlined by *N. meningitidis* infections associating with complement deficiencies[30], of which most prominently are deficiencies of the terminal pathway components, including C5, C7 and C9[31–34]. *N. meningitidis* also interacts with FH by expressing proteins capable of 'hijacking' human FH from circulation, the most prominent being FH-binding protein (fHbp)[35–37]. FH-binding protein binds FH at domain 6-7 through molecular mimicry, similar to how domain 6-8 would bind to human cell surfaces[38]. This confers the complement inhibitory function of FH to the meningococcal surface, effectively increasing its survival. Although potentiation of FH via anti-FH.07, which binds to domain 18, would still allow binding of FH to fHbp, we did not observe any protective effect of enhanced FH over normal FH (**Chapter 3**). This suggests that anti-FH.07 does not increase the intrinsic inhibitory capacity of FH in domains 1-4, as this would also result in increased protection of *N. meningitidis*. This supports our results that FH function is enhanced through increased binding to human cell surfaces. As enhanced FH also did not protect *E. coli*, it seems that the discrimination between bacterial and human surfaces is unaffected. However, as indicated, we did not investigate the effects of FH potentiation on Gram-positive bacteria. Gram-positive bacteria are protected against direct complement-mediated lysis, but can be

cleared via C3b-mediated opsonophagocytosis. As FH inhibits C3b deposition, it is possible that FH potentiation negatively affects opsonophagocytosis of Gram-positive bacteria. It is important to further investigate if increased FH function, either by FH potentiation or with the use of mini-FH constructs, affects the C3b deposition on Gram-positive bacteria. In fact, FH potentiation seems to be an evasion strategy already used by Gram-positive *Streptococcus pneumoniae*. While we have established that FH can be potentiated with a monoclonal antibody, a fragment of the FH binding protein of *S. pneumoniae*, pneumococcal surface protein C (PspC), was recently demonstrated to also enhance FH function[39,40]. PspC binds FH at domain 8-10, with very high affinity, and has similar effects on FH function as anti-FH.07 (increased binding to C3b, enhanced complement regulation). While it indirectly suggests that FH potentiation via an antibody could indeed protect Gram-positive bacteria, there is still a crucial difference between PspC and the anti-FH antibody we describe. PspC is a surface bound, bacterial protein, and is thus already attached to the bacteria. This locates the PspC-potentiated FH directly to the bacterial surfaces. It is unknown if PspC would also result in increased binding of potentiated FH to the bacteria if it would bind FH in fluid phase. In other words, it is unknown if PspC-potentiated FH retains its specificity towards human cells similar to anti-FH.07-potentiated FH.

It is important to remain cautious with enhancing FH function, either through the construction of mini-FH's or through antibody-mediated potentiation. While both approaches are very promising in protecting human cells from complement activation under disease conditions, extensive research in possible side-effects, including protection of pathogens or human cells that should be attacked by complement (e.g. apoptotic cells, necrotic cells, tumor cells) is required. FH is known to bind to apoptotic and necrotic cells, where it helps to control the degree of opsonisation and the release of highly inflammatory nucleosomes[41–43]. This is again a delicate balance. Dying human cells need to be opsonized to be marked for clearance, but too much complement activation would result in (too) strong inflammation and direct lysis of the cell and consequently release of damage-associated molecular patterns (involving factor seven activating protease) that could trigger strong auto-immune responses[44]. While FH binds to dying cells to inhibit the complement system, some opsonisation needs to occur. This is achieved by increased expression of complement activating triggers that mostly initiate the classical and lectin pathway. In addition, the FHR proteins might compete with FH for the binding to necrotic cells, consequently shifting the balance of activation and regulation, and allowing C3b deposition to occur (discussed below). As our potentiating anti-FH antibody increases binding of FH to human cells, it may be speculated that this might also increase binding to necrotic and apoptotic cells. As such, chronic FH potentiation might disturb the balance of activation and regulation, decreasing the opsonisation of necrotic cells and subsequently the opsonophagocytic clearance of these cells. Prolonged disturbed clearance of dying cells has been associated with auto-immune diseases such as systemic lupus

erythematosus[45,46].

Gaining further insight to better understand the delicate balance of complement regulation and activation, and (systemic) consequences of altering that balance are paramount to proceed with the development and application of complement-targeting therapeutics, especially considering FH and its role within the complement system. Much knowledge has been gained over the recent years with the use of eculizumab, demonstrating that systemic therapeutic complement inhibition is relatively safe. Eculizumab treatment has been associated with increased risk for *N. meningitidis* infection, and therefore patients are vaccinated. However, as current vaccines do not cover all *N. meningitidis* serogroups, vaccinated, eculizumab-treated patients are still at an increased risk for MD[47]. Eculizumab targets C5, which is downstream of C3 activation in the complement cascade. Thus, eculizumab tells us little about the effects of interfering at the level of C3 or with specifically targeting activating enzymes like FB, FD, C1s or MASP-2. Studies and clinical trials investigating new possible therapeutics that target these complement proteins are currently ongoing, with variable success. However, the results of these studies are not easily extrapolated towards the use of a potentiating anti-FH antibody. The use of a potentiating antibody to inhibit the complement system is an unique strategy compared to the current approaches in clinical trials in which the compounds tested rely on inhibition of their target. The efficacy of such therapeutics depends on full inhibition of their target protein. Furthermore, in case of eculizumab-treated PNH patients, excessive complement activation results in breakthrough hemolysis despite the high levels of eculizumab maintained in these patients[48]. Potentiation of an endogenous complement regulator might not be so prone to breakthrough events or loss of efficacy when not all of the target is potentiated. Potentiating a part of the FH present might already be sufficient to restore the balance of complement activation and regulation. This could even be done transiently, after which normal FH and other complement regulators can maintain the balance within the complement system and prevent relapses.

FHR proteins in health

Exploring the therapeutic possibilities of FH also involves the FHR proteins. These relatively recent discovered proteins are homologs of FH, but their exact function within the complement system remains elusive. The current hypothesis places FHR proteins as positive regulators of complement activation, by acting as antagonists of FH[3]. The FHR proteins lack physiologically relevant complement regulatory activity, but do interact with the same ligands as FH. It is straightforward to envision competition between FH and FHR proteins which would dictate whether complement activation will be inhibited (FH binding) or can proceed (FHR binding) on the targeted surface. While research on the FHRs over the recent years has

focused on their function, identifying their ligands and mapping the domains responsible for these interactions, little was known regarding their physiological concentration. This was for a large part due to the lack of specific reagents, which in turn are difficult to obtain due to the high sequence identity among the FH family proteins. In **Chapter 4** and **Chapter 5** we describe newly generated and characterized monoclonal antibodies directed against FHR proteins.

FHR-3 and FHR-4A levels are relatively low

The first FHR monoclonal antibodies we obtained were raised against recombinant FHR-3 (**Chapter 4**). FHR-3 has been implicated in meningococcal disease, by the identification of a SNP in *CFHR3* associating with MD susceptibility[49]. We obtained five monoclonal antibodies, of which two monoclonal antibodies were found to be suitable to develop a FHR-3 specific ELISA. This first ever FHR3-specific ELISA allowed us to establish normal levels in healthy individuals. In the previous years, estimates of FHR-3 levels were set on 1.0-1.6 μM, close to the normal range of FH (~2 μM)[50]. The normal levels measured with our FHR-3 ELISA deviated significantly, finding concentrations around 14 nM, which is 144-fold lower compared to FH (**Fig. 2**). It remains unclear how Fritsche *et al.* (2010) estimated the FHR-3 levels as they do not describe the method or antibodies used[50]. It is therefore difficult to assess the validity of their estimation. Possibly, their estimation was based on results with cross-reactive antibodies, causing an overestimation of the true FHR-3 levels in the circulation. Our results were supported by a second report, in which the investigators made use of one of our FHR-3 antibodies to develop their own FHR-3-specific ELISA, reporting normal levels around 21 nM in healthy individuals[51]. Note that the concentrations described here are averages of the whole healthy donor cohort measured, without stratification based on *CFHR3* copy numbers. FHR-3 levels are highly influenced by genetic variation (**Chapter 4** and **Chapter 6**). *CFHR3* is commonly deleted within the healthy population as a result of non-allelic homologous recombination, with allele frequencies of around 20% in the Western population[52–60]. This results in approximately 5% of all healthy individuals in the Western population being homozygous for the *CFHR3* deletion and thus no FHR-3 is present in their blood plasma. Indeed, with our ELISA, we did not detect any FHR-3 signal in individuals homozygous for the deletion, and found a clear gene-dosage effect between the number of *CFHR3* copies present and the serum concentration of FHR-3. Healthy individuals with one copy of *CFHR3* have approximately half of the FHR-3 found in healthy individuals with two *CFHR3* copies. This demonstrates that it is important to take into account the copy number variation in *CFHR3* of an individual when assessing FHR-3 levels. The frequency of the *CFHR3* deletion is highly variable among ethnic groups as well as in patients, for instance aHUS, in which the homozygous deletion is more frequent[53–55,60] or age-related macular degeneration (AMD), in which the frequency of the deletion is decreased[52,54,56,61]. Thus, simply measuring and comparing average FHR-3 levels between patients and healthy individuals

without stratifying for *CFHR3* gene copies biases the results obtained.

In **Chapter 5** we describe thirteen novel monoclonal antibodies raised against recombinant FHR-4A. With the use of recombinant FHR-4A fragments, we were able to characterize the antibodies and map the binding site to domains within FHR-4A. Obtaining specific anti-FHR-4A antibodies is challenging, as *CFHR4* is described to express a splice variant: FHR-4B[62,63]. The smaller FHR-4B comprises domains 1 and 6-9 of FHR-4A and is thus completely identical to that part of FHR-4A. The unique four domains in FHR-4A (domains 2 to 5) have originated from an internal gene duplication, followed by additional mutations. This results in almost complete sequence identity (>85%) with the other domains of FHR-4A and thus also FHR-4B. Thus, in order to obtain anti-FHR-4A specific antibodies, antibodies require to recognize an epitope within domains 2 to 5 of FHR-4A which is not present in its other domains, nor in FHR-4B. Of the thirteen anti-FHR-4A antibodies we obtained, five antibodies bind to such an epitope. Characterization of these antibodies revealed that four of them all bind to the same, or overlapping epitope, while the other one binds to an unique epitope in FHR-4A domain 5. This antibody was used to develop a FHR-4A specific ELISA. The normal levels established with this FHR-4A-specific ELISA were within the range of the levels found for FHR-3, finding an average of approximately 33 nM. This is a 60-fold difference with FH levels (**Fig. 2**). One previous report used an ELISA for the measurement of FHR-4A and FHR-4B together, finding ten-fold higher concentrations as compared to our results[64]. It remains unclear what causes this difference. However, our results are supported by a recent attempt using mass spectrometry to quantify FHR proteins in serum, finding average concentration of approximately 28 nM[65]. We could not detect FHR-4B in human serum with any of the other, cross-reactive anti-FHR-4A antibodies. All were able to bind and precipitate FHR-4A and other FHR proteins to which they were found to be cross-reactive. As all did bind to unique epitopes in recombinant FHR-4B, it seems unlikely that the lack of detection of serum-derived FHR-4B is caused by shielding of the epitopes. In addition, we could not find any indication that FHR-4B is bound to another protein in serum. Rather it seems FHR-4B levels are extremely low, or FHR-4B is even absent in human circulation.

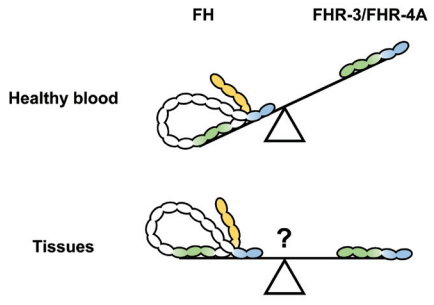

Figure 2 Balance between the relative levels of factor H (FH) and FH-related protein 3 (FHR-3) and FHR-4A as described in this thesis. In healthy blood, FH is approximately 140-fold and 60-fold more abundant compared to FHR-3 (**Chapter 4**) and FHR-4A (**Chapter 5**), respectively. It is unknown what the balance between FH and FHR-3 or FHR-4A is within other body fluids or tissues where FHR-3 and FHR-4A (or other FHR proteins) might play a bigger role within the complement system.

FHRs as antagonists of FH

Having established that both FHR-3 and FHR-4A circulate at 14 nM and 33 nM, respectively, provided valuable insight in the proposed hypothesis of FHR proteins as complement deregulators through competition with FH. FH average concentrations have been well established to be approximately 2 μM[66–69]. Thus, FH molecules in the circulation outnumber FHR-3 and FHR-4A molecules approximately 144 and 67 times, respectively. This challenges the hypothesis of FHR proteins acting as antagonists of FH, as it seems unlikely that, with such a ratio, relevant competition could take place.

The levels of FHR-1, FHR-2 and FHR-5 have also recently been established[70,71]. These FHR proteins circulate as dimers, which are formed via a dimerization motif located in the first two domains of FHR-1, FHR-2 and FHR-5[72]. Although all possible dimer combination between these FHR proteins have been predicted *in vitro*, only FHR-1 homodimers, FHR-2 homodimers, FHR-5 homodimers and FHR-1/FHR-2 heterodimers have been confirmed in human plasma, and no heterodimers containing FHR-5 were found. This is likely explained by small differences in the dimerization motif in FHR-5 compared to FHR-1/-2[72]. Total FHR-1, -2 and -5 levels are 367 nM, 99 nM and 23 nM, respectively[70]. Thus, the total FHR protein content in circulation, including FHR-3 and FHR-4A, is 536 nM on average. As a whole, FHR proteins are only outnumbered roughly six times by FH and FHL-1 (together ~3 μM[73]) and systemic regulation of FH by FHRs seems feasible. However, FHR protein levels with regard to possible competition with FH must be considered individually, as the FHR proteins exhibit different binding properties to various ligands and surfaces. All FHR proteins bind, like FH, to C3b and polyanionic residues, albeit with different affinities. FHR-1, FHR-2 and FHR-3 also bind C3d, whereas FHR-5 binds to both iC3b and C3d[64,74–78]. Furthermore, FHR-1, FHR-3 and FHR-5 bind to heparin, a substitute for polyanionic residues present on human surfaces[77,79–84]. FHR-2 also binds heparin, but only at low, non-physiological, ionic strength[70]. This also affects the binding of FHR-1/FHR-2 heterodimers, having a lower binding affinity for heparin compared to FHR-1 homodimers. FHR-4A does not exhibit any affinity towards heparin[85,86]. Binding of FHR proteins to extracellular matrix, necrotic cells, oxidation-related neoepitopes and pentraxins has also been reported[75,81,84,87–90]. FH, FHR-1 and FHR-5 all interact with monomeric C-reactive protein (CRP), whereas FHR-4A binds to the pentameric CRP[87,88]. The difference in ligand specificity suggests that each of the FHR proteins has specialized to act as FH antagonist during certain conditions or at specific sites. Thus, FHRs are unlikely to function as a group, but rather act individually.

Location determines competition?

While in circulation the relevance of FHR proteins as antagonist of FH is challenged by their apparent low concentration, it remains a possibility that the FHR proteins do act as antagonist

within tissues. It is conceivable that within tissues, the ratio between FH and FHR proteins is different as compared to plasma, favoring FHR proteins and thus allowing competition to take place and determine local complement activation. FHR proteins have been identified at specific sites of the human body. For instance, middle ear effusions contain FHR-3[86]. Others have shown that FHR-4A is present in necrotic tissues[87]. It is unknown if other FHR proteins are also present at these sites. Another site in the human body that has gained increasing interest for FHR biology is the eye. The *CFHR3-CFHR1* deletion has been shown to decrease the risk for developing AMD[52,54,56,61]. In the eye the Bruch's membrane acts as a barrier, through which proteins need to diffuse in order to reach the inner cell layers. FH was found to be unable to pass the Bruch's membrane, while its smaller splice variant, FHL-1, could[91]. It is hypothesized that (some) FHR proteins, as they are of comparable size as FHL-1, might also pass the Bruch's membrane. However, dimerization of FHRs (1, 2 and 5) might prevent them from passing through such biological filters due to their increase in size. FHR-3 has been identified in cells close to the Bruch's membrane[51]. This could greatly affect the ratio's between the complement inhibitors, FH and FHL-1, versus their antagonists, the FHR proteins. This might also explain why the deletion of *CFHR3* and *CFHR1* is associated with a decreased risk for AMD. Lack of FHR-3 and/or FHR-1 would favor increased complement regulation in the eye, resulting in lower complement activation and decreased inflammation and consequently decreased risk for AMD. However, the presence of FHR proteins and the levels of FH, FHL-1, and FHR proteins within the eye have not yet been established. Extravascular levels of FHRs may be defined by local production within the tissue, inflammation (vascular permeability), molecular size and extent of dimerization. The hypothesis that FHR proteins might act locally, within tissues, as antagonists of FH and/or FHL-1, might also provide an explanation why humans possess five FHR proteins. Possibly, each FHR protein has specificity for certain tissues or surfaces within the body, and thus acts as an FH antagonist at only these sites (as suggested for instance for FHR-3 in the eye). At these local sites, FHR proteins might contribute in maintaining tissue homeostasis by facilitating the complement-mediated removal of death cells or debris through competition with FH. With the development of the first FHR-specific ELISA's and well-characterized antibodies, some of which described in this thesis, addressing these questions has now become possible.

The FH and FHR balance in disease

In **Chapter 6** we describe the levels of FHR-3 in 230 aHUS patients from the Spanish aHUS registry and 49 healthy controls. The frequency of the homozygous deletion of *CFHR3-CFHR1* is increased in aHUS patients[53–55,60]. This was confirmed in our study in which homozygous deletion of *CFHR3* was found in 22 patients (9.6%) and two controls (4.1%). This suggests that the absence of FHR-3 is a risk factor for aHUS. Instead, we show that an increase in

FHR-3 levels is associated with increased risk for developing aHUS. The association of the homozygous *CFHR3-CFHR1* deletion is explained by the strong association with the presence of autoantibodies against FH, due to the absence of FHR-1[54,55,83,92,93]. The increased FHR-3 levels were found to be associated with a risk allele for the development of aHUS, *CFHR3*B*[60]. FHR-3*B differs at one amino acid in domain 3 from FHR-3*A (p.P241S) although functional consequences are as yet unknown. Having linked increased FHR-3 levels to *CFHR3*B* and thus risk for developing aHUS, may suggest that FHR-3 has a pathological role in aHUS. However, this could also be an epiphenomenon as the risk allele *CFHR3*B* is in strong linkage disequilibrium with FH haplotype *CFH(H3)*, which was shown to be associated with decreased FH levels[60]. Decreased FH levels could also explain why increased complement activation occurs in aHUS, but seem unlikely to be the only factor considering the decreased levels were still within the normal range. More likely, in line with the proposed antagonistic function of FHR-3, increased expression of FHR-3 on one side together with decreased FH levels on the other side, shifts the balance between FHR-3 and FH and consequently the balance in complement regulation. Possibly, the relative higher ratio of FHR-3 versus FH allows more competition of FHR-3 with FH on human surfaces, including the glomerular base membrane, thus leading to decreased complement inhibition which may be critical in the kidney. While it is unknown whether increased FHR-3, or decreased FH, or a combination of both, is the cause for the development of aHUS, it is evident that disturbed complement inhibition on human surfaces contributes to the disease. It is unclear whether the increased FHR-3 levels originate from increased expression in the liver, the main production site for all FH family members, or whether local production (possibly in the kidney) contributes. It is unknown if FHR-3 is produced locally in the kidney. Thus far, local FHR-3 production has only been identified in microglia and macrophages in retinal tissues[51].

In **Chapter 7** we describe the levels of FH and FHR-3 in former meningococcal disease (MD) patients. MD is a severe, life threatening disease affecting mainly young children and is caused by *N. meningitidis*. Upon infection, *N. meningitidis* is known to recruit FH by fHbp (discussed above) in order to evade complement-mediated destruction (e.g. opsonophagocytosis and lysis)[36]. In 2010 a genome-wide association study elucidated a possible link between MD and FHR-3, identifying a genetic association between SNPs in *CFH* and *CFHR3*, and MD susceptibility[49]. Moreover, FHR-3 domains 1 and 2 have high sequence similarity (>85%) with FH CCP 6 and 7, respectively, which are the domains in FH bound by meningococcal fHbp[38]. Together, this led to a model hypothesizing binding of FHR-3 to fHbp, preventing binding of FH. As FHR-3 does not have complement inhibitory activity, binding of FHR-3 would result in normal complement activation and subsequent clearance of the bacteria. Indeed, binding of recombinant FHR-3 to fHbp was confirmed by others[94]. More importantly, addition of recombinant FHR-3 to a complement-dependent bacterial survival assay increased bacterial killing by approximately 50%. However, a 10-fold molar excess of FHR-3 over FH was

required to achieve this. The physiological ratio is closer to a 150-fold molar excess of FH over FHR-3 instead. The supra-physiological concentration of FHR-3 required to decrease *N. meningitidis* survival may partly be explained by differences in binding affinity of FH and FHR-3 for fHbp[94]. While fHbp variant 2 and 3 have overall equal affinity for FH and FHR-3, the more commonly expressed fHbp variant 1 has up to a 33-fold higher affinity for FH over FHR-3[94,95]. In addition, until recently, it remained unclear why the *CFHR3-CFHR1* deletion did not strongly associate with MD, considering the lack of the hypothesized protective FHR-3.

In summary, it seems highly unlikely that FHR-3 truly rules disease susceptibility through binding to fHbp, and other mechanisms are underlying the association of *CFHR3* with MD. Nonetheless, we could not rule out that FHR-3 levels, as a result of MD-associated SNPs, would be greatly increased in MD patients and thus would affect MD susceptibility. Of note, the SNPs in *CFH* and *CFHR3* associating with MD susceptibility were previously identified during a genome-wide association study which did not include all SNPs in the *CFH* locus.

To identify the causal genetic variation in the *CFH* locus that affects MD susceptibility, we deep-sequenced a selection of Caucasian MD patients and healthy controls and validated this in a larger cohort of MD patients and healthy controls. To elucidate the mechanism, we measured FH and FHR-3 levels in 367 genotyped patients and 124 healthy controls. The previously identified associating SNP, rs426736, was found to be in high linkage disequilibrium with another SNP in intron 1 of *CFHR3*, rs75703017, which was found to be the most significantly associated with MD susceptibility. Overall, FHR-3 levels were not increased in MD patients, while FH levels were higher than control levels. Rather unexpectedly, rs75703017 correlated strongly with FH levels, suggesting a direct genetic control of *CFHR3* over *CFH* expression levels. Using CRISP/Cas9, we confirmed the presence of a cis-regulatory element in intron 1 of *CFHR3* that controls *CFH* expression. This intron is lost as part of the common *CFHR3-CFHR1* deletion, and consequently the deletion also associates with increased expression of FH. This association has been found by others, but thus far a mechanism remained unknown[66,96]. The control of *CFH* expression by *CFHR3* also explains the poor penetrance of the *CFHR3-CFHR1* deletion in MD. No increased frequency of the *CFHR3-CFHR1* deletion was found in MD cohorts, contradictory to the competition hypothesis between FHR-3 and FH. If FHR-3 would indeed compete with FH for binding to fHbp and affect survival of *N. meningitidis* upon infection, lack of FHR-3 due to the deletion would favor binding of FH to fHbp, promoting *N. meningitidis* survival and infection, and consequently put individuals without FHR-3 at a higher risk for MD. Instead, the poor penetrance of the *CFHR3-CFHR1* deletion in MD is an indication that FHR-3 is not a major player in MD. The deletion of *CFHR3* does result in increased expression of *CFH* due to the loss of the regulatory element in intron 1. This is, however, only one of the possibilities to increase *CFH* expression and thus

the *CFHR3-CFHR1* deletion itself does not associate with MD. When correcting for the SNP in *CFHR3* that also affects *CFH* expression, the *CFHR3-CFHR1* deletion does associate with MD susceptibility. This is indirect evidence that a loss of FHR-3 is not detrimental for the risk for MD, but that FH, and not FHR-3, rules MD susceptibility.

In **Chapter 7** we measured FH and FHR-3 levels after MD patients had recovered, with a median time span of two months after disease onset. Furthermore, only risk for MD was investigated. If there would indeed be competition between FH and FHR-3 for binding to the meningococci, this would occur immediately and might affect severity of the disease during the acute stage of infection. In order to investigate this, we measured FH and FHR-3 during the first days of admission to the hospital in 90 patients, as described in **Chapter 8**. As expected, FH and FHR-3 levels were significantly decreased during the first day of infection. The decreases in FH and FHR-3 correlated well with each other, although FHR-3 levels recovered more quickly compared to FH. There are various possible underlying causes that could have resulted in the lower FH and FHR-3 levels. The decrease of both FH and FHR-3 could originate from consumption by the meningococci, overall consumption due to (systemic) complement activation, erroneous measurements due to the presence of fHbp and/or bacteria, aberrant production or loss due to increased vascular permeability, or any combination of these. Nonetheless, it is remarkable that low FH levels at the acute stage were found to be more frequently associated with adverse values of several severity parameters, including CRP levels, platelet count, white cell count and both the pediatric risk of mortality score (PRISM) and the Glasgow meningococcal septicemia prognostic score (GMSPS), whereas low FHR-3 levels only associated with a few of these parameters. This supported our previous results described in **Chapter 7**, that FH instead of FHR-3 is most relevant in MD. Our study only included surviving patients. As such, it is unknown if FH (and/or FHR-3) levels would be even further decreased during the acute stage in non-surviving patients, although considering the results in **Chapter 8** this may be expected. The functional consequences of low FH or low FHR-3 in these patients is unknown. Lower FH levels would likely cause increased C3 activation and decreased protection of human cells against complement.

It has to be noted that we only studied FH and FHR-3 in blood serum samples of MD patients. It is unknown what the levels of FH and FHR-3 are in for instance cerebral spinal fluid (CSF) and how local concentrations are affected by the genetic variations. Furthermore, *N. meningitidis* resides in the nasopharynx. It is thus conceivable that FHR-3 plays a role there, locally, and aids in preventing *N. meningitidis* entering the body. This would be in line with the previously discussed hypothesis that FHR proteins might be more relevant with regard to the complement system at local sites and specific tissues. However, this assumes that complement evasion by *N. meningitidis* in the nasopharynx plays an important role. There is one indication *N. meningitidis* does not need FH to survive in the nasopharynx. The expression of fHbp

is temperature dependent, due to the presence of a RNA thermosensor in its 5'-UTR[97,98]. These secondary RNA structures control translation of mRNA at specific temperatures by sequestering the ribosome binding site and allow translation of mRNA and expression of protein upon temperature shifts[99]. The RNA thermometer of fHbp allows expression at 37°C, while it is low at 30°C. It seems likely *N. meningitidis* would only invest in expression of fHbp when needed, thus when it is being exposed to the complement system upon entering the blood (37°C). As fHbp expression is expected to be low in the relatively colder nasopharynx (~30°C), this suggests that complement evasion in the nasopharynx is not required for the survival of *N. meningitidis*. Consequently, it is unlikely FH and FHR-3 are involved in the survival of *N. meningitidis* in the nasopharynx via binding to fHbp.

In our studies, we focused on *N. meningitidis* and MD. MD is a severe disease with rapid onset, and despite successful vaccines, *N. meningitidis* remains a serious threat, especially considering the increasing incidence of the hypervirulent *N. meningitidis* serogroup W in Western countries, including The Netherlands[100,101]. Furthermore, it is a relatively well-characterized pathogen of which the role of complement in its survival, and the evasion strategies it uses, have been well-studied. However, *N. meningitidis* is not unique in its strategy to evade complement by hijacking one of the systems most pivotal regulators. Many pathogenic bacteria express specialized proteins or have other strategies to bind FH (see reviews [102–105]). Depending on the FH domains bound by these bacteria, some of these proteins are also prone to bind FHR proteins. Binding of FHR proteins has been reported for *Neisseria gonorrhoeae*[106], *Staphylococcus aureus*[107], several *Borrelia* species[108–110], *Leptospira interrogans*[111], *Pseudomonas aeruginosa*[112] and *Fusobacterium necrophorum*[113], although functional consequences have not been unequivocally identified. While we could not confirm that FHR-3 truly competes with FH for binding to meningococcal fHbp, we cannot rule out that competition between FH and FHR proteins on other bacteria might affect susceptibility and severity. Furthermore, as the *CFHR3* SNP rs75703017 affects FH levels, this might be a common risk factor for infections by bacteria that exploit FH to evade the human complement system. In general, higher FH levels will allow FH-binding bacteria to better protect themselves. A study is currently ongoing to investigate if increased FH levels ,as a result of the SNP in *CFHR3,* also associate with other infectious diseases.

Concluding remarks and outlook

In summary, we describe novel antibodies against FH and FHR proteins and used these to further characterize the FH protein family. We established normal values for FHR-3 and FHR-4A under healthy conditions, demonstrating relatively low levels of either one compared to FH. Under healthy conditions, the FH:FHR ratio favors complement regulation

on healthy host surfaces by FH, but this balance can shift during disease as demonstrated with the increased FHR-3 levels found in aHUS patients (**Fig. 3**). Future research is required to elucidate the (patho)physiological consequences of this finding. In addition, the balance of FH and FHRs proteins in local tissues and other body fluids, such a CSF, should now be investigated to elucidate whether the FH:FHR ratio differs locally, and whether FHR proteins play a bigger role in the complement system within the human tissues.

Furthermore, we showed FH function can be enhanced using an anti-FH antibody, highlighting the complexity of the FH molecule and its biology. This antibody opens a new therapeutic possibility, in which an endogenous inhibitor is used to control or correct pathological complement activation strictly on human surfaces (**Fig. 3**). This has a major advantage over current strategies, which focus on blocking entire pathways of the complement system, as it will leave the beneficial side of the complement system, attacking and eliminating pathogens, intact. Determining the precise epitope of the antibody, in combination with information on the structures of FH, in complex with the antibody, will help to elucidate and possibly optimize the mechanism underlying FH potentiation.

Finally, FH remains the most physiologically relevant FH family member in context of MD.

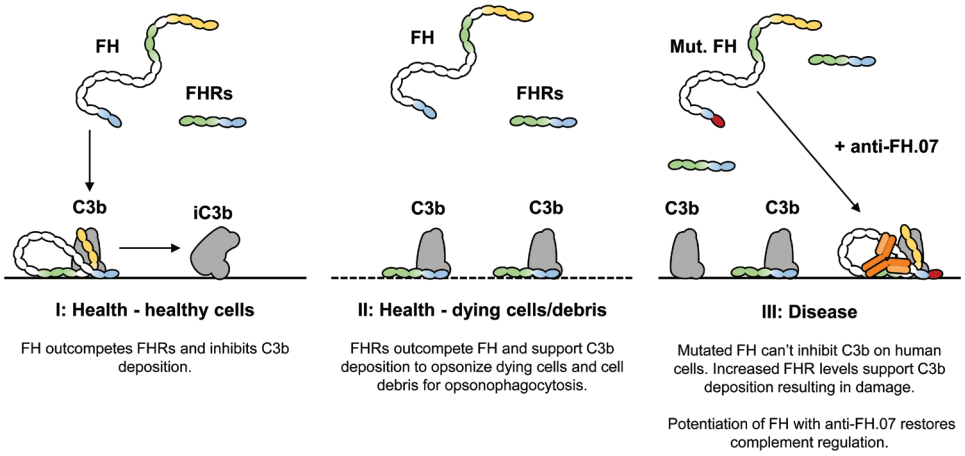

Figure 3 Proposed model of the interplay between FH and FHR proteins within the complement system. During health, FH protects healthy human cells from complement mediated damage, while FHRs compete with FH on dying cells and cell debris to support complement activation and complement-mediated clearance (**panel I** and **II**, respectively). During disease (like aHUS), mutations in FH may limit its ability to bind to cells, resulting in increased C3b deposition (**panel III**). Alternatively, increased FHR levels may support C3b deposition by increased competition with FH and contribute to or cause disease. Potentiation of FH with anti-FH.07 may restore complement regulation and resolve the disease.

Its expression is influenced by intergenic interactions between *CFH* and *CFHR3*, explaining the association of *CFHR3* with MD. It is possible this mechanism is relevant for multiple other infectious diseases, as many pathogens exploit human FH to avoid complement-mediated destruction upon entering the human circulation. FH, and not FHR-3, plays a major role in MD, affecting both the susceptibility and severity of MD. These results help to understand why some individuals are more at risk for disease than others, due to changes in the balance between activation and regulation within the complement system.

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