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Genetic, molecular and functional analyses of factor I

an inhibitor of the complement system

Sara Nilsson

Doctoral Thesis



LUND UNIVERSITY

Faculty of Medicine

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Title and subtitle Genetic, molecular and functional analyses of factor I - an inhibitor of the complement system		
<p>Abstract</p> <p>Factor I (FI) is a serine protease that inhibits the complement system by cleaving activated C3 and C4 complement proteins, in the presence of cofactors. Mutations in the gene coding for FI have been identified in complete FI deficient- and atypical hemolytic uremic syndrome (aHUS) patients.</p> <p>The mutations were introduced in recombinantly expressed proteins, purified and analyzed in functional assays. The mutations are homozygous or compound heterozygous in patients with complete FI deficiency and the mutations mostly affect the folding of the FI protein, thereby hindering secretion. In contrast, the mutations in aHUS patients are heterozygous. One of the mutations, G243D, identified in three unrelated aHUS patients did not have any effect on the expression, secretion or function of FI. It was shown later that these three patients have additional mutations, deletions or autoantibodies against other complement proteins. In the other two studies we detected five mutants that showed impaired function for degrading C3b present on cell surfaces. Only two of these five mutants also had reduced activity in the cleavage of C3b and C4b in the fluid-phase. The rest of the mutations, with pre-mature stop codons and some missense mutations, resulted in no secretion of the protein.</p> <p>So far, no crystal structure of FI is available and therefore we have predicted the structures of the individual domains of the heavy and light chains of FI using homology-based modeling. Based on these models, putative binding patches have been analyzed experimentally regarding their involvement in binding and cleavage of C3b. We found, in addition to the C-terminal serine protease domain, that all domains of the heavy chain are involved, especially the FIMAC and LDLr2 domains, but also the CD5 and LDLr1 domains. These results demonstrate the complexity of the FI protein; all domains contribute to its function.</p> <p>Overall our data indicate that the mutations found in patients suffering from a complete FI deficiency and aHUS are located in all the domains of FI. The main difference between these two patient groups is that the mutations in complete FI deficient patients lead to no secretion of FI, which results in C3 depletion. In contrast, aHUS patients that still have FI, even at reduced concentrations, or have FI with impaired function, but no C3 depletion. This difference could result in the different clinical presentation observed in these patients. Complete FI deficient patients mainly suffer from recurrent bacterial infections due to lack of C3b opsonization, whereas aHUS patients have renal failure because of impaired regulation of complement in their kidneys.</p>		
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Genetic, molecular and functional analyses of factor I

an inhibitor of the complement system

Sara Nilsson

Doctoral Thesis



LUND UNIVERSITY

Faculty of Medicine

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Department of Laboratory Medicine, Malmö
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Lund University

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*Till mamma, pappa,
Robert och vår ofödda son*

*“Det är vad vi tror att vi redan vet
som hindrar oss från att lära oss nytt”*

-Claude Bernard-

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List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

- I. **Nilsson SC**, Trouw LA, Renault N, Miteva M, Genel F, Zelazko M, Marquart H, Muller K, Sjöholm AG, Truedsson L, Villoutreix BO and Blom AM. Genetic, molecular and functional analyses of complement FI deficiency. *Eur. J. Immunol.* 2009. 39:310-323.
- II. **Nilsson SC***, Karpman D*, Vaziri-Sani F, Kristoffersson A-C, Salomon R, Provot F, Fremeaux-Bacchi V, Trouw LA, and Blom AM. A mutation in factor I that is associated with atypical hemolytic uremic syndrome does not affect the function of factor I in complement regulation. *Mol Immunol.* 2007. Mar;44(8):1835-1844.
* These authors contributed equally.
- III. **Nilsson SC**, Trouw LA, Fremeaux-Bacchi V, Villoutreix BO, and Blom AM. Mutations in complement factor I as found in atypical hemolytic uremic syndrome lead either to altered secretion or altered function of factor I. *Submitted to Eur. J. Immunol*
- IV. Bienaime F, Dragon-Durey M-A, Régnier CH, **Nilsson SC**, Kwan WH, Blouin J, Jablonski M, Renault N, Rameix-Welti M-A, Loirat C, Sautés-Fridman C, Villoutreix BO, Blom AM, and Frémeaux-Bacchi V. Factor I associated atypical hemolytic uremic syndrome: Complement genetic background influences the disease outcome. *Submitted to Kidney International*
- V. **Nilsson SC**, Månsson L, Groeneveld TW, Trouw LA, Villoutreix BO, and Blom AM. Analysis of binding sites on complement factor I that are involved in complement inhibition. *Manuscript*

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Abbreviations

ANA	Anaphylatoxin domain
AMD	Age-related macular degeneration
aHUS	Atypical hemolytic uremic syndrome
BCR	B-cell receptor
C3Nef	C3 nephritic factor
CCP	Complement control protein (domain)
C4BP	C4b-binding protein
CR	Complement receptor
CRP	C-reactive protein
CUB	Uegf and Bmp1 domains
DAF	Decay-accelerating factor
DFP	Diisopropylfluorophosphate
ESRD	End-stage renal disease
FB	Factor B
FD	Factor D
FH	Factor H
FHL-1	Factor H like protein 1
FHR	Factor H related protein
FI	Factor I
FIMAC	Factor I membrane attack complex (domain)
GBM	Glomerular basement membrane
GPI	Glycosylphosphatidylinositol
HUS	Hemolytic uremic syndrome
HUVEC	Human umbilical vein endothelial cells
IC3b	Inactivated C3b
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
LDLr	Low-density lipoprotein
LHR	Long homologous repeat
LNK	Linker domain
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MCP	Membrane cofactor protein
MG	Macroglobulin domain
MPGN	Membranoproliferative glomerulonephritis
PNH	Paroxysmal nocturnal hemoglobinuria
PS	Protein S
RCA	Regulators of complement activation
SCR	Short consensus repeat
SLE	Systemic lupus erythematosus
SP	Serine protease
STP	Region rich in serines, threonines and prolines
TED	Thioester containing domain

Introduction

The immune system can be divided into the innate and acquired immune systems. We are born with the innate immune system and it is our first line of defense against pathogens. The first barriers that pathogens must breach to be able to invade the body are the skin, mucosa, saliva and tears. If the pathogen was ingested, the next step of protection is the low pH of the stomach. There are also enzymes in the mucosa that can digest the bacteria. Additionally, some bacteria cannot grow at our body temperature, especially when the temperature is raised during fever. The complement system, which is an important part of the innate immune system, can recognize bacteria and enhance their phagocytosis. When the complement system gets activated it produces chemoattractant factors that recruit inflammatory cells to the site of infection, which can further eliminate pathogens. The last step of complement activation is direct killing of the bacteria by lysis. The complement system can also promote clearance of apoptotic cells and immune complexes. The innate immune system also activates the acquired immune system through presentation of foreign antigens. Although the acquired immune system responds slowly to invading pathogens, it has a life long immunological memory. It can discriminate between self and non-self antigens during antigen presentation thereby inducing antibody production to non-self antigens. These antibodies neutralize the pathogen, which is then eliminated. Specialized cells of the acquired immune system can also direct kill pathogens.

The focus of this thesis is the complement inhibitor factor I, which is a serine protease that degrades activated complement proteins. We have studied mutations in the gene coding for FI in two patient groups: those who show a complete FI deficiency and those with atypical hemolytic uremic syndrome (aHUS). The consequences of the mutations are analyzed and discussed. Structures of the individual domains of FI have been predicted by homology-based modeling and the involvement of the heavy chain of FI in substrate binding and cleavage is analyzed.

Complement system

In the end of the nineteenth century, complement was described as a heat-labile factor that "complemented" the bacteriolytic activity of antibodies. The complement system is composed of over 40 plasma and membrane bound proteins and when activated it is involved in many different events e.g. clearance of apoptotic cells, foreign cells and molecules, killing pathogenic microorganisms, inducing inflammatory responses and enhancing the response of the adaptive immune system. The complement system is involved in the pathogenesis of many diseases eg. arthritis, auto-immune diseases such as systemic lupus erythematosus (SLE), Alzheimer's disease and renal diseases. In order to participate in all these events, the complement system is activated through three different pathways, the classical, the lectin and the alternative pathways [1], Fig 1.

Classical pathway

The classical pathway is initiated by the recognition of immune complexes on target surfaces by C1q, which is in complex with two molecules each of C1r and C1s (this complex is named C1). The association of the C1 complex is dependent on Ca^{2+} ions. The classical pathway can also be triggered by aggregates of immunoglobulin G (IgG) or IgM, C-reactive protein (CRP), certain bacteria and viruses and apoptotic cells. The binding of C1q to its targets leads to a conformational change in C1q, which activates the serine protease C1r that cleaves C1s. Activated C1s cleaves C4 into the larger C4b and smaller C4a fragments. The internal thioester buried in C4 is exposed in the C4b molecule, which enables covalent binding of C4b to target surfaces, initiating the cascade. C2 then binds to C4b and is cleaved by activated C1s to generate C2a. The C4b2a complex constitutes the classical C3 convertase and C2b is released into solution.

Lectin pathway

The lectin pathway is initiated when mannan-binding lectin (MBL) or ficolins bind to carbohydrate moieties on bacterial surfaces. This binding promotes the activation of MBL-associated serine proteases (MASPs), which leads to the subsequent cleavage of C4 and C2 and the generation of the classical C3 convertases.

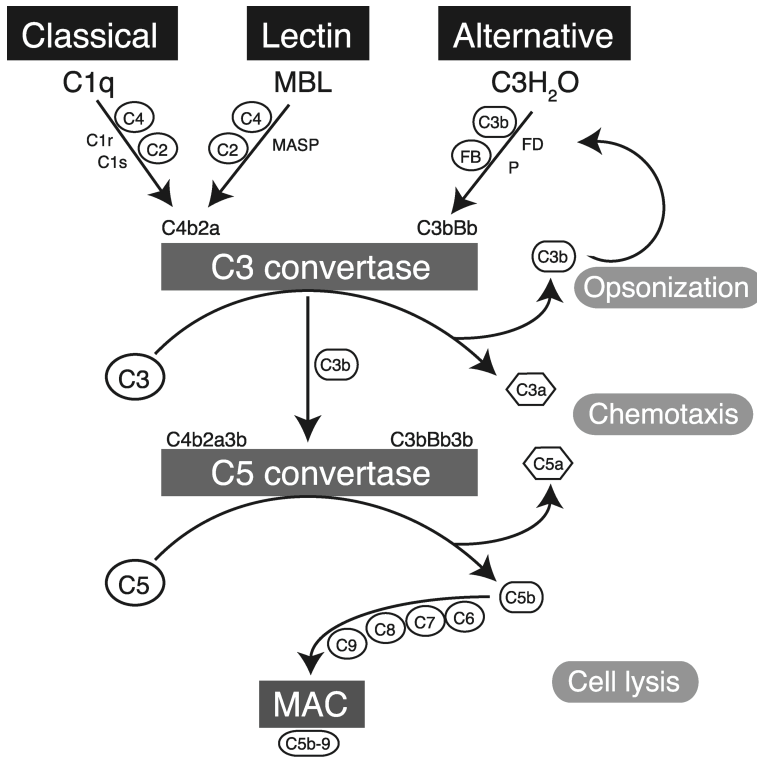


Figure 1. Overview of the complement system.

Alternative pathway

The alternative pathway is initiated by spontaneous hydrolysis of an internal thioester bond in C3, which generates C3(H₂O). This molecule resembles C3b and can bind factor B (FB), which is cleaved by the serine protease factor D (FD) to generate a variant of the alternative C3 convertase, C3(H₂O)Bb and Ba. This C3 convertase can cleave C3 molecules into C3b that contain the reactive thioester bond, which becomes covalent attached to nearby surfaces. The other C3 cleavage product is the anaphylatoxin C3a. The thioester bond in C3b can also be hydrolysed by water and is thereby inactivated. When formed, C3b molecules are able to bind to FB, resulting in the assembly of the C3 (C3bBb) convertase. This part of the alternative pathway constitutes the amplification loop to the classical and lectin pathway that also generate C3b. The alternative pathway can be also initiated by C3b binding to surfaces of bacteria and viruses, which also leads to formation of the C3 convertases. The alternative C3 convertase, which is very unstable, is stabilized by properdin. It has been reported that properdin itself can initiate the alternative pathway [2]. The C3b molecule bound to cell surfaces (eg. pathogens and damaged cells) can act as an opsonin and thereby enhance phagocytosis of the marked cells.

Terminal pathway

When an additional C3b molecule binds to the classical (C4b2a) or the alternative (C3bBb) C3 convertase, the C5 convertase (C4b2aC3b, C3bBbC3b) is formed on the surface of cells. This C5 convertase cleaves C5 into the smaller C5a and the larger C5b fragments. C5b binds to C6 to produce a soluble, stable C5b-6 dimer. C7 then binds and the affinity of the resulting complex for hydrophobic environments increases and the binding of C5b-7 to cell surface occurs. Thereafter, a C8 molecule binds, giving C5b-8, which possesses a binding site for C9. Several C9 molecules associate to form the final C5b-9 or membrane attack complex (MAC). The insertion of the MACs in cell membranes changes the osmotic pressure, which lead to lysis of the cells.

Anaphylatoxins

The anaphylatoxin cleavage products C3a and C5a have pro-inflammatory properties when they are bound to their receptors, C3aR for C3a and C5aR and C5L2 for C5a. The C5a fragment is much more potent than the C3a fragment. C3a and C5a function as chemoattractants for neutrophils, monocytes and macrophages, they induce oxidative bursts and the release of granular enzymes in neutrophils and histamine release from mast cells. These anaphylatoxins also enhance phagocytosis, smooth muscle cell contraction, vasodilation, expression of adhesion molecules and they are involved in cytokine expression. Both C3a and C5a are converted to C3a-desArg and C5a-desArg by the enzyme carboxypeptidase-N, which cleaves off the carboxy terminal arginine of both C3a and C5a. C3a-desArg lacks anaphylatoxic properties and can no longer bind to the C3aR, whereas C5a-desArg can still bind C5aR and C5L2 has mild pro-inflammatory effects [3, 4]. It is thought that C4a also acts as an anaphylatoxin [5] but no specific receptor in humans has yet been discovered [4].

Complement proteins

C3

Complement component C3 is a glycoprotein of 183 kDa and it is synthesized as a single-chain precursor that is proteolytically processed into two polypeptide chains (α -chain of 115 kDa and β -chain of 75 kDa), which are connected via a disulfide bridge [6]. C3 is composed of several domains: macroglobulin domains (MG1-8), a linker domain (LNK), an anaphylatoxin domain (ANA), a loop in the N-terminal part of the α -chain (α' NT), complement C1r/C1s domains, Uegf and Bmp1 domains (CUB), a thioester containing domain (TED), an anchor region and the C345C domain, Fig 2.

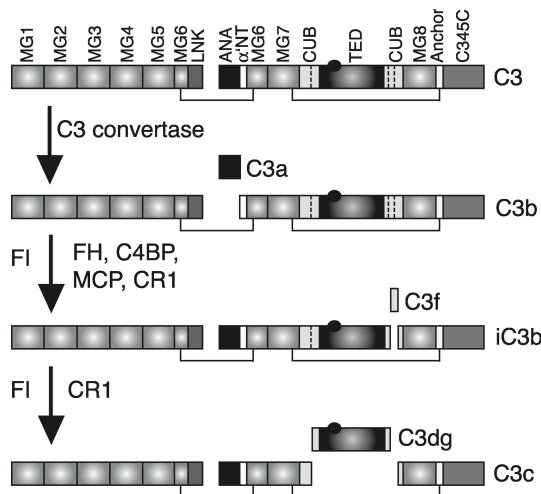


Figure 2. Schematic overview of C3 and its domains and cleavage pattern by FI.

The 3D-structure of C3 and its degradation products have been recently solved [7-9]. When C3 is cleaved by C3 convertase there is a large conformational change in the molecule. The internal thioester bond that is hidden between the TED and MG8 domains becomes exposed due to unraveling of the C3 molecule when it is cleaved by the C3 convertase to generate C3a and C3b. C3a is released whereas C3b is able to bind to nearby surfaces via the reactive thioester bond [10] in the TED domain, Fig 3. The half-life of the C3b, with the accessible thioester bond is very short, restricting the activation of the alternative pathway to activating surfaces [11]. The C3b molecule can take part in the formation of the C3 convertases together with FB, FD and properdin to continue the complement cascade. C3b can bind to immune complexes, enhancing their clearance from circulation and also bind to pathogens and

apoptotic cells, enhancing their phagocytosis or be inactivated by the serine protease factor I (FI), thus inhibiting the cascade [12].

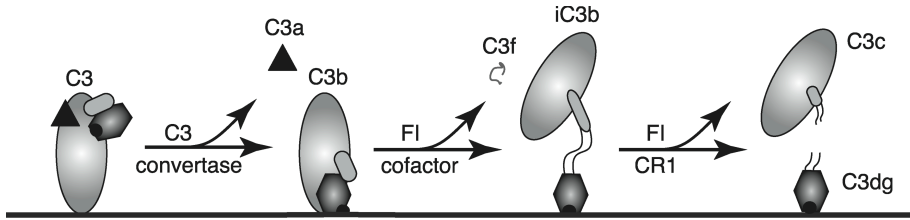


Figure 3. Structural view of the degradation of C3.

C3b has three cleavage sites (scissile bonds) in the CUB domain for FI, Fig 4 [8]. Cleavage of C3b by FI only occurs in the presence of specific cofactors; factor H (FH) [13], C4b-binding protein (C4BP) [14], membrane cofactor protein (MCP/CD46) [15] and complement receptor 1 (CR1/CD35) [16]. The inactivated C3b (iC3b) is formed and C3f is released. In iC3b the CUB domain is unraveled and elongated, which enables further degradation by FI and CR1 to form the surface-bound C3dg and the soluble C3c, Fig 2 and 3. C3dg can be further degraded by proteases to form C3d and C3g. iC3b can also act as an opsonin and enhance phagocytosis whereas the surface bound C3dg/C3d binds to receptors on B- and T-cells, which facilitates production of antibodies against the pathogens.

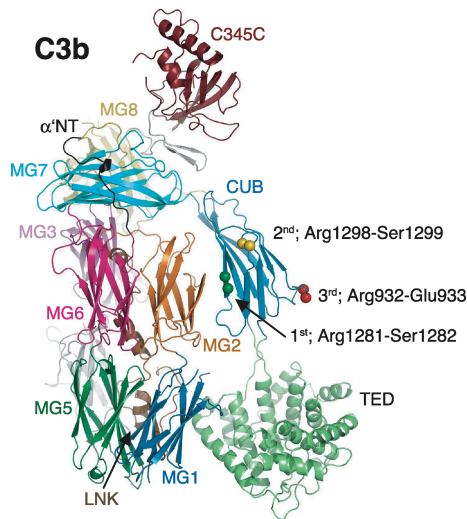


Figure 4. Cleavage sites for FI in C3b [8].

Three regions of C3b in particular have been implicated by Janssen *et al.* [8] to be involved in the binding to FH and CR1: the α' NT region and the MG6 domain for FH and CR1 and the TED domain for FH only. In the C3b structure, the α' NT and the MG6 domain lie adjacent to each other and form a continuous region that can also bind FB. This indicates that the binding sites of FB and FH/CR1 overlap and that dissociation of the alternative C3 convertase by FH and CR1 is due to steric hindrance. FH binding to the TED domain is probably involved in cofactor activity for FI. FI needs cofactors for orienting the protease domain and unraveling the CUB domain.

C4

Complement component C4 is synthesized as a single chain precursor that is proteolytically processed by cleaving off four positively charged amino acids at two sites. The mature C4 protein contains an α -chain (93 kDa), a β -chain (75 kDa) and a γ -chain (33 kDa), which are connected via disulfide bridges [17]. There are two different isoforms of C4, named C4A and C4B and they are transcribed from two different genes located close to each other. The isoforms differ from each other by four amino acids in the C4d region and this has been implicated in different binding specificity; the acidic form (C4A) binds more efficiently to amino groups (proteins) and the basic form (C4B) binds more efficiently to hydroxyl groups (carbohydrates). The C4Ab form binds more efficiently to the immune complexes than C4Bb. These differences in binding properties affect the clearance of immune complexes that contain a lot of amino groups. On the other hand, C4Bb has been proposed to have better hemolytic activity than C4Ab [18].

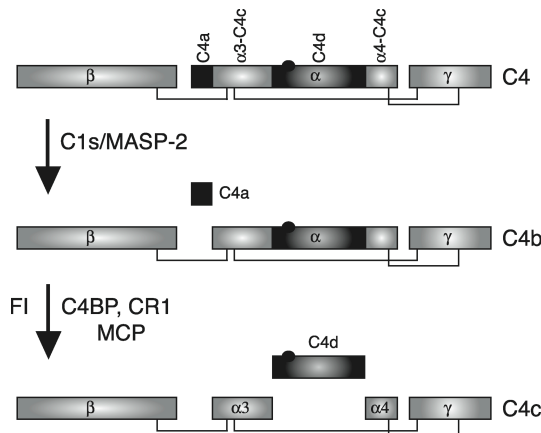


Figure 5. Cleavage pattern of C4.

C4 is cleaved by activated C1s, generating the fragments C4a and C4b. C4, like C3, contains an internal thioester bond that is hidden in the intact C4 molecule but becomes exposed in C4b, where it can covalently attach to close surfaces [10]. C4b can form part of the classical C3 convertase (C4b2a), bind to immune complexes, enhance their clearance through interaction with CR1, or become inactivated by FI. C4b is cleaved by FI only in the presence of specific cofactors such as C4BP [14], MCP [15] and CR1 [16]. Cleavage results in the formation of soluble C4c and surface bound C4d, Fig 5. Unfortunately, there is no available structure of full-length C4. Only the C4d domain has been crystallized [19]. The structure of C4d closely resembles that of C3d [9], although the surface around the thioester bond seems to be more negatively charged in C4d. In contrast, there are more positively charged or neutral residues in C3d.

Complement regulation

Since the complement system is activated constantly it has to be tightly regulated by both soluble and membrane bound regulators to protect self-tissues from complement-mediated damage. Many of these regulators are located in chromosome 1q32 and they are collectively called regulators of complement activation (RCA). The RCA proteins inhibit the complement system by inhibiting the formation, by accelerating the decay of the C3 and C5 convertases or by acting as a cofactor for FI in the degradation of C3b and C4b, Fig 6. Decay accelerating factor (DAF/CD55)

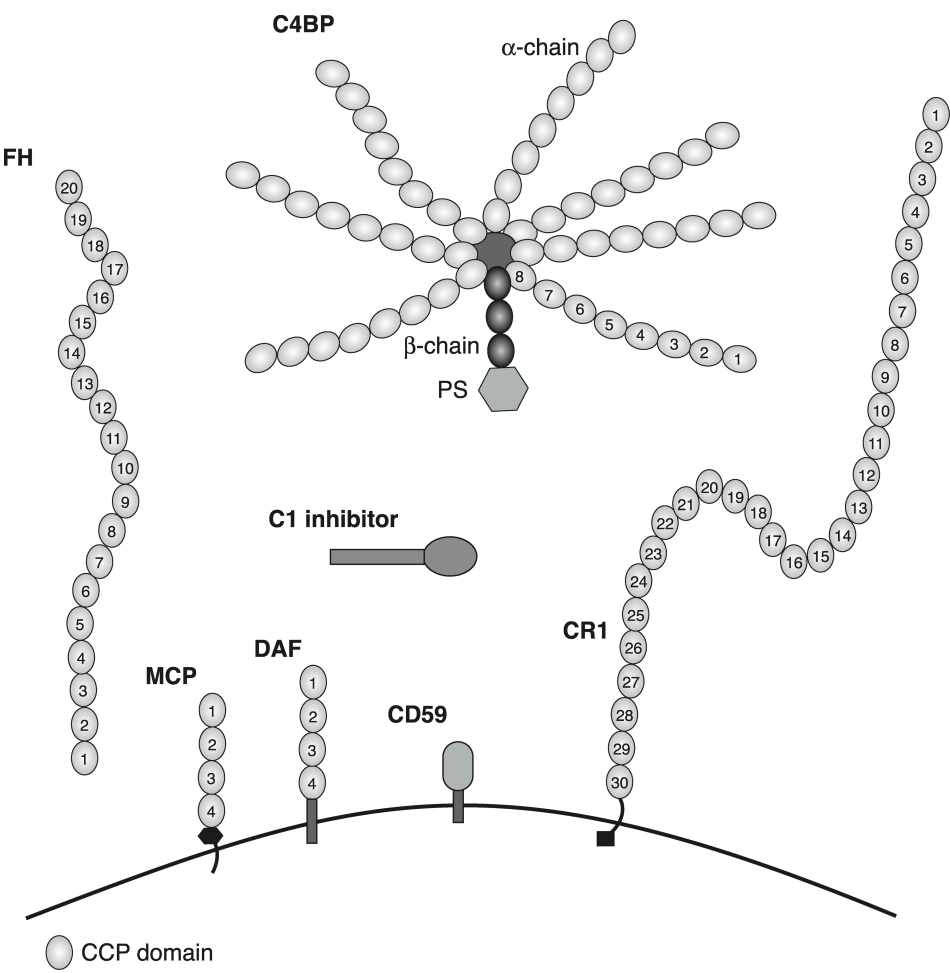


Figure 6. Schematic presentation of inhibitors of complement activation.

posses only decay activity, MCP only has cofactor activity, whereas C4BP, FH and CR1 have both decay and cofactor activities. These regulators are predominantly composed of complement control protein (CCP) domains, also known as short consensus repeats (SCR). Each CCP, containing approx. 60 amino acids, is composed of a hydrophobic core comprising mainly β -strand structure, with protruding loops that may be involved in ligand binding. The domains contains four conserved cysteine residues, of which the first and third, and the second and forth, form disulfide bridges. The sequence homology among different RCA proteins is not very high (30-40%), but the folding and the inner core show much higher identity [20].

C1 inhibitor belongs to the family of serine protease inhibitors and it inhibits the activation of the classical pathway by dissociating C1r and C1s from the C1 complex through binding mainly to C1r, Fig 6. Also MASP-2 can be inactivated by C1 inhibitor, thereby inhibiting the lectin pathway [21].

The terminal pathway can be inhibited by membrane bound protein CD59, also called protectin, Fig 6. CD59 can interact with C8 and C9, thereby inhibiting the insertion of MAC in the cell membrane. This will prevent lysis of the cell [22].

The soluble complement proteins are mainly produced in the liver but they can also be produced locally by monocytes, fibroblasts and endothelial cells. The membrane bound proteins are expressed on different cells and tissues and this is described in more detail later in the thesis.

Factor I

Factor I (FI) is an inhibitor of all three pathways of the complement system. FI is an 88 kDa glycoprotein that circulates in the blood at a rather low concentration, 35 $\mu\text{g/ml}$ [23]. As with many other complement proteins, FI is mainly synthesized in the liver by hepatocytes [24] but it is also produced by monocytes [25], fibroblasts [26], keratinocytes [27] and human umbilical vein endothelial cells (HUVEC) [28]. FI is an acute-phase protein and the production of FI is upregulated by Il-6 in hepatocytes [24] and by interferon- γ (IFN- γ) in keratinocytes [27] and HUVEC cells [28]. The regulation of synthesis of complement proteins such as FI is cell-type specific. The *CFI* gene (63 kb) is located on chromosome 4q25 [29] and consists of 13 exons with a very large intron (35 kb) between exon 1 and 2 [30]. The exon arrangement corresponds well to the domain structure of the protein, Fig 7.

FI is a multidomain protein synthesized as a single polypeptide chain, but four positively charged amino acids (RRKR) are then cleaved out to give the heavy (50 kDa) and light (38 kDa) chains [31]. These chains are covalently linked via a disulfide bridge [32]. The protein also undergoes glycosylation and each chain contains 3 *N*-linked glycosylations. Since the protein is secreted, the signal peptide is cleaved off. It has been shown that FI can be expressed recombinantly in eukaryotic or insect cells, but only partial proteolytic processing occurs. The four central residues from immature FI are removed by paired basic amino acid cleaving enzyme in only

half of the FI molecules [33]. The FI protein is made up of several unique domains some of which share sequence similarity with domains found in complement and non-complement proteins. The heavy chain consists of the FI membrane attack domain (FIMAC), a CD5-like domain (also known as the scavenger receptor cysteine-rich domain), low-density lipoprotein receptor 1 and 2 domains (LDLr1 and 2) and a region of unknown homology, Fig 7 (schematic overview of FI). The FIMAC and LDLr domains are also found in components C6 and C7. The light chain comprises the serine protease (SP) domain [34] where the catalytic triad in the active site is formed by His362, Asp411 and Ser507 [35]. FI contains 40 cysteines and 36 of them are involved in intra-domain disulfide bridges, and the remaining four cysteines (Cys15-Cys237, Cys309-Cys435) connect the FIMAC and LDLr1 domains and the SP domain with the heavy chain. FI contains 40 cysteines and 36 of them are involved in intra-domain disulfide bridges, and the remaining four cysteines (Cys15-Cys237, Cys309-Cys435) connect the FIMAC and LDLr1 domains and the SP domain with the heavy chain.

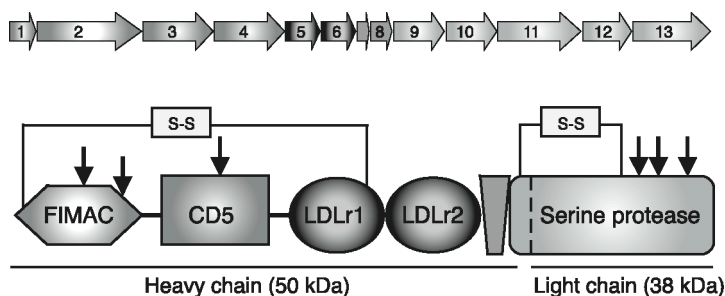


Figure 7. Schematic overview of the exons and domains of FI. The glycosylations are marked with arrows.

FI is a serine protease [36] that cleaves C3b (Fig 2-4) and C4b (Fig 5) in presence of specific cofactors such as FH [13], C4BP [14], MCP [15], CR1 [16], which will inhibit the complement system. FI can degrade C3b and C4b both in the fluid-phase and when they are deposited on the surfaces of cells.

Serine proteases require a serine residue in the active site. FI shows the most structural homology to the serine protease trypsin, which is a protease of the chymotrypsin-like family. Trypsin cleaves the scissile bond following a positively charged amino acid such as lysine or arginine. In the base of the pocket of the active site there is an aspartic acid residue that can interact with the positively charged substrate residue, which enables the enzyme to cut at the scissile bond. FI is a unique serine protease, since it circulates in its active form in the blood stream and does not have any known circulating inhibitor, as is the case for other serine proteases. In contrast, synthetic inhibitors such as suramin, have been shown to inactive FI [37].

Both electron microscopy [38], X-ray and neutron scattering [34, 39] have been used to elucidate the low-resolution 3D-structure of FI, but so far no high-resolution structure of FI has been obtained by NMR or X-ray spectroscopy. Electron

microscopy images suggest that FI is a bilobal protein. The two different lobes that could be distinguished were a crescent and a dumbbell shape, which may resemble the two different chains, the heavy and the light chain, respectively [38]. According to the molecular modeling study of Perkins *et al.* [39] FI cannot assume a linear extended arrangement of domains since the observed length of the protein is too short. These authors suggested that the domains could be arranged in a zig-zag conformation or a V-shaped structure. More recently, Chamberlain *et al.* [34] suggested that Cys15 in the FIMAC domain and Cys237 in the LDLr 1 domain form a disulfide bridge, which brings the FIMAC, CD5 and LDLr1 domains together, perhaps forming a compact triangular arrangement. Together with the SP domain, these globular entities could constitute a bilobal structure in FI as proposed in the electron microscopy study of DiScipio *et al.* [38].

The affinity of FI for C3b is very low but increases in the presence of FH. It is thought that when FH binds to C3b it induces a conformational change in the C3b molecule, which exposes a binding site for FI that enables FI to cleave C3b. The proteins dissociate after the cleavage, probably because the affinity of FH for iC3b is much lower than for C3b [40]. There is probably also a conformational change in FI to make the active site available upon interactions with C3b and FH. This has been shown by Ekdahl-Nilsson *et al.* [41], where they used diisopropylfluorophosphate (DFP) to inhibit the activity of FI. The cleavage of C3b was inhibited when C3b and FI were incubated with DFP before the addition of FH. In contrast, no inhibition of C3b cleavage was seen when FI and FH were pre-incubated with DFP. Ekdahl-Nilsson *et al.* [41] also noticed that the SP domain bound DFP in the presence of C3b, but in the presence of FH no binding of DFP was detected. Since FH could inhibit binding of DFP to FI, their suggestion was that the catalytic site of FI is not exposed to the surroundings to the same extent in the tri-molecular complex as in the complex between C3b and FI. The interactions between FI, FH and C3b were analyzed by Soames and Sim [42] by ligand blotting using ¹²⁵I-labeled proteins. They observed that FH binds to the heavy chain and not the light chain of FI and that both FI and FH bind to both the α' - and β -chains of C3.

As with many other proteins in the immune system, bacteria and virus can take advantage of FI and use it to prevent activation of the complement system. *Staphylococcus aureus* uses FI to inactivate C3b on their surface to escape phagocytosis [43]. Kaposi sarcoma-associated herpesvirus (KSHV) produce a KSHV complement control protein that can act as cofactor for FI-mediated degradation of C3b and C4b, thereby avoiding further complement activation [44].

Factor H family

Complement factor H (FH, 155 kDa) is a soluble glycoprotein composed of 20 CCP domains [45], Fig 6. The concentration of FH varies between individuals but the average concentration is 550 μ g/ml. Variation could be due to genetic and

environmental influences [46]. The main function of FH is to regulate the alternative pathway. It does so by inhibiting the assembly of the C3 convertase by binding to C3b, accelerating the decay of existing C3 and C5 convertases by binding to Bb and displacing it and finally by acting as a cofactor for FI in the degradation of C3b [47]. FH can bind to different ligands via different CCP domains. The decay [48] and cofactor activities [49] are concentrated to the four N-terminal CCP domains, CCP1-4, where C3b binds. In order to protect self-tissues from complement activation, soluble FH binds to surfaces rich in sialic acid polyanions [50] and glucosaminoglycans [51]. Self-tissues lacking polyanions cannot be protected by FH. The CCP7-8 and CCP19-20 of FH are responsible for binding to heparin [52]. The importance of interactions mediated by CCP19-20 is shown by the fact that in diseases such as atypical hemolytic uremic syndrome (aHUS) the patients often have heterozygous mutations in CCP19-20 and show impaired regulation of the alternative pathway [53]. In a recently published study by Pangburn *et al.* [54] it was shown that clusters of FH molecules binds to surface polyanions, protecting broad areas of the surface due to the flexibility of FH.

CRP, which activates the classical pathway upon interaction with C1q, can also bind to FH via CCP7 and this interaction inhibits the C3/C5 convertases. This dual interaction of both activators and inhibitors by different proteins is a common phenomenon in the complement system, ensuring tight regulation and thus preventing damage to host cells [55].

In addition to FH, the FH family contains several FH like/related proteins (FHL/FHR). The protein FHL-1 is a product of alternative splicing of the *CFH* gene and is composed of only seven CCP domains. FHL-1 has the same properties as the N-terminal part of full-length FH, but it cannot bind to surfaces because it lacks CCP19-20. In contrast, FHR-1 to FHR-5 are the products of individual genes and are composed of between four and nine CCP domains. The N-termini of the FHR proteins share similarities to CCP6-9 of full-length FH and therefore able to bind to heparin and CRP. FHR proteins can also bind to surfaces because the CCP domains in their C-termini show similarities to CCP19-20 of full-length FH [47]. Several pathogens such as *Borrelia burgdorferi* [56, 57], *Streptococcus* species [58], *Neisseria* sp. [59, 60] have developed strategies to evade elimination by the complement system and one of them is through binding to FH, FHL-1 and/or FHR proteins.

C4b-binding protein

C4b-binding protein (C4BP) is the major inhibitor of the classical and lectin pathways, but it can also inhibit the alternative pathway to some extent. C4BP is a glycoprotein (500 kDa), composed of seven identical α -chains (70 kDa) and one β -chain (45 kDa) linked together by a central core by disulfide bridges [61], Fig 6. The α -chains each contain eight CCP domains and the β -chain contains three CCPs. C4BP has a spider-like shape, where the α -chains resemble the legs [62]. In blood,

C4BP circulates in complex with the vitamin K dependent anti-coagulant protein S (PS), but free PS is also found in the circulation. PS is bound via hydrophobic interactions to CCP1 of the β -chain of C4BP. PS directs C4BP to apoptotic cells via binding to negatively charged phospholipids and this decreases inflammatory responses that could be evoked by the apoptotic cells [63]. During the acute phase, the level of C4BP is upregulated four-fold and a different isoform of C4BP, containing only the α -chains and no β -chain, is produced. This ensures that the PS which would otherwise become bound to C4BP is available for participating as an anti-coagulant in the coagulation cascade [64].

C4BP inhibits the complement system at the level of the C3 convertases. C4BP can either prevent the assembly of the C3 convertases, enhance their dissociation by binding to C4b [14] and C3b [65], or act as a cofactor to FI for cleavage of C4b [14] and C3b [65]. C4b and C3b probably undergo conformational changes upon binding of C4BP, which make the scissile bonds available to FI. CCP1-3 in C4BP is enough to allow binding to C4b [66], whereas CCP4 is also needed for binding to C3b [65]. The interactions are ionic in nature and a cluster of positively charged amino acids between CCP1 and 2 in C4BP is important for binding. C4BP also binds to heparin and the binding site overlaps with the one for C4b, since heparin can inhibit C4b binding to C4BP [67]. C4BP can bind to several pathogens e.g. *Neisseria gonorrhoeae* [68, 69], *Streptococcus pyogenes* [70, 71], *Bordetella pertussis* [72], and *Candida albicans* [73], which protects these pathogens from complement attack and inhibits their phagocytosis.

Membrane cofactor protein

Membrane cofactor protein (MCP, CD46) is a transmembrane protein composed of several domains: 4 CCP domains with *N*-glycosylations, a region rich in serines, threonines and prolines (STP domain) that is *O*-glycosylated, a 12-amino acid segment of undefined homology, a hydrophobic transmembrane domain and a charged cytoplasmic anchor [74], Fig 6. MCP consists of four different isoforms (51-68 kDa), which arise from alternative splicing of the STP domain and the anchor region [75]. The protein is expressed on the surface of all cells except for erythrocytes [74]. MCP was first identified as a C3b and C4b binding protein on human peripheral blood cells, and soon thereafter, it was demonstrated to be a cofactor for the FI mediated degradation of C3b and C4b, [15, 76]. CCP 2-4 is necessary for binding and cleavage of C3b, whereas for C4b binding and cofactor activity, CCP1 is also needed to some extent [77, 78]. MCP works only intrinsically, i.e. it protects only the cells that it is attached to and not neighboring cells [79] and does not possess any decay accelerating activity towards the C3 convertases [15]. MCP is also a receptor for several pathogens e.g. measles virus [80], group A *S. pyogenes* [81], pathogenic *Neisseria* sp. [82] and human herpesvirus 6 [83].

Complement receptor 1

Complement receptor type 1 (CR1, CD35) is a type 1 integral membrane glycoprotein (160-250 kDa) [84, 85]. CR1 is composed of an extracellular region of 27-44 CCPs, a transmembrane region and finally a cytoplasmic region. The CCP domains, except for the two C-terminal CCPs, are divided into long homologous repeats (LHR), each containing seven CCP domains per LHR. There are four common allelic variants of CR1 and they differ in the number of LHR regions. Type A, B, C and D have 4, 5, 3 and 6 LHR regions (30, 37, 23 and 44 CCPs), respectively. These variants are not created from alternative splicing but probably arise through unequal crossing-over between the highly homologous regions. The most common variant is type A, with 30 CCPs [85], Fig 6. CR1 has an extended linear structure with a folded-back arrangement according to electron microscopy [86] and x-ray scattering studies on soluble CR1 [87]. CR1 is expressed on erythrocytes [88], polymorphonuclear leukocytes [88], monocytes [88], follicular dendritic cells [89], B-cells [88], glomerular podocytes [90] and also found on a subset of T-cells [91]. A soluble form of CR1 (sCR1) has been found in serum at rather low levels, 30 ng/ml [85].

One of the most important functions of CR1 (on erythrocytes) is to bind C3b- and C4b-opsonized immune complexes and transport them to the liver and spleen. One CR1 molecule can interact with several C3b/C4b molecules and because erythrocytes contain many CR1 molecules per cell, this interaction becomes very efficient. Patients with “immune complex” diseases such as SLE show reduced expression of CR1 during the active phase of the disease [92, 93]. CR1 on phagocytic cells interacts with pathogens and damaged cells that have been opsonized with C3b and C4b, leading to enhanced phagocytosis. When neutrophils and monocytes become activated, CR1 stored intracellularly in secretory vesicles [94] is transported to the surface, initiating phagocytosis [95, 96]. CR1 also links the innate and adaptive immune systems, via involvement in antigen-presentation to T-cells, through CR1 expression on B-cells and follicular dendritic cells [97].

Another interesting function of CR1 is that it is a cofactor for the FI-mediated cleavage of C3b and C4b. CR1 also inhibits the formation and enhances the decay [98] of the classical and alternative C3 and C5 convertases by binding to C3b and C4b. Furthermore, CR1 is the only cofactor that is able to further degrade iC3b to C3c and C3dg [99]. CCP1-3 in the LHR A region of CR1 are responsible for binding to C4b and give the decay accelerating activity. CCP8-10 in the LHR B region and CCP15-17 in the LHR C region bind to both C3b and C4b and are also important for cofactor activity [100, 101].

Complement receptor 2

Complement receptor type 2 (CR2, CD21) is a type I transmembrane glycoprotein comprising of 15 or 16 CCP domains, a 28-amino acid transmembrane domain and a 34-amino acid cytoplasmic tail [102, 103]. The 15 and 16 CCP variants result from alternative splicing of one exon in the *CR2* gene [104]. The extracellular part of CR2, containing the 16 CCP domains, can be divided into four large repetitive units, each containing four CCPs. CR2 is expressed on various cells: mature B-cells [105], follicular dendritic cells [89], a subpopulation of T-cells [106, 107], basophils [108], mast cells [109], keratinocytes [110] and epithelial cells [111]. CR2 has four different classes of ligands: C3 fragments (iC3d, C3dg and C3d) [102], the glycoprotein 350/220 of the herpesvirus Epstein-Barr virus [112], IFN- α [113] and the immunoregulatory protein CD23, also named Fc ϵ RII, which is a receptor for IgE [114].

The structure of CR2 CCP1-15 in solution has been predicted using electron microscopy [115], constrained scattering and ultracentrifugation modeling [116]. The CR2 molecule assumes an extended but flexible conformation, with folded back regions. The structure resembles a “fish-hook”, where the “hook” is CCP1-2. CCP1-2 of CR2 is responsible for the interaction with C3d, EBV and IFN- α and the binding seems to be both charge-dependent and charge-independent [113, 117, 118]. The ligand CD23 interacts with both CCP1-2 and CCP5-8 of CR2 [114].

One of the most important functions of CR2 is to enhance B-cell activation. CR2, together with CD19 and CD81, functions as a co-receptor for the B-cell receptor (BCR) on B-cells. The BCR initially recognizes the antigen, which is part of a complex containing immune complexes and C3d molecules. When the foreign antigen binds to the BCR, C3d binds to CR2, cross-linking the BCR with the co-receptor and in turn allowing phosphorylation of CD19. This generates further signal transduction inside the B-cell, which lowers the threshold of B-cell activation [119].

Complement receptor 3

Complement receptor 3 (CR3, CD11b/CD18) is a type I membrane glycoprotein. CR3 belongs to a family of surface receptors called integrins, which are involved in cellular adhesion and cell-cell interactions. CR3 is a β_2 -integrin and is composed of an α -subunit (CD11b, 160 kDa) and a β -subunit (CD18, 95 kDa), held together non-covalently. The α -subunit contains an I-domain, a C-terminal domain, a transmembrane domain and a cytoplasmic domain. In contrast, the β -subunit contains an extracellular domain with four cysteine-rich repeat regions, a transmembrane domain and a cytoplasmic domain [120]. The I-domain contains a metal ion-dependent adhesion site that is involved in binding to different ligands e.g. iC3b, intercellular adhesion molecule 1 (ICAM-1), fibrinogen [121, 122]. In the C-terminal domain of the α -subunit there is a lectin site, which functions to promote

signaling between endogenous membrane glycoproteins and CR3 [123]. Like CR1, CR3 is stored in cytoplasmic granules and upon activation of the neutrophils, CR3 is transported to the cell surface [124]. CR3 is expressed on leukocytes e.g. NK-cells [125], monocytes [126] and neutrophils [127].

The CR3 works in co-operation with CR1 when bound to opsonized particles. CR1 on neutrophils binds to C3b-opsonized particles and functions as a cofactor for FI cleavage of C3b. When iC3b is formed affinity for CR1 is lost but at the same time, is increased for CR3. The interaction between iC3b and CR3 can now lead to further downstream signaling inside the neutrophils and phagocytosis of the opsonized particle is enhanced [128].

Complement receptor 4

Complement receptor (CR4, CD11c/CD18) is a type I membrane glycoprotein composed of two subunits: an α -subunit (CD11c, 150 kDa) and a β -subunit (CD18, 95 kDa, as found in CR3). The two subunits are connected via non-covalent interactions. The α -subunit contains the following structural elements: an I domain, a C-terminal region, a transmembrane segment and a cytoplasmic domain [129]. CR4 is expressed at low levels on neutrophils [130], monocytes [130], activated B-cells [131] and at higher levels on macrophages [132] and dendritic cells [133]. CR4 binds similar ligands to CR3: iC3b [134], ICAM-1 [135], fibrinogen [136] and lipopolysaccharide [137]. The interactions between CR4 and these ligands mediate adhesion of neutrophils and monocytes to the endothelium [138]. CR4 expression on macrophages enhances phagocytosis whereas CR4 expression on dendritic cells plays a role in priming and regulating the immune response [139]. The interaction between the I domain of CR4 and iC3b has been elucidated by Vorup-Jensen *et al.* [140]. The I domain of CR4 undergoes conformational change upon binding to iC3b.

Complement deficiencies

Plasma protein deficiencies can be classified as type 1, 2 and 3. Type 1 is characterized by no production or secretion of the protein in question. In a type 2 deficiency, the protein is produced and secreted but it is not functional. In the third type of deficiency, the protein is produced and secreted but it is inactivated, for example, by autoantibodies such as anti-FH autoantibodies [141] or anti-C1 inhibitor autoantibodies [142].

The inherited deficiencies are caused by mutations in the genes coding for the proteins. There are several different kinds of mutations that can affect the transcribed protein, table 1. The difference between a mutation and a polymorphism is that a polymorphism is more common, occurring in more than 1% of the population. If a single nucleotide is changed it can generate a missense mutation, which means that an amino acid is changed, or a nonsense mutation, resulting in a stop codon. A silent mutation does not give any change in amino acid, but it can introduce an alternative splicing site in the DNA sequence that can lead to skipping of an exon or intron retention (the intron is not excised). In such a case the gene product cannot be expressed since pre-mature stop codons are generated. Homology recombination of closely related genes or of repetitive sequences can sometimes lead to the deletion or fusion of genes. This has been seen in the FH protein family, where deletion of the *CFHR1* and *CFHR3* genes is the result of recombination [143]. An insertion or deletion of one or several nucleotide generates pre-mature stop codon

Table 1. Vocabularies used in genetics.

Name	Explanation
Allele	Alternative forms of the same gene
Mutation	A change in a nucleotide sequence
Point mutation	Substitution, insertion or deletion of a single nucleotide
Polymorphism	A variation (mutation) more frequent than 1% of the population
SNP	Single nucleotide polymorphism
Missense mutation	The amino acid is changed to another amino acid
Nonsense mutation	The amino acid is changed to a stop codon
Silent mutation	The nucleotide change is not changing the amino acid
Insertion mutation	A nucleotide or a larger sequence are inserted/deleted in the DNA, generating pre-mature stop codon
Deletion mutation	
Heterozygous mutation	Is a mutation of only one allele
Homozygous mutation	Is an identical mutation of both the paternal and maternal alleles
Compound heterozygous	Two different mutations, one mutation in the paternal and one mutation in the maternal allele
Autosomal recessive	A mode of inheritance that requires a homozygous mutation or a compound heterozygous mutation to cause a disease

that results in a truncated protein or a protein that is degraded inside cells because it is misfolded.

An individual with only one mutation, present on one gene allele is heterozygous whereas a person with two different heterozygous mutations (one mutation in the paternal and one mutation in the maternal allele) is called compound heterozygous. In contrast, a person homozygous for a mutation has the same mutations in both alleles.

Deficiencies in complement proteins lead to increased susceptibility to bacterial infections, autoimmune and renal diseases.

Bacterial infections

A properdin deficiency was initially associated with meningococcal disease [144] but it is now associated with recurrent otitis media and pneumonia also [145]. Properdin stabilizes the alternative C3 convertases but it is also regarded as an initiator of the alternative pathway. Properdin binds to bacterial surfaces and promotes C3b-deposition, generating alternative C3 convertases. It has been shown that C3b can only bind to the surface of *N. gonorrhoeae* if properdin is already bound [2]. This suggests that activation through the alternative pathway is essential for the complement response against Neisserial infections [146]. Deficiencies in the other proteins in the alternative pathway such as FD also lead to increased susceptibility of Neisserial infections [147].

A MBL deficiency is associated with recurrent bacterial infections during childhood, especially in the time period between loss of passively transferred maternal IgG and generation of an acquired immune system. This is most likely due to impaired recognition of carbohydrate structures on bacteria surfaces because MBL is not present [148].

Deficiencies of the terminal pathway components (C5 [149], C6 [150], C7 [151], C8 [152] and C9 [153]) are associated with recurrent infections by *N. meningitides*. The MAC complex plays an important role in neutralizing *N. meningitides* and deficiencies therefore predispose to infection.

Autoimmune diseases

Deficiencies in the classical pathway proteins (C1, C4 and C2) are associated with an increased susceptibility to SLE, which is characterized by a defect in the clearance of immune complexes and apoptotic cells [154, 155]. A deficiency in C1q leads to an accumulation of apoptotic cells, providing a source of autoantigens that drives the autoimmune responses seen in SLE [156].

Renal diseases

Glomerulonephritis is the name for inflammation of the glomerulus, Fig. 8, the small blood vessels in the kidney, and it is a common pathological feature in complement deficient patients. Glomerulonephritis is caused by immune complex and complement (C3, C4) deposition within the glomerular basement membrane (GBM). Glomerulonephritis is found in complete FI [157, 158] and FH [159-161] deficient patients. The renal inflammation observed in a proportion of SLE patients, lupus-nephritis, is caused by the deposition of immune complexes. Currently this is explained by the deposition of nuclear components to glomerular structures, followed by the binding of autoantibodies directed against these nuclear components, in addition also pre-formed immune complexes can be trapped in the glomerulus. Finally these immune complexes will activate complement that will induce damage by itself but will also attract inflammatory cells, together contributing to the pathological changes of lupus-nephritis [155, 162]. Symptoms of glomerulonephritis are hypertension, proteinuria, hematuria, and impaired filtration by the glomerulus. This leads to impairment of kidney function and can ultimately develop into end-stage renal disease (ESRD) [162].

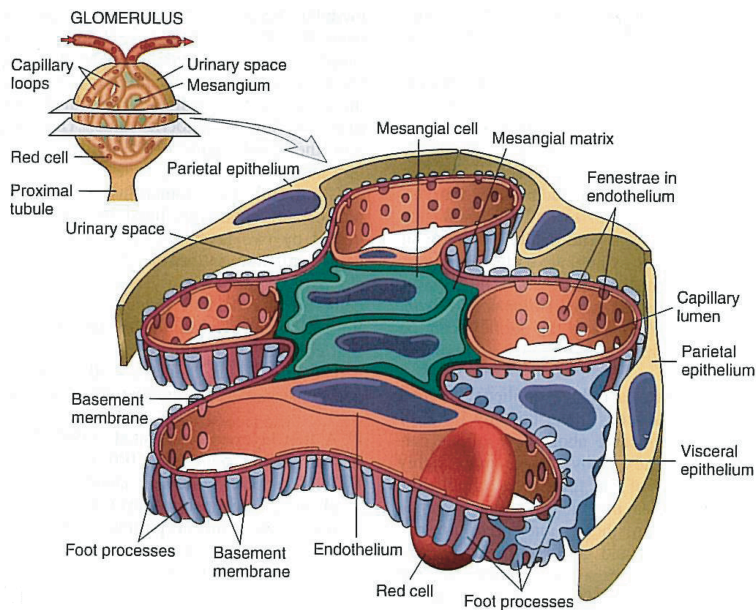


Figure 8. Schematic picture of the glomerulus [163].

Membranoproliferative glomerulonephritis (MPGN) is a variant of glomerulonephritis that can be divided in three types, type 1, type 2 and type 3, where type 1 is the most common. MPGN is characterized by a thickening of the

GBM, proliferation of the mesangial cells and leukocyte infiltration [164]. In MPGN 1, there are deposits of immune complexes and C3, and sometimes also C1q and C4, in the subendothelium. MPGN 2, also known as dense-deposit disease, is characterized by the deposition of dense material in the GBM. Additionally, C3 is present on the other side of the basement membrane but not within the dense deposits. C3 is also present in the mesangium [165]. Unlike MPGN 1, there is no immune complex deposition in MPGN 2. MPGN 3, is rare and is a combination of types 1 and 2. It is thought that the classical pathway is involved in the pathogenesis of MPGN 1, while the alternative pathway is involved in MPGN 2 [164]. Most patients suffering from MPGN 2, have a circulating autoantibody called C3 nephritic factor (C3Nef) that is directed against the alternative C3 convertase. C3Nef stabilizes and increases the half-life of this C3 convertases [166], which leads to depletion of C3 molecules [167]. MPGN 2 is also associated with homozygous mutations in FH, which also results in low levels of C3 [160, 161]. Heterozygous mutations of FH are not associated with MPGN 2 but are instead involved in another renal disease, aHUS [159].

Age-related macular degeneration

Patients with MPGN 2 may also develop the eye disease, age-related macular degeneration (AMD) [168]. AMD is a leading cause of irreversible vision loss among elderly people in developed countries. In the eye, immune deposits (drusen) are formed between the retinal pigmented epithelial cells and the Bruch's membrane. This drusen contains complement proteins and complement activation products. It has been reported by several groups that the polymorphism Y402H in FH and FHL-1, strongly increases the risk for developing AMD. This may be because the FH-H402 variant has reduced binding to heparin and CRP and can therefore not inhibit complement activation to the same extent as FH-Y402. Also polymorphisms in C3 [169, 170] and 3' upstream of the *CFI* gene [171], have been implicated in AMD. Studies by Wong *et al.* showed that when FI interacts with amyloid β , it could not cleave C3b [172] and that the production of FB was indirectly upregulated by amyloid β [173]. Amyloid β is found in both drusen and senile plaques in the brain of Alzheimer's patients.

FI deficiency

Complete FI deficiency is a rare disease and around 40 cases have been reported so far in the literature [26, 157, 158, 174-191]. The first case of a complete FI deficiency was first described in 1970 by Alper *et al.* [175, 176]. The patient was first suspected of being C3-deficient due to hypercatabolism of C3, but he was later diagnosed with a complete FI deficiency [174]. Recently, a link between partial FI deficiencies and aHUS has been identified and this is discussed later in this thesis.

A complete FI deficiency results in an uncontrolled activation of the amplification loop of the alternative pathway, leading to the consumption and depletion of C3. Many patients also have reduced levels of FH and FB, since these proteins are also consumed [177, 187]. Patients suffering from a complete FI deficiency often show an early onset of recurrent pyogenic infections of the upper and lower respiratory tract, for example otitis media, sinusitis, pneumonia and bronchitis. Meningitis and septicaemia are also common among complete FI deficient patients [181-183, 185]. This increased susceptibility to infections by encapsulated bacteria is due to impaired opsonization of these pathogens with C3b, resulting from low C3 levels. This is the reason why Alper *et al.* [175, 176] at first hypothesized that the FI deficient patient was deficient in C3. Also, the decreased production of chemotactic factors e.g. C5a, results in fewer inflammatory cells at the site of infection. These patients, who show diminished C3 levels also have impaired B-cell memory since the cleavage products of C3b, C3dg and C3d, enhance the activation of B-cells during an infection.

In some cases, complete FI deficient patients, have rheumatological diseases such as SLE [177], arthralgia [184], vasculitis [157, 184] and renal diseases like glomerulonephritis [157, 158, 184]. One of the biological functions of C3 is to solubilize and clear immune complexes from the circulation. Low levels of C3 in those complete FI deficient patients who suffer from autoimmune diseases would increase the exposure of auto-antigens to the immune system. In three patients renal disease was also reported. These patient suffered from glomerulonephritis, but with different associations such as focal segmental glomerulonephritis with glomerular deposits of immunoglobulins and C3 and C4 fragments [158], diffuse proliferative glomerulonephritis [177] or immune complex glomerulonephritis with glomerular deposits of immunoglobulins and C3 [157]. The involvement of renal diseases in FI deficient patients may be explained by the lowered or complete loss of expression of CR1 on erythrocytes and epithelial cells (podocytes) in the kidney [158]. When FI is missing there is an over-production of C3b molecules that bind CR1, leading to a consumptive loss of CR1. Reduced expression of CR1 results in impaired clearance of immune complexes. This is also seen in patients with SLE [192, 193].

FI deficiency is an autosomal recessive condition where patients have a homozygous mutation or combined heterozygous mutations in the *CFI* gene [184]. Consanguinity is most often involved in cases of homozygous mutations.

So far, there is no good treatment for FI deficient patients. Plasma infusion can help in the beginning but since the half-life of FI is short the treatment has to be repeated often. If the patient suffers from recurrent infections, the patient can be vaccinated against the pathogen in question. If there are renal diseases involved, treatments must be tailored to the individual patient.

Atypical hemolytic uremic syndrome

Hemolytic uremic syndrome (HUS) is characterized by microangiopathic hemolytic anemia, thrombocytopenia and renal failure caused by platelet thrombi in the renal microcirculation [194]. The most common form of HUS is caused by infection of shiga toxin producing *Escherichia coli* (stx-*E. coli*) and it is associated with bloody diarrhea. This form of HUS is called the diarrhea positive HUS (D+ HUS) or typical HUS. It is mostly children that are affected. The prognosis is rather good and there is often no recurrence of the disease. The stx-*E. coli* is spread through bad hygiene and is common in undeveloped countries. After oral ingestion, the stx-*E. coli* reaches the gut and closely adheres to the epithelial cells of the gastrointestinal mucosa through the outer membrane protein intimin. Stx is then taken up by gastrointestinal cells and translocated into the circulation by neutrophils. When it reaches the kidney, which has a high blood flow, stx is then transferred and binds to endothelial cells. The endothelial cells become damaged by the toxin, which leads to renal impairment, manifested as proteinuria, hematuria and sometimes renal failure [195]. Typical HUS patients can be treated with antibiotics and the earlier they are treated the better their prognosis [194].

The other form of HUS only accounts for 10-20% of HUS cases and is called non-diarrhea HUS or atypical HUS (aHUS). aHUS affects both children and adults, and the prognosis is bad. Around 50% of the patients develop ESRD and the mortality is up 25% during the acute-phase of the disease. Many patients also have recurring disease. aHUS can be initiated by several stimuli such as infections, drugs, tumors and pregnancy but during the last decade it has become clear that dysregulation of the alternative pathway of the complement system plays a major role [194]. Around 50-60% of the aHUS patients have mutations in one or more regulators of the alternative complement pathway. The majority of these patients carry mutations and polymorphisms in *CFH* (20-30%) [53, 159, 196-204] followed by mutations in *MCP* (10-13%) [53, 198, 199, 205-209], deletion of *CFHR1-CFHR3* (10%) [210], *CFI* (5-10%) [53, 198, 211-217], *C3* (5%) [218], *CFB* (1-3%) [219] and *C4BPA* [220]. The mutations seen in aHUS patients are mostly heterozygous, while the mutations found in complete FI deficient and MPGN 2 patients are homozygous or compound heterozygous [159, 184].

Most of the mutations in *CFH* are located in the C-terminal part of FH, which is important for host cell recognition [200, 201, 221, 222]. Some aHUS patients have auto-FH antibodies directed against the C-terminal part of FH [141, 223, 224]. In aHUS, patients with mutations or autoantibodies show impaired protection of endothelial cells because FH cannot bind properly to the cell surface ligands C3b, sialic acids and heparin-like glycosaminoglycan polyanions [225]. This generates continuous activation of the alternative pathway, which leads to insertion of MAC that will later destroy the endothelial cells. The insertion of MAC into the

membrane upregulates the expression of P-selectin, which binds neutrophils recruited by anaphylatoxins. The neutrophils release reactive oxygen species and proteases that further damage the endothelial cells and eventually the GBM becomes exposed. Platelets become activated, aggregate and form a thrombus on the basement membrane. Additionally, erythrocytes are lysed when they are trapped in the thrombus and because platelets are consumed, their systemic levels are decreased [194]. The cofactor activity in the fluid-phase of FH is normal in these mutants since this function is located in the N-terminal part of the molecule. But those mutations which are located outside the C-terminal part of FH can give impaired cofactor activity, both on the cell surface and in the fluid-phase. Caprioli *et al.* [197] have shown that several polymorphisms in *CFH* can predispose to aHUS.

The effects of mutations in the C-terminal part of FH have been characterized in a mouse model by Pickering *et al.* First, they showed that mice totally lacking FH have low C3 levels and develop MPGN 2 [226]. They also produced mice expressing a truncated form of FH (CCP1-15) and these mice developed aHUS [227]. Later they also showed that C3b cleavage by FI is necessary for the development of MPGN2 in their FH deficient mice [228].

The *CFI* gene was screened for mutations in patients in whom no mutations in the *CFH* or *MCP* genes could be detected. So far, around 14 mutations in the *CFI* gene have been identified in aHUS patients and an additional 15 novel mutations are presented in this thesis [198, 211-217]. These mutations are mainly missense or nonsense mutations but there are also some deletion and insertion mutations. The mutations are spread among the different domains but most of them are found in the SP domain. As with the mutations in *CFH* and *MCP*, the mutations in *CFI* are also heterozygous. Around half of the mutated proteins are not secreted, which leads to decreased levels of FI. Other mutations express at normal levels but they show impaired function. There are also some mutations that express at normal levels and have the same known function as wild type FI; no impairment in these mutations has been detected so far [215, 216].

MCP is expressed on endothelial cells but not on the basement membrane. Mutations in *MCP* generate either protein with reduced expression on these cells, or MCP molecules that are expressed normally but with impaired C3b binding and cofactor activity [206, 208, 209]. Most of the mutations are located in the CCP domains. Patients with *MCP* mutations who underwent renal transplantation had a very good outcome. There are very few recurrences of aHUS in transplanted kidneys because there is expression of wild type MCP that is locally produced in the donor kidney. This is enough to give protection against complement activation. Most aHUS patients with *MCP* mutations do not develop ESRD [206, 208, 209].

In addition to the mutations in *CFH*, *CFI* and *MCP*, mutations have also been identified in other complement proteins such as C4BP [220], FB [219] and C3 [218]. The polymorphisms found in *C4BPA* showed impaired binding to C3b and reduced cofactor and decay activity [220]. The mutations in FB and C3 are called

“gain-of-function” mutations since they result in enhanced formation of the C3bBb convertase, or increase resistance to inactivation by complement regulators [218, 219].

So far, no mutations have been found in the *CR1* gene, but one mutation in the *DAF* gene has been identified in an aHUS patient [229]. This mutation had both normal expression and decay acceleration activity and the authors suggest that the cofactor activity and not the decay accelerating activity are important in the pathogenesis of aHUS.

Plasma exchange and plasma infusion are the first choice when treating aHUS patients. Plasma exchange is more effective since waste products, the mutated protein or autoantibodies are all removed. Additionally, this treatment is beneficial for the patient because of the large volume of material exchanged. Patients with *CFH* or *CFI* mutations often develop ESRD and they need a renal transplantation. Unfortunately, almost all of these patients get recurring disease in the transplant [198, 212, 214, 217, 230-232]. In recent years, combined liver and renal transplantation have been performed with good results. Although some patients developed aHUS in the transplanted kidney [233], those who received extensive plasma exchange before and during the operation had a successful transplantation without any recurrence of the disease after several months or years [234, 235]. Transplantations of only the kidney have also been successful when extensive plasma exchange has been used before, during and now also after the operation. These patients need recurrent plasma exchange for the rest of their lives in order to prevent development of aHUS in the transplanted kidney [236, 237]. A donation from a live-related donator should be avoided since the donor himself could develop aHUS after the kidney donation [238].

The mutations in complement regulators may not be the direct cause of aHUS, but predispose to the disease. It is thought that several mutations and also environmental factors play a role in the development of aHUS.

The present investigation

Aims of the thesis

FI is a serine protease that cleaves its substrates in the presence of specific cofactors, thereby inhibiting the complement system. Exactly how the interactions between FI, substrates and cofactors takes place is not known. The mutations identified in complete FI deficient and aHUS patients may shed some light on these interactions and on structure-function relationships in FI. One of the aims of this thesis was to elucidate how FI is affected by the mutations identified in complete FI deficient and aHUS patients. We have also introduced mutations at putative binding patches in FI to further analyze the consequences of FI's interaction with its substrates and cofactors.

Results and discussion

FI mutations in FI deficient patients (Paper I)

Complete FI deficiency is a rare disease and around 40 cases have been reported so far. At the Department of Clinical Microbiology and Immunology, Lund University Hospital, Dr. Anders Sjöholm and Dr. Lennart Truedsson collected DNA and some serum samples from five FI deficient patients and some family members. This was a part of their ongoing large project investigating different complement deficiencies. After sequencing the genomic DNA of these five patients we found one homozygous mutation (C237Y) in a patient (patient 1) whose parents are first cousins. Some of her siblings also carry the same mutation, in homozygous or heterozygous forms. Another patient from Argentina (patient 3) carries a homozygous mutation (Q336X). The mother carries the same mutation but in a heterozygous form and she is healthy. Since we did not have a DNA sample from the father we could not exclude the possibility that the patient may have a deletion of the complete *CFI* gene in the paternal allele. The results from southern blot analysis showed that the patient does not have any partial deletion of the *CFI* gene. In the three remaining patients compound heterozygous mutations are identified. In patient 4, one of the mutations is located in the 3'-end of exon 5 that leads to an interruption of the donor splice site for the mRNA and skipping of exon 5. The same mutation has been reported

previously by two unrelated studies by Vyse *et al.* [26] and Ponce-Castro *et al.* [189]. The rest of the mutations are point mutations where an amino acid is substituted.

The FI concentration was undetectable or very low in all patients and no autoantibodies against FI could be detected. According to the western blot results FI could only be detected at a low level in patient 2 and 4. In the C3b/C4b degradation assay of serum samples, a small amount of cleavage product of C3b could be detected in the serum sample from patient 2. This means that the small amount of FI detected in this patient was sufficient for partial inhibition of the complement system and this correlates well with the clinical presentation of patient 2. This patient did not have as many infections as the other FI deficient patients in this study.

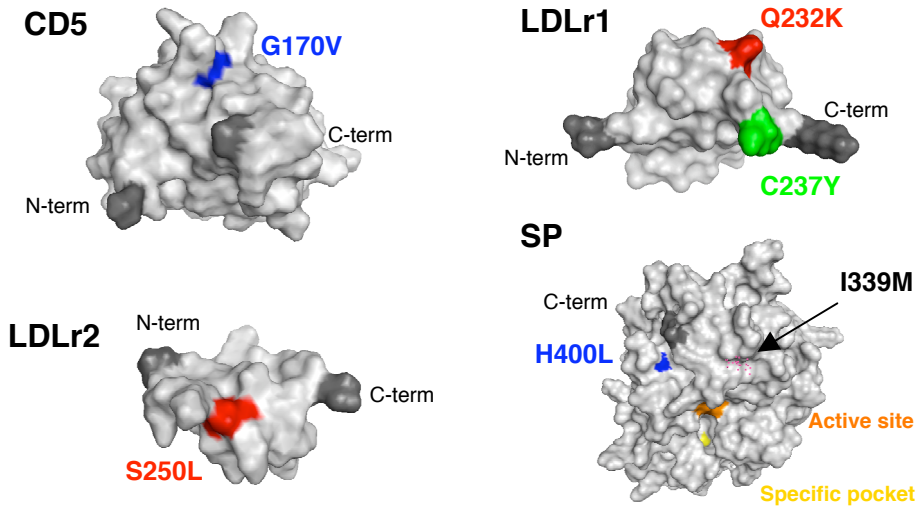


Figure 9. Putative models of the domains of FI. The mutations in the complete FI deficient patients are highlighted: patient 1; green, patient 2; red, patient 4; black, patient 5; blue, active site; orange; specific pocket; yellow. The I339M mutation is buried in the SP domain and are therefore marked in dots in magenta.

To further analyze the consequences of the mutations, they were introduced in a vector containing the cDNA of FI, then human embryonic kidney (HEK 293) cells were transfected in both a transient and stable manner. All mutants were expressed and secreted at a lower level than the wild type, except for the truncated mutant (Q336x). In the patient with this mutation, no FI could be detected and the explanation for this inconsistent result may be that the truncated form of FI was degraded rapidly in the patient compared to the simple cell system that we used to express proteins. We managed to partially purify four mutants and test them in C3b and C4b degradation assays. The mutants (Q232K and S250L) identified in patient 2 were both able to degrade C3b and C4b, whereas the mutations in patient 1 (C237Y)

and 3 (Q336x) were non functional. The C237Y mutant disrupts an interdomain disulfide bridge between the FIMAC (Cys15) and the LDLr1 (Cys237) domain and may alter the structure of FI in such a way that it is no longer functional. The Q336x mutant is truncated and lacks almost the whole SP domain and can therefore not function. The mutations from patient 4 (I339M) and 5 (G170V and H400L) did not secrete and therefore we could not analyze their function.

The mutations in FI deficient patients identified in the current study are located in different domains and they mainly affect the expression and secretion of the FI protein, therefore the levels of FI are low in these patients. The mutants Q232K and S250L in particular were functional but showed impaired expression and secretion.

FI mutations in aHUS patients (Papers II-IV)

This study was initiated as a collaboration with Dr. Diana Karpman from the Department of Pediatrics at Lund University Hospital who treated a young girl with severe aHUS. After sequencing the *CFH*, *MCP* and *CFI* genes, she identified three heterozygous SNPs in the *CFH* gene that are associated with aHUS and a heterozygous mutation in *CFI*, G243D. The mother carried the SNPs in *CFH* gene and the father carried the *CFI* mutation. Simultaneously, Dr. Veronique Fremeaux-Bacchi at the Service d'Immunologie Biologique, Hopital European Georges Pompidou in Paris, identified the same heterozygous mutation in *CFI* in two unrelated patients in the French cohort of aHUS patients. All three patients had FI plasma levels within the normal range. The only complement protein tested that was lower than normal was C3. For patient 1, the hemolytic activities of the classical and alternative pathways were normal.

In order to elucidate if and how the G243D mutation affected FI, this mutant was expressed recombinantly in HEK 293 cells and successfully purified. There were no differences in expression, secretion or processing of the mutant compared to wild type FI. The function of this mutant was normal in the degradation of both C3b and C4b in the fluid-phase. Since many aHUS patients show impaired regulation of the alternative pathway on the cell surfaces, the activity of this mutant was tested in an assay where C3b was deposited on the surface and then cleaved by FI and cofactor. This mutant also showed the same activity as wild type FI. Even though three unrelated patients had the same heterozygous mutation in *CFI*, no impaired expression, secretion or function could be detected for this mutation. Later, Kavanagh *et al.* [215] confirmed our results.

All three patients underwent renal transplantations because they suffered from ESRD, but unfortunately they all developed a recurrence of aHUS in the transplanted kidney. Finally, Patient 2 died due to ESRD. After this article was published further gene sequence analyses were performed in the patients investigating in genes that were just identified to be related to development of aHUS. In patient 1,

an additional mutation in the *CFB* gene was identified (personal communication with Diana Karpman). The function of the mutation has not been analyzed yet, but other mutations in the *CFB* gene identified in aHUS patients have been shown to give gain-of-function mutations that stabilize the C3 convertase, resulting in the depletion of complement. In patient 2, a missense mutation in the *C3* gene, K155Q was identified. This mutation is located in the MG2 domain and is very close to the 1st scissile bond (CUB domain) for FI in the C3b molecule, Fig 10. However, so far there is no functional analysis for this particular mutation, but considering its location we hypothesize that it could interfere with the cleavage of C3b by FI. We have reported in our original publication that patient 3 developed autoantibodies against FH, which are known to impair FH function in aHUS [143, 200]. Furthermore, we know now that this patient also has a homozygous deletion of the *CFHR1* gene, also frequently found in aHUS patients [210].

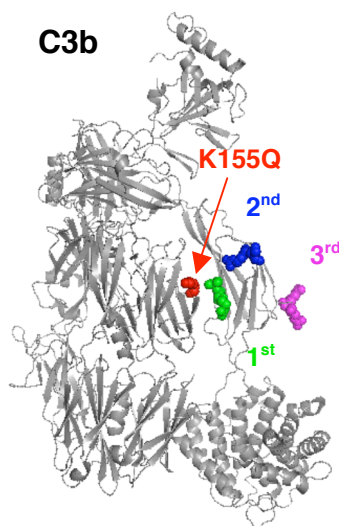


Figure 10. The C3b structure with the K155Q mutation identified in patient 3 in Paper II. The structure is downloaded from NCBI homepage, reference number 2I07.

Taken together, all three patients carrying the same mutation in *CFI* and investigated in our study have additional mutations or show deletion of other complement proteins. These findings imply that this particular mutation in *CFI* is simply not associated strongly with the development of aHUS. Perhaps, it has certain effects in the presence of additional mutations in complement proteins, which would strengthen the theory that multiple-hits are necessary to develop aHUS. Since therapeutic strategies must be devised using information for all the mutated complement proteins, it is important to characterize the effects of each mutation at the protein level. Only then we can make rational choices guiding therapy. However,

discovering a potential defect in a gene does not necessarily equate to a functionally impaired protein.

Several studies have reported *CFI* mutations in aHUS patients but the functions of many of these mutations have not been elucidated. In paper III, we decided to further analyze the consequences of 14 *CFI* mutations identified in aHUS patients. Six of the 14 mutations were introduced in recombinant FI, the protein purified and their function analyzed. Three of these mutations (M120V, A222G and R299W) showed impaired secretion. From the six mutants that were tested functionally, one of them (D501N) had no activity when degrading C3b and C4b in the fluid-phase or C3b deposit on surfaces. The remaining five mutants (P32A, M120V, H165R, A222G and R299W) were functional when cleaving C3b and C4b in the fluid-phase but two of them, P32A and A222G, had impaired activity when cleaving surface bound C3b.

The mutants that did not express and secrete were mutations that generated stop codons and two missense mutations, C25F and N133S. The mutation where a cysteine is lost probably generates a FI protein that does not folded correctly due to the loss of the disulfide bridge and is degraded inside the cell. Cys25 forms a disulfide bridge with Cys36 and they are both located close to the N-terminal part of the FIMAC domain, Fig 11.

When looking at the CD5 domain that contains the M120V, N133S and H165R mutations, one can see that the mutations are located in different parts of the domain, Fig 11. His165 is not conserved between various species and it could be solvent-exposed since both the N- and C-termini are located on the other side of the domain. The mutation to an arginine should therefore be well tolerated since it does not interrupt any interactions with the other domains of FI. This mutant functioned in a similar way to wild type FI. Met120, which is semi conserved, is located closer to the C-terminus and this residue could be involved in binding to other domains of FI. The other mutation, where an asparagine is changed to a serine, is located on the surface of the CD5 domain when modeled alone, but the surface of this domain could sit close to another domain in the full-length FI protein, so folding could be disrupted by this mutation.

The three mutations at solvent-exposed positions that showed impaired activity on the surface are located on different domains, FIMAC, LDLr1 and SP. Pro32 in the FIMAC domain may be involved in binding to surfaces when degrading C3b, since the P32A mutant showed impaired surface-bound activity. It is difficult to draw any conclusions from the A222G mutation that had impaired secretion and function on surfaces. The semi conserved Ala222 is solvent-exposed in the LDLr1 model, but we cannot say how the residue is oriented with respect to the rest of the FI protein Fig 11. The residue Asp501 is part of the specific binding pocket that interacts with the substrate and directs it to the active site, Fig 9 and 11. When asparagine replaces this aspartate the interaction cannot take place and the FI protein becomes inactive.

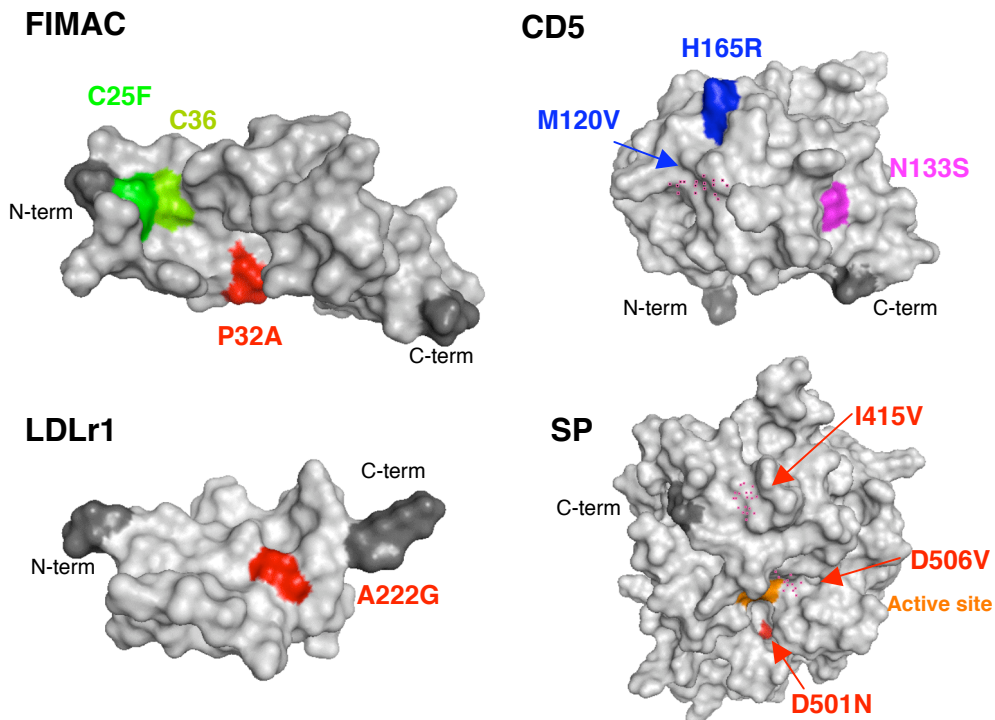


Figure 11. Models of the FIMAC, CD, LDLr1 and SP domains with CFI mutations in aHUS patients highlighted. Mutations with normal activity is marked in blue, impaired surface activity is marked in red, the mutations that did not secrete are marked in green or magenta, the Cys36 that is forming a disulfide bridge with Cys25 is marked in light green, the active site is marked in orange. The M120V (dots colored in magenta) is located on the other side of the CD5 domain. The I415V and D506V (dots colored in magenta) are buried in the SP domain.

The fourth paper describes a large study of the French cohort of aHUS patients, organized by Dr. Veronique Fremeaux-Bacchi. Among the 202 aHUS patients, 23 patients had a mutation in the *CFI* gene. Sixteen missense mutations, one non-sense mutation and one deletion mutation were identified in these patients and all except for one mutation were heterozygous. Nine of the mutations were identified in the SP domain and the rest were spread throughout the heavy chain. The remarkable finding when sequencing these patients was that seven patients also had additional mutation(s) in other complement genes (*CFH*, *MCP*, *C3*, *CFB*, or *CFH* and *MCP*), one patient had a SNP in the *C4BPA* gene and five patients had a deletion of the *CFHR1* gene.

The clinical outcome between the two patient groups, those with only a *CFI* mutation (10 patients) and those with a *CFI* mutation together with an additional

mutation or deletion (13 patients), were different. Patients with more than just the *CFI* mutation develop ESRD more often (77%) than patients with only the *CFI* mutation (50%).

When analyzing the effect of the mutations in an *in vitro* system using transient transfection of HEK293 cells, most of the mutations lead to decreased secretion of FI. The patients carrying these mutations had lower levels of FI or were in the low range of the normal interval. The sub-cellular localization of two of the mutations that had impaired secretion was examined using confocal microscopy. The wild type FI was localized in the trans-Golgi apparatus, whereas the two mutants (I398L and A413T) were localized in the endoplasmic reticulum. The mutants may show impaired interactions with those chaperon proteins in the endoplasmic reticulum that help the protein to fold correctly. Two other mutants (I415V and D506V) had no effect on secretion so they were tested in functional assays, Fig 11. Both the mutants degraded C3b and C4b as efficiently as wild type in the fluid-phase but both had impaired function when cleaving C3b deposited on sheep erythrocytes.

The *CFI* mutations identified in these aHUS patients mainly affected the production/secretion of the FI protein but the patients had almost normal FI levels. This emphasizes that even if an aHUS patient has normal FI concentration a mutation in the *CFI* gene cannot be excluded and therefore it is important to sequence the *CFI* gene in all aHUS patients.

Structural investigation of FI (Paper V)

In the last study (paper V), we attempted to elucidate the importance of the heavy chain in the function of FI. Serine protease activity is located in the light chain but so far the function of the heavy chain is not clear. Currently, it is hypothesized that the heavy chain blocks the active site of the light chain but upon interaction with its substrates and cofactors there is a conformational change in the FI protein that makes the active site accessible.

The structures of the domains of the heavy chain (FIMAC, CD5, LDLr1 and 2) have been predicted using homology-based modeling. The importance of several putative binding patches on these domains was investigated in mutagenesis studies, where between two and five amino acid residues were changed simultaneously. In total 18 mutants were produced, but only 16 of them were expressed and secreted and therefore the binding and function of only these mutants could be analyzed. The mutations located in the FIMAC and the LDLr2 domains resulted in both reduced binding to C3met and an almost complete loss of function for degradation of C3b and C4b in the fluid-phase and for C3b deposited on surfaces. Some mutations in the CD5 and LDLr1 domains also affected binding and function but not to the same extent as the mutations in the FIMAC and LDLr2 domains. The mutations with reduced and normal binding and function are highlighted in different colors in Fig 12.

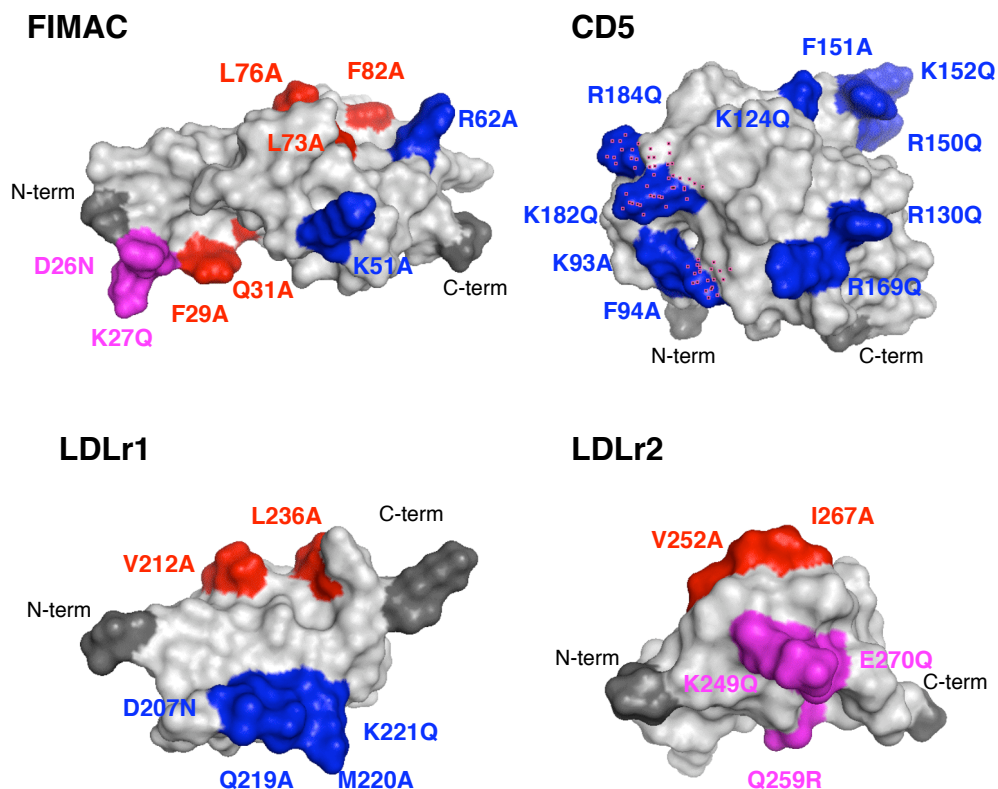


Figure 12. Mutations of FI analyzed regarding binding and function. Mutations marked in magenta are not secreting, red are not functional and blue are functional (but some have imparied function). The mutant consisting of F94A/K182Q/R184Q was not functional and it is marked with dots in magenta.

It seems that large portions of the FIMAC and LDLr2 domains and to some extent the CD5 and LDLr1 domains are involved in the binding and cleavage of C3b and C4b. These results show the complexity of the FI protein that all domains contribute to its function.

Conclusions

Mutations in the *CFI* gene have been identified in patients completely lacking in FI and from patients suffering from aHUS. The mutations in the patients with a complete FI deficiency mainly affect the expression and secretion of the FI protein, whereas the mutations in aHUS patients could affect the expression and secretion but also the function of FI. Therefore, the patients with a complete FI deficiency have very low or undetectable levels of FI in contrast to aHUS patients, who often have normal levels of FI. The mutations in the patients completely lacking in FI are homozygous or compound heterozygous, but in the aHUS patients the mutations are only heterozygous. Some patients completely lacking in FI have parents with only one heterozygous mutation, but they are healthy and have not develop aHUS. One reason for this could be that to develop aHUS, the patient must carry several mutations or polymorphisms in different complement proteins. According to the multiple-hit theory these mutations predispose to disease, but are not a direct cause of disease and the threshold required for disease development decreases with an increasing number of these minor mutations/polymorphisms. Initiation of aHUS in patients with *CFI* mutations may also be associated with infections or a pregnancy but this has not been studied in detail.

It seems that the mutations in both complete FI deficient and aHUS patients are located in all the domains of FI, there is no single domain that is mainly involved in one type of disease as seen for the aHUS mutations in the *CFH* gene. The disease-causing *CFH* mutations are predominantly located in the C-terminus of the protein. It appears that a complete deficiency of FI also leads to the consumption of complement, thus rendering patients susceptible to infections. However, no tissue damage should occur since there is no complement. In aHUS, mutations in *CFI* cause relatively small defects leading to a slight tipping of the balance towards too much complement activity. This ultimately leads to damage in sensitive tissues, such as the kidneys.

In the last study we have identified several solvent-exposed amino acids in the FIMAC, CD5, LDLr 1 and 2 domains that are involved in the function of FI. It appears that part of the heavy chain blocks the active site in the SP domain and upon interaction with the substrate and the cofactor, a conformational change in the FI protein occurs, making the active site available for substrate cleavage.

Future perspectives

Today, there is no satisfactory treatment for patients with a complete FI deficiency. These patients can be vaccinated against several pathogens that give rise to recurrent infections and when they succumb to infections antibiotics can be given. So far, there is no effective treatment to replace the missing FI in order to restore complement system inhibition. One potential therapeutic possibility would be to treat these patients with recombinantly produced FI. The major drawback of this approach would be that the patients entirely lacking FI could develop autoantibodies against FI, which could inactivate FI or cause faster degradation/clearance of the FI protein from the circulation.

When it comes to the treatment of aHUS patients, the first step is plasma infusion or plasma exchange. This helps the patients to survive and in some cases they are even cured. However, in many patients this therapy is not sufficient and they develop ESRD, which in some cases leads to death. aHUS patients may have normal levels of complement proteins but a protein can still be mutated and dysfunctional. Therefore, it is very important to screen several genes (*CFI*, *CFH*, *MCP*, *C3* and *CFB*) of the complement system in every patient since an aHUS patient can have more than one mutation or polymorphism predisposing to the disease. The treatment also depends on which mutations are found. Those aHUS patients with *MCP* mutations have a good outcome following renal transplantations but patients with *CFI* or *CFH* mutations have a bad outcome, since the majority of FI and FH is produced in the liver, while MCP is expressed in the kidney. Recently, combined liver and renal transplantations have been performed in aHUS patients with *CFH* mutations. The first transplantations were unsuccessful and a recurrence of aHUS was seen in the transplanted kidney. But in most recent reports patients have shown better outcomes if they also underwent plasma exchange before and during the combined transplantation. So far no recurrence in the transplants has been reported.

In the beginning of 2009, two case reports were published describing the effect of treating two patients with severe aHUS with Eculizumab [239, 240]. Eculizumab is a humanized monoclonal antibody directed against the C5 component that blocks the cleavage of C5 and inhibits the formation of MAC. Eculizumab has been successfully used as a treatment for patients with paroxysmal nocturnal hemoglobinuria (PNH) [241]. PNH patients are deficient for glycosylphosphatidylinositol (GPI) anchors, including the GPI-linked complement regulator CD59 that inhibits the formation of MAC. This leads to lysis of erythrocytes and thus to hemolytic anemia. The first aHUS patient, described in the paper, has a heterozygous mutation in the *CFH* gene and a homozygous deletion in the *CFHR1* gene, while the second patient has congenital aHUS but no mutations were found in the *CFI*, *CFH* or *MCP* genes. Both patients were unresponsive to plasma therapy but when given Eculizumab to completely block complement activity

their platelet levels increased, creatinine levels were reduced and kidney function was recovered. These two cases are the first report of a successful treatment for aHUS patients who do not respond to plasma therapy and get recurring disease following transplantation. In the future, maybe this will be the preferred treatment for aHUS patients, but it will require larger studies before any recommendations can be made. Perhaps this treatment is also going to be effective in patients with a complete FI deficiency, since these patients will also benefit from complement inhibition. This could be especially true for patients that also have renal disease, since their kidneys are destroyed by complement. One advantage of using Eculizumab is that it is already an approved drug for PNH patients and already available on the market.

In the future, probably more *CFI* mutations will be identified in both complete FI deficient and aHUS patients, which will hopefully give greater insight into the function of the individual domains of FI. However, in order to understand exactly how the heavy chain functions the complete 3D-crystal structure is needed. Then, putative models of the interaction between FI, C3b and cofactors can be constructed. During my PhD studies I have tried to make deletion mutants of FI, in which one domain was deleted at the time. Unfortunately, none of these deletions mutants could be successfully expressed and secreted, despite the fact that the constructs were planned based on intron/exon boundaries. This implies that the structure of full length FI is more complicated than anticipated.

During the last years several X-ray crystal structures of complement proteins such as C3, C3b, FB and part of the MAC have been solved. Last year, the structure of C3b in complex with the N-terminus of FH (CCP1-4) was solved, revealing details of how these proteins interact. FH binds in an extended fashion to C3b and the C3b-FH complex exposes residues that are putatively important for cofactor activity and thus form the binding site for FI that may then degrade C3b. In the future the co-crystallization of C3b-FH together with FI may also be possible, giving us very valuable information about this tri-molecular complex.

Populärvetenskaplig sammanfattning på svenska

Människans immunförsvar är livsnödvändigt och gör det möjligt för oss att bekämpa olika bakterier, infektioner och sjukdomar. Immunförsvaret är uppdelat i två delar; det medfödda, där komplementsystemet är en viktig del, samt det förvärvade immunförsvaret. Det förvärvade immunförsvaret bygger man upp under hela sitt liv och produktionen av antikroppar, som ser till att bekämpa bakterier och virus, är en väsentlig del. Ett exempel på det förvärvade immunförsvaret är vid vaccinering där ett minne för att producera en viss typ av antikroppar aktiveras, vilket resulterar i att man blir immun mot t.ex. mässlingen eller röda hund och alltså inte blir sjuk. Självklart så interagerar dessa två system med varandra, som allt annat i kroppen.

Komplementsystemet, som den här avhandlingen handlar om, består av flera lösliga och membranbundna proteiner, som antingen cirkulerar i blodet eller sitter bundna på olika celler i kroppen. Detta är vårt första försvar mot sjukdomsframkallande (patogena) bakterier och virus och det är ett potent och starkt försvar. Komplementsystemet är som en kaskad, där det kan aktiveras på tre olika sätt beroende på vad som känns igen som främmande i kroppen och som sedan eskalerar för varje protein som involveras. Många proteiner i komplementsystemet är proteaser, dvs. proteiner som kan klyva andra proteiner. Det är inte bara bakterier eller virus som kan aktivera komplementsystemet utan även skadade kroppsegna celler eller immunkomplex mellan antikroppar och antigener. Effekten av komplementsystemet är att aktivera de vita blodkropparna, vilka attraheras till infektionsstället och där kan kroppens egna celler lättare "äta upp", bryta ner och därmed bekämpa bakterierna. Dessa vita blodkroppar kan även aktiveras och börja producera en massa små molekyler, som i sin tur kan hjälpa till att aktivera det förvärvade immunförsvaret att producera antikroppar eller döda bakterierna. En annan konsekvens är att komplementsystemet kan bilda hål i bakterierna, som gör att de spricker p.g.a. att det blidas obalans i trycket, vilket kan jämföras med att man sticker hål på en ballong. För att reglera komplementsystemet så att det inte äter upp eller gör hål i våra egna friska celler finns det även lösliga och membranbundna hämmare. Hämmarna reglerar komplementsystemet på olika nivåer i kaskaden och det är för att ibland kan det vara en fördel att få en liten aktivering, för att t.ex. städa undan skadade celler som bildats under olika processer i kroppen, men att det inte ska leda till hål i cellerna. Patienter kan sakna ett eller flera proteiner i komplementsystemet, vilket kan leda till att de inte kan aktivera eller reglera komplementsystemet i samma mån som i vanliga fall och detta kan leda till olika typer av sjukdomar. Vissa får autoimmuna sjukdomar där kroppens egna celler känns igen som något främmande och därmed framkallar ett immunförsvar mot sina egna celler. Andra patienter kan få fler och upprepande infektioner, speciellt orsakade av bakterier. Anledningen till att dessa patienter saknar något protein beror ofta på att de har en mutation i genen, som kodar för det proteinet. Mutationen kan leda till att proteinet inte kan produceras, att

det bryts ner för att det inte kan veckas på rätt sätt eller att proteinet kan produceras som normalt men att det inte är funktionellt. En tredje orsak kan vara att kroppen har börjat producera antikroppar mot proteinet, vilket resulterar i att det bryts ner eller blir inaktivt.

I den här avhandlingen har jag studerat ett protein som kallas för faktor I och det hämmar komplementsystemet genom att klyva två viktiga proteiner. Patienter som helt saknar detta protein p.g.a. mutationer i både genen från modern och från fadern, kommer ofta till sjukhuset för att de får upprepade lung-, öron- och hjärnhinneinflammationer. Detta är en ganska ovanlig bristsjukdom och hittills är det endast ca 40 patienter beskrivna i litteraturen. Patienter med atypiskt hemolytiskt uremiskt syndrom (aHUS) är en annan grupp av patienter, som har mutationer i faktor I genen. aHUS är en njursjukdom, där det bildas små blodproppar i de minsta artärerna i filtreringsenheten (glomeruli) i njuren. Detta leder till att de röda blodkropparna förstörs och att blodplättarna fastnar i blodpropparna och därmed får patienterna låga nivåer av både röda blodkroppar och blodplättar. Detta i sin tur leder till akut njursvikt och i värsta fall till döden. Vanligtvis behandlas dessa patienter med plasmaersättning men njurtransplantation är ofta nödvändigt. Vissa aHUS patienter har även mutationer eller antikroppar mot andra komplementproteiner. I fyra manuskript har jag analyserat konsekvensen av de olika mutationerna i faktor I genen, som har identifierats i dessa två patientgrupper. Genom att producera faktor I proteinet med de olika mutationerna har jag sedan kunnat testa deras funktion i lösning och på cellytan. Det visade sig att mutationerna i patienter med klassisk faktor I brist påverkar proteinet på ett sådant sätt att strukturen förändras och därmed bryts ner innan det kommer ut från cellerna. När det gäller mutationerna i aHUS patienter kan dessa dels påverka hur mycket proteinet som produceras men även resultera i att proteinets funktion är nedsatt, speciellt på cellytan. Det har även visats att aHUS patienter, som får en njure transplanterad, nästan alltid får tillbaka sjukdomen inom kort tid och att den transplanterade njuren går förlorad. På senare år har man dock lyckats att transplantera både njure och lever samtidigt (levern är den största källan till komplementproteiner) hos aHUS patienter och det har visats sig vara en lyckad kombination om de även får plasmaersättning innan och under operationen. Senast i år har en annan behandling testats på två aHUS patienter, som blev allt sämre och som inte svarade på plasmaersättning. De behandlades med en kommersiellt tillverkad antikropp, som hämmar komplementsystemet. De återhämtade sig inom de första dygnet och deras njurfunktion återgick till det normala. Detta är en banbrytande förändring i hur man kan behandla aHUS patienter, som inte svarar på plasmaersättning eller njurtransplantation.

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