Complement-mediated kidney diseases: Genotype, phenotype and inhibition studies

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Complement-mediated kidney diseases: Genotype, phenotype and inhibition studies

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Abstract:
Complement-mediated kidney diseases are ultrarare conditions characterized by excess complement activation related in most cases to complement gene variants or circulating autoantibodies. These conditions are chronic and can lead to kidney failure. They include atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy (C3G), and, to a certain extent, immune complex-membranoproliferative glomerulonephritis (IC-MPGN). In this thesis, a panel of genes was studied including CFH, C3, FB, F1, CD46/MCP, C5, CFHR1-5, CFP, CLU, DGKE, THBD and PLG.

The phenotype of three heterozygous CFB variants was characterized in Paper I in which one variant (D371G) was shown to have gain-of-function properties and form excess C3 convertase. The phenotype was compared to a well-characterized CFB variant, D279G, and studies showed that a factor D inhibitor, Danicopan, could inhibit cleavage of factor B and excess complement activation as determined by hemolysis of rabbit red blood cells and release of C5b-9 from human glomerular endothelial cells. In Paper II the CFB D371G variant was further studied in a large pedigree in which three family members were affected by aHUS and seven were carriers of the variant. Two of the carriers were adult monozygotic twins but only one was affected by the disease. As they did not carry other variants this suggests that the CFB D371G variant predisposes but is not the sole factor associated with the development of the aHUS phenotype. In Paper III a large cohort (n=141) of Nordic patients with the three kidney diseases was investigated. Patients (73% aHUS and 38% C3G) were found to have genetic variants with a minor allele frequency <1% or with known association with these conditions. Twenty-six of the variants were novel. Importantly, many patients had more than one genetic variant, and 17 variants occurred in both patients with aHUS and C3G. The latter indicates that genotype per se does not predict phenotype in these conditions. In Paper IV the phenotype of a heterozygous variant in CFHR5, M514R, was investigated. The variant was found in a child with aHUS with a deletion of CFHR3/CFHR1 as well as antibodies to factor H. The variant was minimally secreted from cells and the patient had low levels of circulating factor H-related protein 5 (FHR5). The addition of FHR5 to patient serum reduced hemolysis of rabbit red blood cells, and at higher concentrations, this even occurred in normal sera. We, therefore, suggest that this genetic variant could contribute to complement activation.

In summary, this thesis describes many novel variants in genes encoding complement proteins associated with aHUS, C3G and IC-MPGN, and describes the phenotype of several variants to better understand how they cause disease. Furthermore, a factor D inhibitor was effectively shown to block factor B degradation and down-stream complement activation.

Key words: complement, factor B, factor D, danicopan, atypical hemolytic uremic syndrome, C3 glomerulopathy, membranoproliferative glomerulonephritis, genes, monozygotic twins

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Cover photo: Immunofluorescence microscopy image of C3 deposition on glomerular endothelial cells in the presence of serum from patient with atypical hemolytic uremic syndrome associated with a gain-of-function CFB variant, by Sigríður Sunna Aradóttir

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Every man should be moderately wise, let him never be too wise; let no one have foreknowledge of his fate, one’s mind is [then] freest from sorrows (1)

Hávamál verse 56, unknown author (800-900 AC)
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List of Papers

This thesis includes the following papers:


Papers I-III are open access publications.
Abbreviations

aHUS  Atypical hemolytic uremic syndrome
AMD  Age-related macular degeneration
C3GN  C3 glomerulonephritis
C3G  C3 glomerulopathy
C3NEF  C3 nephritic factor
C4BP  C4-binding protein
C5aR1  C5a-receptor 1
C5aR2  C5a-receptor 2
CR1  Complement receptor 1
CR2  Complement receptor 2
DAF  Decay accelerating factor (CD55)
DDD  Dense deposit disease
FHL-1  Factor H-like protein 1
FHR  Factor H-related protein
MAC  Membrane attack complex
MCP  Membrane cofactor protein (CD46)
MPGN  Membranoproliferative glomerulonephritis
MBL  Mannan binding lectin
MASPs  Mannan-binding lectin-associated serine proteases
MAF  Minor allele frequency
NK-cells  Natural killer cells
PAMP  Pathogen associated molecular pattern
PNH  Paroxysmal nocturnal hemoglobinuria
PRR  Pattern recognition receptor
RCA  Regulators of complement activation
SNP  Single nucleotide polymorphism
TMA  Thrombotic microangiopathy
Preface

Genotyping has become an important part of clinical practice in a broad spectrum of specialties. The human genome project generated the first sequence of the human genome. Genome-wide association studies have revealed previously unknown genotype-phenotype associations. Genes known to be associated with a certain condition can be studied with targeted gene sequence panels. This evolution has created data on several rare variants, and functional testing of these can elucidate the mechanism of disease, paving the way for the development of specific therapies, and precision medicine.

Rare disease is defined as a condition affecting less than 1 in 2000 persons. Rare diseases are a challenge for the clinician and are often difficult to diagnose. It has been shown that ~80% of rare diseases have a genetic cause (2). There are no approved treatments for the majority of rare diseases (3). It is estimated that there are more than 30 million individuals living with rare diseases in the European Union and more than 300 million globally.

Atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G) are examples of rare diseases that are severe and associated with genetic variants, that result in excess activation of the complement system. The pathophysiology is associated with dysfunction of complement proteins caused by rare genetic variants or acquired autoantibodies. The penetrance of the phenotype is incomplete, and thus there are other factors determining the development of disease.

In this thesis, I will introduce the complement system and the kidney diseases associated with its excess activation, describe the genetic background studies, the available treatments and the state of current knowledge including my scientific contributions.
Abstract

Complement-mediated kidney diseases are ultrarare conditions characterized by excess complement activation related in most cases to complement gene variants or circulating autoantibodies. These conditions are chronic and can lead to kidney failure. They include atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy (C3G), and, to a certain extent, immune complex-membranoproliferative glomerulonephritis (IC-MPGN). In this thesis, a panel of genes was studied including *CFH*, *C3*, *FB*, *FI*, *CD46/MCP*, *C5*, *CFHR1-5*, *CFP*, *CLU*, *DGKE*, *THBD* and *PLG*.

The phenotype of three heterozygous *CFB* variants was characterized in **Paper I** in which one variant (D371G) was shown to have gain-of-function properties and form excess C3 convertase. The phenotype was compared to a well-characterized *CFB* variant, D279G, and studies showed that a factor D inhibitor, Danicopan, could inhibit cleavage of factor B and excess complement activation as determined by hemolysis of rabbit red blood cells and release of C5b-9 from human glomerular endothelial cells. In **Paper II** the *CFB* D371G variant was further studied in a large pedigree in which three family members were affected by aHUS and seven were carriers of the variant. Two of the carriers were adult monozygotic twins but only one was affected by the disease. As they did not carry other variants this suggests that the *CFB* D371G variant predisposes but is not the sole factor associated with the development of the aHUS phenotype. In **Paper III** a large cohort (n=141) of Nordic patients with the three kidney diseases was investigated. Patients (72% aHUS and 38% C3G) were found to have genetic variants with a minor allele frequency <1% or with known association with these conditions. Twenty-six of the variants were novel. Importantly, many patients had more than one genetic variant, and 17 variants occurred in both patients with aHUS and C3G. The latter indicates that genotype per se does not predict phenotype in these conditions. In **Paper IV** the phenotype of a heterozygous variant in *CFHR5*, M514R, was investigated. The variant was found in a child with aHUS with a deletion of *CFHR3/CFHR1* as well as antibodies to factor H. The variant was minimally secreted from cells and the patient had low levels of circulating factor H-related protein 5 (FHR5). The addition of FHR5 to patient serum reduced hemolysis of rabbit red blood cells, and at higher concentrations, this even occurred in normal sera. We, therefore, suggest that this genetic variant could contribute to complement activation.

In summary, this thesis describes many novel variants in genes encoding complement proteins associated with aHUS, C3G and IC-MPGN, and describes the phenotype of several variants to better understand how they cause disease. Furthermore, a factor D inhibitor was effectively shown to block factor B degradation and down-stream complement activation.
The immune system

The host immune system has an important role in maintaining homeostasis when confronted with external and internal challenges. Invading pathogens are an example of an external threat and protection requires an intact barrier of the host, a response if the invader breaks through, and finally elimination of the pathogen and repair of tissue. The ideal immune response would be to perform these actions, without any deleterious effect on host tissues (4). The importance of the immune system is demonstrated by the consequences of its dysfunction. Hypofunction leads to infections and an increased risk of developing malignancies, while overactivation leads to autoimmune diseases.

The immune system consists of the innate and the acquired adaptive immune systems that are intertwined.

The innate immune system

The innate immune system is the evolutionarily most conserved part of the immune system. Its main function is to rapidly prevent infection by elimination of invading pathogens, and activation of the adaptive immune response when required (5). It performs this by recognizing common pathogen-associated molecular patterns (PAMPs) on pathogens, and danger-associated molecular patterns (DAMPs) on damaged host cells.

Disruption of homeostasis is sensed by a variety of proteins termed pattern recognition receptors (PRRs), that recognize and interact with components of the pathogen or damaged host cell. PRRs are mostly expressed by macrophages and dendritic cells and endothelial cells. They can be soluble or membrane-bound, recognizing pathogens such as bacteria or viruses extracellularly or intracellularly (6, 7). Recognition of “non-self”- PAMP surfaces initiates a signalling pathway. A prototypical example of a PAMP is lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria. LPS is recognized by Toll-like receptor 4 that triggers an intracellular signalling pathway resulting in the generation of pro-inflammatory mediators. Similarly, endogenous molecules released from stressed, injured, or dying host cells are examples of DAMPs (6). These can be altered phospholipids, heat shock proteins, adenosine triphosphate (ATP), deoxyribonucleic acid (DNA),
ribonucleic acid (RNA), or molecules usually located within lysosomes or mitochondria that spill out to the extracellular compartment as a result of stress.

The innate immune system consists of physical and chemical barriers as well as cellular and humoral elements. The first-line barrier in the mammalian host is the epithelial surface of the skin and mucous membranes. This barrier contains three main hinders for invaders: The normal bacterial flora, that inhibits excessive growth of other microorganisms, a mechanical barrier formed by tight junctions between epithelial cells, the mucosal layer of mucosal membranes, and a chemical barrier including antimicrobial peptides (8, 9). The cellular part involves granulocytes, macrophages, dendritic cells, mast cells, and natural killer cells, and the humoral part is composed of specific effector proteins such as cytokines, chemokines and the complement system. The complement system is a complex surveillance system in defense against pathogens, as well as in maintaining host homeostasis, initiating inflammation, and activating the adaptive immune system (10). The complement system will be elaborated on below.

The adaptive immune system

The main function of the adaptive immune response is to distinguish between host and foreign antigens, generating an adapted pathogen-specific immunologic effector mechanism, that eliminates the pathogen or infected cells and develops immunological memory for quick elimination of a specific pathogen. It consists of a cellular response mediated by T lymphocytes and a humoral response mediated by antibodies produced by B lymphocytes. The development of this specific response takes days to weeks (11). Lymphocytes are activated by non-self antigens in mucosal membranes or peripheral lymphoid organs, including lymph nodes, the spleen, and tonsils (12).

Auto-antibodies are formed if tolerance mechanisms are dysfunctional, and this leads to the maturation of auto-antibody producing B-cells as well as their subsequent differentiation into antibody-secreting plasma cells (13).
The complement system

The complement system was first identified by Jules Bordet as a heat-sensitive component of human plasma that “complemented” antibodies in the killing of bacteria (14). The complement system includes approximately 50 proteins, mainly synthesized in the liver (5). Complement proteins are found circulating as inactive zymogens, or as membrane-bound proteins. Complement activation occurs in the fluid phase, on the cell membrane, and as well as intracellularly (5). The complement system responds through a well-coordinated sequence of enzymatic reactions, resulting in the elimination of microorganisms, immune complexes, and cells that are damaged, altered, or undergoing apoptosis. This elimination can occur through processes such as opsonization and phagocytosis, cellular lysis, or by triggering the activation of the adaptive immune system (15, 16). During this process, the activation of complement leads to inflammation through the liberation of anaphylatoxins (17). Furthermore, the complement system serves as a connection between the innate and adaptive immune systems, participating in the stimulation of B cells, the removal of B cells that react to self, and the facilitation of T cell responses (16). Complement participates in crosstalk with both the coagulation system and the contact system (18).

Depending on the trigger, the complement cascade is initiated via the classical, the lectin, or the alternative pathway (Figure 1) (19-21). Activation via the classical and lectin pathways necessitates the presence of pattern recognition molecules (such as C1q, mannan-binding lectin, and collectins) capable of distinguishing between self and non-self surfaces, by attaching to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). The alternative pathway is triggered by activated non-self surfaces and does not rely on a recognition molecule. All three pathways converge in the activation of the C3 convertase (22). This pivotal element of the complement system consists of fragments from activated C3 and factor B. The pathways are described below.
Overview of the complement system

Complement is initiated through three pathways: the classical, lectin, and alternative pathways, which converge at the level of C3 in the cleavage of C3 to its fragments C3b and C3a. The C3b fragment can attach to surfaces via a thioester bond, and the soluble C3a fragment binds to its receptor C3aR. Progression of the complement cascade results in the cleavage of C5 to its fragments C5b and C5a. C5b bound to C6-C9n forms the membrane attack complex. C5a is a chemoattractant and an anaphylatoxin.

**Classical pathway activation**

The initiation of the classical pathway occurs when the recognition molecule C1q interacts with PAMPs on pathogens or DAMPs on apoptotic cells in a calcium-dependent manner (23). C1q in complex with the serine proteases C1s and C1r (C1qr2s2) binds to the Fc domain of an antibody in complex with an antigen (20). The classical pathway can also be triggered in an immune complex independent manner (5) for example by LPS of bacteria (24) and surface molecules of dying cells (25). Upon binding its target, the C1qr2s2 complex undergoes a conformational change that triggers the activation of serine proteases. As a result, C1s cleaves C4 and C2 into their subunits, enabling the formation of the classical/lectin pathway C3 convertase C4bC2a (26, 27).
**Lectin pathway activation**

The lectin pathway is initiated by ficolins especially mannose-binding-lectin (MBL) (28), and collectins, that recognize and bind to PAMPs on the cell membranes of bacteria, viruses, and on and DAMPs damaged or dying cells (29). The serine proteases of the lectin pathway are called MBL-associated serine proteases (MASPs). MASP-1 or MASP-2 form a complex with ficolins or collectins (30). MASP-1 cleaves C2, and simultaneously activates MASP-2 an in a Ca++-dependent process (31). MASP2 cleaves both C2 and C4, leading to the formation of the classical/lectin pathway C3 convertase C4bC2a (32, 33).

**Alternative pathway activation**

The alternative pathway is constitutionally active and can therefore respond rapidly to invading pathogens (5). Hydrolysis of C3 results in a C3(H2O) conformation that enables the binding of the serine protease factor B (34). On binding factor B undergoes a conformational change allowing factor D to attach in a Mg++-dependent reaction (35). Factor D enzymatically cleaves factor B, resulting in the generation of the smaller Ba fragment and the larger Bb fragment. Bb remains bound to C3(H2O), giving rise to the alternative pathway C3 pre-convertase, designated as C3(H2O)Bb. This pre-convertase holds the capability to cleave C3 into C3b and C3a. This cleavage action exposes a highly reactive thioester bond in C3b, facilitating its swift attachment to activating surfaces (36). This process is referred to as the tick-over theory (37). When an activating surface is not present, the liberated C3b undergoes rapid degradation.

Activating surfaces enable the binding of deposited C3b to factor B. Subsequently, factor D catalyses the cleavage of factor B into its fragments, facilitated by the presence of Mg++. This process culminates in the formation of the C3 convertase termed C3bBb (38). The surface-bound C3bBb convertase is stabilized by properdin (39). This gives rise to a cycle of amplified C3b production, constituting the amplification loop of the alternative pathway (Figure 2). Properdin is the main activator of the alternative pathway (40). In contrast to most complement proteins, properdin is expressed extra-hepatically by leukocytes and neutrophils release properdin upon activation (41). The amplification loop accounts for nearly all deposited C3b irrespective of the initiating pathway of complement activation (42, 43).

In addition to the above C3b has an opsonizing role in labelling pathogens as well as immune complexes for clearance by phagocytosis (44).

C3a is a mediator of inflammation, an anaphylatoxin, as binding to the C3a receptor (C3aR) stimulates mast cells to release histamine, inducing smooth muscle contraction, vasodilatation, and cytokine release (45). Cytokine release results in the chemotaxis of leukocytes to the sites of inflammation.
Terminal pathway activation

C3bBb or C4b2a convertases possess the ability to attach extra C3b molecules, resulting in the formation of C5 convertases C3bBb3bn or C4b2a3bn (46). This event marks the commencement of the terminal pathway of the complement system. Subsequently, the C5 convertases cleave C5, releasing the subunit C5a, a highly potent chemoattractant and anaphylatoxin (47). C5b undergoes a change in structure upon engaging with C6 and C7, resulting in its detachment from the convertase. The C5b-7 complex features a hydrophobic surface that adheres to external surfaces and recruits C8 (48). The C5b-8 complex can create a pore-like structure within the membrane of the target cell, leading to cell lysis. However, this process becomes more efficient in inducing osmotic lysis when multiple copies of C9 are added, resulting in the formation of the C5b-9 complex, also known as the membrane attack complex (MAC). This complex acts as a channel for the outward movement of ions and the inward influx of water, enhancing the process of osmotic lysis. (49). Erythrocytes and gram-negative bacteria are easily subject to lysis, whereas nucleated cells and thick-walled gram-positive bacteria exhibit resistance and require multiple impacts for the lysis process to occur (50). sC5b-9 (TCC) molecules signify unsuccessful insertions and serve as indicators of terminal complement activation (51).

Furthermore, the membrane attack complex (MAC) possesses pro-inflammatory characteristics that lead to the expression of pro-inflammatory mediators and cytokines (52).

The highly potent chemoattractant and anaphylatoxin C5a can attach to two distinct G-protein coupled receptors known as C5a receptor 1 (C5aR1)(53) and C5a receptor 2 (C5aR2) (54). These receptors, C5aR1 and C5aR2, are present on various cell
types in tissues, as well as on myeloid cells such as monocytes/macrophages, neutrophils, basophils, eosinophils, and mast cells (55). C5a's binding to C5aR1 results in the release of cytokines. C5aR2, on the other hand, plays a modulatory role in the activity of C5aR1 (56).

Complement factor B

Factor B is an essential component of the C3 convertase. It is transcribed by the \( CFB \) gene on chromosome 6 (6p21.33) localized within the major histocompatibility complex class III region gene cluster (57). Factor B is mostly expressed by hepatocytes, but can even be expressed by immune cells, fibroblasts, intestinal epithelial cells and kidney tubular cells (58-61). Factor B is a single-chain polypeptide with a molecular weight of 93 kDa, made up of 764 amino acids (depicted in Figure 3). The N-terminal fragment Ba (30 kDa) consists of three complement control proteins (CCPs) also termed short consensus repeats or sushi domains (referred to as CCPs in this thesis). The larger C-terminal fragment Bb (73 kDa), contains a von Willebrand type A (VWA) domain, and a serine protease domain (62). The serum concentration is approximately 200\( \mu \)g/mL, compared to C3 which circulates at a concentration of approximately 1200 \( \mu \)g/mL (58).

![Figure 3: Structure of complement factor B](image)

Factor B undergoes a conformational change upon binding to C3b(H2O) or C3b. This alteration affects the MIDAS (Metal-ion-dependent adhesion) site in the VWA domain region, facilitating the attachment of factor D, which subsequently can cleave factor B (34). Once Bb becomes detached from C3b, it loses the ability to reattach (38).

Factor B deficiency is an exceptionally uncommon condition, documented in only two individuals. In both cases, this deficiency was linked to reduced defense against encapsulated bacteria. This underscores the critical role of factor B in complement-mediated defense against invading pathogens (63, 64).
Overview of complement regulation

Maintaining a balance between complement activation and inhibition is crucial. This equilibrium is essential to prevent excessive complement activation. Control mechanisms are required at every stage of the cascade, involving both soluble and membrane-bound regulators present in human cells (65).

Factor H, C4-binding protein (C4bp), decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP or CD46), complement receptor 1 (CR1, CD35), and complement receptor 2 (CR2, CD21) are among the regulators (66). These proteins are part of the superfamily called regulators of complement activation (RCA), and they are encoded by genes located in the RCA cluster on chromosome 1q32 (67). Additionally, other significant regulators encoded independently include factor I and regulators specific to the terminal pathway. This thesis will focus on regulators of the alternative pathway as they will be investigated.

Figure 4: Overview of complement regulation

Complement regulation is performed at all steps of the cascade and in all pathways as depicted. The main regulators of the alternative pathway are factor H and factor I. The main regulators of the classical and lectin pathway are C1 inhibitor, C4bp and factor I. The carboxypeptidase N inactivates C3a and C5a. Vitronectin, Clusterin, and Protecin regulate the terminal pathway. C4bp in the classical and lectin pathway has a corresponding function as factor H in the alternative pathway. MASP: MBL-associated serine protease. CR1: Complement receptor 1. MCP: Membrane cofactor protein. DAF: Decay accelerating factor. FH: Factor H. C4bp: C4 binding protein. Created with BioRender.com
Regulators of the alternative pathway and amplification loop

Complement factor H is the main soluble regulator of the alternative pathway of complement as well as on the endothelial cell surface (68). Factor H is composed of twenty CCPs. It inhibits the formation of the C3 convertase by binding C3b and accelerates the decay of the C3 convertase by displacing Bb from the C3bBb convertase. Factor H is a cofactor to factor I-mediated degradation and inactivation of C3b (69, 70). The N-terminal CCPs 1-4 mediate C3b binding, cofactor activity and decay-accelerating activity. The two C-terminal CCPs 19-20 interact with molecules found on host cell surfaces and are crucial in host cell recognition (71).

Factor H-like protein-1 (FHL-1) is a truncated form of factor H composed of the first seven N-terminal CCPs and transcribed by alternative splicing of the \textit{CFH} gene. It possesses properties associated with the N-terminal of factor H (72).

Factor I mediates the irreversible degradation of C3b to its fragments iC3b and C3f in the presence of cofactors factor H, MCP (CD46), and CR1. C3f is cleaved off while iC3b remains bound to the cell membrane. Further fragmentation of iC3b leads to the release of C3c while C3dg remains anchored to the membrane (73).

DAF (CD55) and CR1 accelerate the decay of the alternative pathway C3 convertase (74).

Thrombomodulin accelerates factor I-mediated degradation of C3b (75). It also acts as a cofactor for the activation of thrombin-activable fibrinolysis inhibitor (TAFI) to TAFIa, which inactivates C3a and C5a (75). Likewise, carboxypeptidase N cleaves C3a and C5a to their desArg forms, preventing binding to their respective receptors (76).

Regulators of the terminal pathway

Protectin, clusterin, and vitronectin block the assembly of the pore-forming MAC in the terminal pathway. Protectin (CD59) is a glycosylphosphatidylinositol (GPI)-anchored regulator that binds C8 and blocks the recruitment of C9 to the complex (77). Clusterin binds to C7, C8, and C9, and inhibits the correct assembly of the MAC and insertion into the membrane (78). Vitronectin (Protein-S) binds to C5b-7 and inhibits the polymerization of C9, thereby blocking the assembly of the pore-forming complex in the terminal pathway (79). Cub and sushi multiple domains 1 (CSMD1) is a cofactor for factor I mediated degradation of C3b, impedes insertion of C7 and generation of the MAC (80).
Factor H-related proteins

Factor H and factor H-related proteins (FHR) 1-5 comprise a family of structurally related proteins. The encoding genes are positioned in the following order: CFH-CFHR3-CFHR1-CFHR4-CFHR2-CFHR5 (67). The five FHRs are synthesized in the liver and composed of CCPs with various degrees of homology to factor H (Figure 5)(81).

Figure 5: Homologous CCPs of factor H and factor H-related proteins
FHRs bear variable homology to corresponding CCPs of factor H. The N-terminal FHRs resemble CCP 6-8 of factor H. The C-terminal part shares homology to CCPs 19–20 of factor H. FHR-5 also shares certain homology to factor H CCPs 10–14 (82). FHR-1, FHR-2 and FHR-5 contain dimerization domains (marked by*). The molecular weight of each protein is depicted to the left. CCP: Complement control protein. GAG: Glycosaminoglycans. For reference see (83, 84).

FHRs can be subdivided into two groups based on their ability to dimerize (85). FHR1, FHR2 and FHR5 show high amino acid homology in the N-terminal (CCPs 1-2) and circulate in dimeric form, either as heterodimers or homodimers (86). FHR5 circulates in heterodimers (87) and homodimers (86). The non-dimerizing FHR3 and FHR4 proteins have homologous C-terminal domains (83).

Precise quantification of the FHR proteins is challenging due to the high structural homology of proteins in the factor H protein family, as well as the presence of dimers. The circulating concentration of the FHRs is much lower than factor H (88). In this thesis, FHR5 was studied.
Factor H-related protein -5

FHR5 is a 65 kDa plasma protein composed of nine CCPs. It is the least conserved FHR protein and is less prone to form dimers compared to FHR1 and FHR2 (86). CCPs 1-2 contain a dimerization motif, CCPs 5-7 bind to glycosaminoglycans, components of the extracellular matrix, heparin, pentraxin, C reactive protein (CRP) as well as DNA, and CCPs 8-9 bind C3b/C3d and glycosaminoglycans (89, 90).

FHR5 was shown to display weak cofactor activity to factor I and inhibit the C3 convertase (89, 91). The significance of this finding has, however, been questioned due to the concentrations used (92).

Other studies have suggested involvement in complement activation. Surface-bound FHR5 has been observed to engage with C3b, properdin, C1q, and pentraxins, acting as a foundation for the generation of the alternative pathway C3 convertase (83, 93). Others did not find direct binding between FHR5 and properdin (93). FHR5 can compete with factor H for ligand binding and interfere with the regulatory function of factor H thereby activating complement (87, 94, 95).

To further complicate the picture FHR5 was reported to activate the C3 convertase but regulate the C5 convertase, this has been explained by differential binding to C3b (93, 94). Thus, the exact role of FHR5 is still a matter of investigation.
Complement-mediated kidney diseases

Complement activation plays a distinct role in the pathophysiology of certain kidney disorders, including systemic lupus erythematosus, post-streptococcal glomerulonephritis, membranous nephropathy, allograft rejection and ischemic injury, antibody-mediated rejection, IgA nephropathy, and vasculitis. This thesis places particular emphasis on exploring the involvement of complement activation in atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy (C3G), and immune complex-associated membranoproliferative glomerulonephritis (IC-MPGN).

Atypical hemolytic uremic syndrome

Hemolytic uremic syndrome manifests as the triad of non-immune hemolytic anemia, thrombocytopenia, and acute kidney injury. aHUS is associated with complement dysregulation via the alternative pathway. The causative abnormality can be aberrant dysfunctional complement proteins associated with genetic variants or autoantibodies to factor H. The alternative pathway of complement is most commonly involved.

aHUS is an ultra-rare chronic disease with an incidence of 0.5-2 cases/million (96). aHUS can present at all ages (97), both sexes are equally represented in childhood, but females are overrepresented as adults (98). Presentation during the neonatal period or in infancy is most probably associated with genetic variants, either in complement genes or other genes, such as DGKE (99) or MMACHC (cobalamin C type methylmalonic aciduria and homocystinuria) (100). The development of disease is most often associated with a trigger, such as an infection, vaccination, or pregnancy together with a dysregulated complement system (101).

aHUS usually presents acutely with the rapid progress of the disease. Patients present with pallor, icterus secondary to hemolytic anemia, purpura indicating thrombocytopenia, edema, hypertension, and oliguria caused by acute kidney injury. Arterial hypertension can be severe (96). Extrarenal manifestations encompass neurological symptoms such as seizures and coma. Ophthalmological symptoms
such as blindness. Other extrarenal manifestations include pancreatitis, myocardial infarction, gastrointestinal involvement, extracerebral artery stenosis and digital gangrene (102).

Laboratory work up of hemolysis shows anemia with a negative direct antiglobulin test (DAT test), increased reticulocytes, lactate hydrogenase, bilirubin, and low haptoglobin due to consumption (Table 1). Blood films show fragmented red blood cells or schistocytes. Complement work-up involves, as a minimum, measurement of C3, C3dg, and autoantibodies to factor H. Despite dysregulation of the alternative pathway, C3 levels can be normal (101).

<table>
<thead>
<tr>
<th>Table 1: Work-up of a patient suspected of aHUS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First evaluation of the patient with kidney injury and clinically suspected HUS:</strong></td>
</tr>
<tr>
<td>History, physical examination, and medical review.</td>
</tr>
<tr>
<td>History penetrated with regards to triggers: infections, vaccinations, drugs, pregnancy and family history.</td>
</tr>
<tr>
<td>Routine lab work up: Complete blood count with differential count, reticulocyte count, blood smear, conjugated and total bilirubin, lactate dehydrogenase, Direct antiglobulin test (Coombs test), coagulation screen. Kidney, hepatic, and pancreatic function.</td>
</tr>
<tr>
<td>Urine for dipstick, microscopy.</td>
</tr>
<tr>
<td><strong>If clinical suspicion of aHUS:</strong></td>
</tr>
<tr>
<td>Complement C3, C3dg. Anti-factor H antibodies.</td>
</tr>
<tr>
<td>ADAMTS13. Homocysteine, methylmalonic acid (plasma and urine).</td>
</tr>
<tr>
<td>Screening for rheumatologic disease.</td>
</tr>
<tr>
<td>Blood culture.</td>
</tr>
<tr>
<td>Stool: Swab/culture or PCR for EHEC genes.</td>
</tr>
<tr>
<td>Genetic aHUS panel</td>
</tr>
</tbody>
</table>

ADAMTS13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13. PCR: Polymerase chain reaction. ECEC: enterohemorrhagic *Escherichia coli*

aHUS is a clinical diagnosis of exclusion. A kidney biopsy is not essential for the diagnosis and is rarely performed. The pathology observed is thrombotic microangiopathy (103), and typically, immunofluorescence staining yields negative results for immunoglobulins, C3, and C5b-9 (104). Differential diagnoses to be excluded are thrombotic thrombocytopenic purpura (105), hemolytic uremic syndrome associated with Shiga toxin-producing enterohemorrhagic *Escherichia coli* (96), or secondary to neuraminidase-producing pathogens such as *Streptococcus pneumoniae* (106), autoimmune diseases, cancers, drugs, hemopoietic stem-cell or solid organ transplantation, malignant hypertension or monoclonal gammopathy (107, 108).

Complete recovery may occur after the presenting episode, but the disease course is relapsing and if untreated most patients eventually develop kidney failure requiring permanent kidney replacement therapy with dialysis or transplantation. The disease tends to recur after a kidney transplant (109).
For patients with aHUS, the rates of kidney failure or death at 3-5 years was ~40% in children and more than 60% in adults before the introduction of anti-complement therapy (110). The outcome of kidney transplantation was also poor with recurrence of disease in 60-70%, in most cases developing within the first year after transplantation (111). With the emergence of complement inhibitory treatments the risk of developing kidney failure is 10-15% (112).

The pathology

aHUS is a form of thrombotic microangiopathy (TMA). The TMA lesion is characterized by endothelial injury and detachment and platelet aggregation. Occlusion of the microvascular lumen leads to ischemia (96). In the kidney both the glomerular capillaries as well as the arterioles are affected with disrupted swollen endothelium that is detached from the basal membrane, leading to thickened vessel walls. The damaged endothelium is pro-coagulant, leading to fibrin deposition and platelet consumption. This results in thrombocytopenia (113). The glomerular filtration barrier is damaged, and glomerular filtration decreased due to luminal occlusion, leading to ischemic damage to the nephron and acute kidney injury.

Patients presenting with symptoms and signs of thrombotic microangiopathy are usually not biopsied at presentation because of the risk of bleeding complications due to low platelet counts. Available biopsies are either from patients with an atypical presentation, unclear diagnosis, or post-mortem (114, 115).

Genetic drivers of disease

More than 50% of aHUS patients have been shown to carry rare pathogenic complement gene variants (98, 116-121). Most variants are heterozygous with incomplete penetrance (122). Homozygous variants are unusual but have been described in CFH and MCP (123, 124). A combination of variants is reported in ~15% of patients (118, 119, 125). Variants have been identified that affect different components and regulations of the complement system. Variants in complement regulators are the most common. Interpreting the significance of a novel variant is a challenge. Prediction can be carried out by analysis of the minor allele frequency, family pedigree (segregation analysis), and expected consequence on the protein structure using prediction models (126, 127). Familial disease is described in ~20% of cases (120). The inheritance is usually autosomal dominant (122).

Functional testing of mutant complement variants by in vitro and/or in vivo models is important in elucidating the phenotype regarding complement activation.

CFH variants account for 25% of cases. Missense variants in the C-terminal of factor H lead to aberrant host cell recognition (128) as well as decreased decay of the C3 convertase and cofactor activity (129). Patients with CFH variants in the
C-terminal usually have normal FH levels. Mutant variants have also been detected in C3 (130-132), CFB (63, 133), CFI (134-136), MCP/CD46 (137, 138) and in non-complement genes such as THMD (75). Based on the functions of these proteins, described above, mutant variants can lead to a gain-of-function in C3 or factor B, thereby enhancing the activity of the C3 convertase, or loss-of-function in complement regulators by decreasing co-factor activity, degradation of C3 (CFI) or inactivation of C3a and C5a (THMD). CFB variants and their phenotype will be investigated in this thesis.

Deletion of FHR3/1 particularly in homozygous form is associated with auto-antibodies to factor H in aHUS (81). Variants at the C terminal of FHR1 may resemble C-terminal variants in CFH (139). Variants in CFHR5 have also been identified in aHUS (120, 140, 141). To my knowledge functional studies of CFHR5 variants in aHUS have not been reported and one such variant will be investigated here.

**Haplotypes**

Certain combinations of genetic variants in the RCA gene cluster tend to be inherited together as a haplotype and have been associated with aHUS (81). Two specific haplotypes in aHUS are CFH(H3)tgtggt and MCPggaac (142, 143).

**Acquired drivers of disease**

Factor H auto-antibodies are associated with aHUS in 5-10% of cases except in India, where anti-FH autoantibodies are the main cause of aHUS and occur in ~55% of aHUS patients typically associated with CFHR3/1 deletions (144). The antibodies preferentially bind to the C terminal of factor H (145) and inhibit the binding of factor H to the endothelial cell leading to complement dysregulation on the cell surface. The level of antibodies is correlated to disease activity (146). aHUS associated with autoantibodies to FH is a recurrent disease leading to kidney failure (144, 147).

**Management of atypical hemolytic uremic syndrome**

Symptomatic treatment of high blood pressure, kidney failure and kidney replacement therapy are administered if needed. Transplantation with organs from living-related donors carries a risk of de novo disease in the donor if they carry the same disease-associated genetic variant, and careful genotyping is, therefore, crucial (148). Combined kidney and liver transplantation was previously attempted (149). This strategy was associated with complications, complement activation and high mortality.
Complement blockade is a gold standard for the treatment of atHUS in children and adults. Eculizumab is a recombinant monoclonal antibody targeting C5, preventing C5 cleavage while allowing for proximal complement functions such as opsonization and phagocytosis (150-153).

The risk of terminal complement inhibition is infection with encapsulated bacteria. Vaccination against Neisseria meningitis is required. Eculizumab is administered intravenously every other week. Ravulizumab a C5 inhibitor with a longer half-life given every 8 weeks. The availability of these treatments is not universal, and the cost is extremely high. The duration of treatment is contingent on the patient’s risk for recurrence. A subgroup of patients with a specific polymorphism in C5 are non-responders to treatment (154).

Treatment duration with complement inhibitors is controversial, some advocate lifelong treatment for patients carrying variants associated with a more severe phenotype while others suggest discontinuation is possible (155, 156).

Treatment of atHUS caused by auto-antibodies to factor H is immunosuppressive including cyclophosphamide, rituximab, and corticosteroids in combination with plasma exchanges, followed by maintenance with mycophenolate mofetil and prednisolone (157).

C3 glomerulopathy

C3G is a chronic kidney disease that can be categorized into two forms: C3 glomerulonephritis (C3GN) and Dense Deposit Disease (DDD) (158). Both conditions arise due to a disruption in the complement system, specifically, the alternative pathway (159) This dysregulation is typically triggered by rare genetic variations in complement proteins or by auto-antibodies, as elaborated below. C3G is an exceedingly rare disorder, with an estimated incidence of 1 to 3 cases per million (158). DDD mainly manifests during childhood, while C3GN can manifest later in life. The prevalence of both forms is consistent across both sexes.

C3G presentation can be triggered by an upper respiratory tract infection or a streptococcal infection (160). Clinical features are heterogeneous but include hematuria, proteinuria, hypertension and affected kidney function. One third of patients with C3G will develop kidney failure within 10 years and the disease recurs in the transplant in 50% of cases within 5 years (161).

There are two extrarenal features in C3G. These are drusen, electron-dense macular complement deposits in the basement membrane of the retina (Bruch’s membrane) (145, 162) and acquired partial lipodystrophy, loss of subcutaneous fat in the face and the upper half of the body (163).
Decreased C3 levels are found in 40-60% of patients with C3G (164). Levels of terminal activation product sC5b-9 (TCC) are increased in approximately 25% of patients with DDD and 50% of patients with C3GN (165).

**Diagnosis based on pathology**

Diagnosis of C3G is based on clinical characteristics combined with kidney biopsy findings. Immunofluorescence is necessary to make a diagnosis of C3G and electron microscopic evaluation is required to distinguish DDD from C3GN (166).

Light microscopy shows a membranoproliferative or mesangioproliferative pattern of glomerulonephritis. Crescents may be present. A membranoproliferative pattern is characterized by cell proliferation, infiltration of inflammatory cells and deposition of products of complement activation in the capillary wall and mesangium, causing changes in the basement membrane and capillary wall thickening (167).

Immunofluorescence microscopy shows dominant glomerular C3 staining of at least two orders of magnitude greater than the intensity for other immune reactants (168). Staining for C5b-9 in glomeruli is also positive (169).

Electron microscopy in DDD reveals thickened glomerular basement membranes and intramembranous electron-dense ribbon-like deposits. The deposits in C3GN are localized to the subendothelial- or subepithelial areas of the basement membrane (170). Deposits are composed of complement components (171).

**Acquired drivers of C3G**

C3 nephritic factors (C3NeF) are autoantibodies that stabilize the C3 convertase resulting in resistance to decay. C3NeFs are found in 50% of patients with C3G (164, 172). Other autoantibodies include C4NeF which stabilize the C3 convertases of the classical and lectin pathway (173) and C5NeFs which stabilize the C5 convertase (174). The latter are more often found in C3GN than in DDD.

**Genetic drivers of disease**

C3G is not always clearly correlated to rare genetic variants. Approximately 25% of patients are found to carry rare variants in retrospective studies (164, 165, 175) and familial cases are rare. Reports indicate the occurrence of both autosomal dominant and autosomal recessive inheritance patterns. The degree of penetrance remains uncertain and the risk to family members is likely low (176). Homozygous and compound heterozygotic variants were reported in C3G (177).
CFH variants have been reported in approximately 11% (178, 179) clustered to the N-terminal SCR1-4 resulting in impaired fluid phase regulation of the alternative pathway. Decay and cofactor activity is decreased as well as binding to C3b, while host cell recognition remains intact. Homozygous CFH variants impair the release of factor H. The result is excessive consumption of C3 in plasma and C3 degradation products deposit in glomeruli (124, 180, 181). Likewise, variants have been found in CFI (5% of C3G patients) (164, 182) and the MCP gene (2%) (182).

C3 variants were found in approximately 11% of C3G patients. Both heterozygous and homozygous. Variants result in an overactive C3 convertase either resistant to decay or reduced factor I-mediated proteolysis of C3b in the presence of factor H (183). CFB variants may also be associated with C3G possibly by causing a stabilized C3 convertase but functional studies are lacking (184).

CFHR variants, rearrangements, duplications, and hybrid and fusion proteins were reported (81, 185-190). One form of C3G is termed CFHR5 nephropathy and was shown to be associated with exon duplication in CFHR5 in a Cypriot family (191).

Rare variants in genes THBD (175) and DGKE (192) have been reported but the functional significance is unclear.

Management of C3 glomerulopathy

Mild cases of C3G (proteinuria <0.5 g/24h and normal kidney function) warrant supportive treatment with renin-angiotensin-aldosterone-system blockade, low-sodium diet and lipid control. In cases of moderate disease (proteinuria >0.5 g/24h, elevated kidney function parameters), immunomodulatory treatment is indicated, combining mycophenolate mofetil and oral glucocorticoids (193). The risk of relapse is high after MMF discontinuation.

In severe cases (proteinuria >2 g/24h, elevated kidney function parameters) intravenous methylprednisolone pulses are given. Cases presenting with rapidly progressive glomerulonephritis are treated with glucocorticoid pulses, followed by oral treatment combined with either cyclophosphamide or mycophenolate mofetil (161). Plasma infusion or exchange has also been suggested for patients with moderate to severe disease (194).

The data on the use of other immunosuppressive agents such as rituximab and calcineurin inhibitors is too scarce to conclude.

Complement inhibitory treatment with an anti-C5 antibody (eculizumab) is reserved for a subset of patients with a more severe phenotype that does not respond to initial treatment with mycophenolate mofetil and glucocorticoid treatment, or as an add-on treatment for patients presenting with rapidly progressive glomerulonephritis. Eculizumab has been evaluated in two studies (195, 196). The results did not report a uniform beneficial effect.
Complement inhibitors of the alternative pathway have been investigated in various trials. These include the factor D inhibitor danicopan in a trial that was discontinued and the factor B inhibitor iptacopan being tested in a phase 3 trial (NCT04817618) (197-199). C3 inhibition with pegcetacoplan influenced complement levels and proteinuria (198, 199). The oral C5aR1 antagonist Avacopan is also currently being investigated in a phase 2 trial (NCT03301467) (198, 199). A lectin pathway inhibitor, the MASP2 inhibitor narsolimab, is also in the phase 2 trial (NCT02682407). Our group has shown that renin can cleave C3 and that patients with C3G, in a limited study, benefited from treatment with a renin inhibitor, aliskiren, showing reduced complement activation in the circulation and in the kidneys (169). This is being further assessed in a phase 2 trial (NCT04183101).

The management of kidney transplantation in patients with C3G is challenging because of the high risk of disease recurrence and graft loss (165).

Immune complex membranoproliferative glomerulonephritis

The clinical picture of IC-MPGN is similar to C3 glomerulopathy. The etiology may be different, as immune complexes are formed during infections, autoimmune diseases and malignancies. Biopsies show MPGN with immunoglobulin deposition in addition to complement (168). Although these conditions are not primarily complement-mediated cluster analysis has shown that a subgroup of patients exhibit complement activation and have C3NeF as well as genetic variants in genes encoding complement proteins suggesting an overlap with C3G (200). The course of disease is dependent on the primary cause and its amenability to treatment.
Complement-targeted therapy

Growing evidence of the involvement of the complement system in the pathogenesis of various diseases has been an incentive for the development of complement-targeted therapy. Nonetheless, the manipulation of the complement system, an ancient evolutionary mechanism, and a vital element of innate immunity, requires careful consideration. The current recommendation for treatment of aHUS is terminal pathway inhibition with intravenous eculizumab or ravulizumab which involves increased risk of meningococcal infections and protocols recommend vaccination against encapsulated bacteria and prophylactic antibiotics (109, 201).

Targeting components of the alternative pathway in both aHUS and C3G has been proposed as a rational strategy. These inhibitors should theoretically allow for the activity of the classical and lectin pathways.

Several potential complement inhibitors in the pipeline can be administered orally or subcutaneously. This offers the advantage of enabling treatments outside of a hospital setting.

Table 2 summarizes the current status of some complement inhibitors approved or in trials.
<table>
<thead>
<tr>
<th>Target</th>
<th>Generic name</th>
<th>Type</th>
<th>Disease</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s, C1r, MASP, FXIIa, kallikrein</td>
<td>C1 inhibitor (human)</td>
<td>Plasma protein rC1-inhibitor</td>
<td>Hereditary angioedema</td>
<td>Approved</td>
</tr>
<tr>
<td>C1s</td>
<td>Sutimlimab</td>
<td>Antibody</td>
<td>Cold agglutinin disease ITP*</td>
<td>Approved</td>
</tr>
<tr>
<td>C1q</td>
<td>ANX005, ANX007, ANX009</td>
<td>Antibody</td>
<td>Huntingtons disease, Guillain-Barré syndrome, GA/AMD, lupus nephropathy, ALS, AIHA</td>
<td>III/II/I</td>
</tr>
<tr>
<td>C1q</td>
<td>RLS0071</td>
<td>Peptide</td>
<td>Hypoxic ischemic encephalopathy, asthma</td>
<td>II/I</td>
</tr>
<tr>
<td>MASP2</td>
<td>Narsoplimab</td>
<td>Antibody</td>
<td>aHUS, C3G, IgAN, HSCT-TMA, Covid-19 membranous nephropathy, lupus nephritis</td>
<td>III/II</td>
</tr>
<tr>
<td>MASP-3</td>
<td>OMS906</td>
<td>Antibody</td>
<td>PNH</td>
<td>I</td>
</tr>
<tr>
<td>C2</td>
<td>ARGX117</td>
<td>Antibody</td>
<td>Multifocal motor neuropathy, dermatomyositis, delayed graft function</td>
<td>II/I</td>
</tr>
<tr>
<td>C3</td>
<td>ALXN2030</td>
<td>SIRNA</td>
<td>C3G, Chronic antibody-mediated rejection</td>
<td>I</td>
</tr>
<tr>
<td>C3</td>
<td>Pegcetacoplan</td>
<td>Pegylated peptide</td>
<td>PNH C3G, GA/AMD, cold agglutinin disease, ALS</td>
<td>Approved II</td>
</tr>
<tr>
<td>C3</td>
<td>AMY-101</td>
<td>Compstatin peptide</td>
<td>C3G, COVID19</td>
<td>II</td>
</tr>
<tr>
<td>C3 convertase</td>
<td>GT005</td>
<td>AVV Factor I plasmid</td>
<td>AMD</td>
<td>II</td>
</tr>
<tr>
<td>C3 convertase</td>
<td>rFH (Gem103)</td>
<td>rFH</td>
<td>AMD</td>
<td>II</td>
</tr>
<tr>
<td>FB</td>
<td>Iptacopan (LNP-023)</td>
<td>Small molecule</td>
<td>aHUS, C3G, PNH, IgAN, cold agglutinin disease, AMD, membranous nephropathy, lupus nephropathy</td>
<td>II/III</td>
</tr>
<tr>
<td>FB</td>
<td>IONIS-FB-Lr6</td>
<td>ASO</td>
<td>GA, AMD, IgAN</td>
<td>II</td>
</tr>
<tr>
<td>Bb</td>
<td>SAR443809</td>
<td>Antibody</td>
<td>C3G, PNH</td>
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<tr>
<td>FD</td>
<td>Danicopan and derivatives</td>
<td>Small molecule</td>
<td>C3G, GA/AMD, PNH, Covid-19, lupus nephropathy, IgAN</td>
<td>III/II</td>
</tr>
<tr>
<td>C5aR1</td>
<td>Avacopan</td>
<td>Small molecule</td>
<td>ANCA-associated vasculitis, granulomatous polyangiitis, microscopic polyangiitis <strong>C3G</strong>&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Approved</td>
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<tr>
<td>C5b-9</td>
<td>AAVCAgSCG59</td>
<td>Gene therapy</td>
<td>AMD</td>
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</tr>
</tbody>
</table>

The present investigation

The overall aim of this thesis was to correlate genotype to phenotype in complement-mediated kidney diseases and evaluate a complement inhibitory treatment, using in vitro models.

Specific aims:

1. To investigate the phenotype of factor B mutations in atypical hemolytic uremic syndrome and membranoproliferative glomerulonephritis and characterize the effect of a factor D inhibitor.

2. To study complement activation in monozygotic twins with a factor B mutation and discordance for disease expression.

3. To describe genetic variants in complement in a cohort of Nordic patients with atypical hemolytic uremic syndrome, C3 glomerulopathy and membranoproliferative glomerulonephritis.

4. To investigate a genetic variant in factor H-related protein 5 in atypical hemolytic uremic syndrome for its effect on complement activation.
Methods

A brief description of the main methods included in this thesis is presented below. The methods and the rationale for the choice are summarized in Table 3. For details, please see the individual papers.

Ethical statement:

The study involved using sensitive patient data, blood samples and DNA from living human subjects. All studies were performed in accordance with the Declaration of Helsinki and with the approval of the Swedish Ethical Review Authority or the approval of the Regional Ethics Review Board of Lund University.

Papers I and II: The project involved blood and DNA from patients, family members and controls in Iceland, Norway and Sweden and was approved by the National Bioethics Committee of Iceland, The Data Protection Officer at Oslo University Hospital and the Swedish Ethical Review Authority. Written informed consent was obtained from all individuals included in the study, patients or their parents, healthy controls as well as individuals included in the deCODE database.

Papers III and IV: The project involved blood and DNA samples from patients with complement-mediated kidney disease living in Sweden and Norway as well as their family members and healthy controls. The studies were approved by the Swedish Ethical Review Authority. In Paper III the Swedish Ethical Review Authority waived the requirement for written consent from patients included retrospectively in this study. All patients included after October 2021 gave informed consent. In Paper IV informed consent was obtained from the patient, her parents and the controls.
<table>
<thead>
<tr>
<th>Description</th>
<th>Paper</th>
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<tbody>
<tr>
<td>Clinical data</td>
<td>I</td>
</tr>
<tr>
<td>History, laboratory results, disease course and familial cases</td>
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<tr>
<td>Genetics</td>
<td>II</td>
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<tr>
<td>Sanger sequencing</td>
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<tr>
<td>Whole exon sequencing</td>
<td>+</td>
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<td>Whole genome sequencing</td>
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<td>Mutagenesis</td>
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<td>CFB</td>
<td>+</td>
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<tr>
<td>CFHR5</td>
<td>IV</td>
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<td>ELISA</td>
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<tr>
<td>Quantification</td>
<td>+</td>
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<tr>
<td>Autoantibody detection</td>
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</tr>
<tr>
<td>Activation products (Ba, C3a, C5a, sC5b-9)</td>
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<td>Immunoblot</td>
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<td>Protein size by immunoblot</td>
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<td>Protein dimer analysis</td>
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<td>C3 convertase formation and visualization of the degradation of factor B</td>
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<tr>
<td>Hemolytic assay(^1)</td>
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<td>Sheep RBCs</td>
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<tr>
<td>Rabbit RBCs</td>
<td>+</td>
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<td>pGECs(^2)</td>
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<td>C3 deposition by immunofluorescence microscopy</td>
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<td>SPR</td>
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<tr>
<td>Factor B binding to C3b</td>
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<td>Assembly of the C3 convertase</td>
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<td>FD inhibition</td>
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<td>C3 convertase formation factor B degradation visualized by immunoblotting</td>
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<td>Two-tailed Mann-Whitney U-test</td>
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<td>Kruskal Wallis multiple comparison tests followed by Dunns procedure</td>
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</tr>
<tr>
<td>Non-parametric Wilcoxon signed rank test</td>
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</tbody>
</table>

\(^1\) The difference between hemolytic assays using sheep and rabbit erythrocytes lies in their susceptibility to complement-mediated lysis. Rabbit erythrocytes lack certain complement regulators and have low membrane sialic acid content, making them easily lysed. On the other hand, sheep erythrocytes are not lysed by human complement under normal conditions. They are used to measure the assembly of C3 convertase, as their complement activation kinetics are slower due to the regulatory activity of factor H, allowing the reaction to be stopped before MAC formation on pre-existing C3-convertases.

\(^2\) Glomerular endothelial cells were activated with ADP before incubation with sera and compared to normal controls in paper I. In paper 2 the aim was to compare the two twins with each other and not to compare them with normal sera. There was deposition of C3 fragments as well as C5b-9 in both twins with or without ADP pre-stimulation in the pilot studies. Final experiments were performed without pre-stimulation with ADP.

**Human subjects**

The laboratory at the Department of Pediatrics, Lund University, is a European reference lab for the detection of genetic variants in complement factors associated with
complement-mediated kidney diseases. The data in the referral may include information regarding the patient sex, age, diagnosis, relevant aspects of the disease course, treatments, transplantation and family history.

**Blood samples**
Collection and shipment of samples were performed according to the standardized local routine of the referring unit. The samples were aliquoted upon arrival to minimize ex-vivo complement activation and stored at -80°C.

**Screening for autoantibodies**
Patients with complement-mediated kidney diseases were screened for factor H autoantibodies and/or nephritic factors. In Paper I patients were also screened for factor B autoantibodies. Antibodies to factor H and nephritic factors were performed in accordance with hospital routines.

C3 nephritic factor was assayed using three methods: ELISA (210), hemolysis (211), or crossed immunoelectrophoresis ((212, 213). A positive ELISA result indicates the presence of an IgG against the alternative pathway C3 convertase (C3bBb). The hemolytic assay is functional. C4 nephritic factor was detected using a previously described modified method (173).

**Genetic analysis and variant screening**
Genetic assays were performed by Sanger sequencing (until 2016) or by whole exome or whole genome sequencing, the latter from 2020. Next generation sequencing was performed in collaboration with the Center for Molecular Diagnostics, Skåne University Hospital in Lund and deCODE in Reykjavik. The genetic panel filtered 17 genes, including complement factor H (CFH), factor I (CFI), membrane cofactor protein (MCP, CD46), C3, factor B (CFB), properdin (CFP), clusterin (CLU), factor H-related proteins 1-5 (CFHR1-CFHR5), a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), thrombomodulin (THBD), diacylglycerol kinase epsilon (DGKE), C5 and plasminogen (PLG). ADAMTS13 variants were not included in this thesis.

**Data analysis**
When a variant was detected, it was annotated using Ensembl Variant Effect Predictor and interpretation was carried out using gnomAD, or a database for complement variants, complement-db.org, and a literature search was performed. Nonsynonymous genetic variants were described as having a minor allele frequency of <1% or variants that were previously associated with aHUS, C3G or IC-MPGN. Prediction of the significance of found variants was performed using Mastermind Genomic Search Engine (https://www.genomenon.com/mastermind). Classification of predicted variant pathogenicity was reported according to international recommendations (145) using ACMG (214).
**Variant protein expression for functional testing**

Recombinant wild-type and mutated proteins were generated for functional studies. Mutagenesis was performed in wild-type *CFB* or *CFHR5*. Competent cells were transformed with plasmids. Sanger sequencing was performed to confirm the introduction of mutations. Wild-type and mutated proteins were synthesized in human embryonic kidney (HEK) cells subject to transient transfection. Supernatants were collected and concentrated, and proteins assayed by immunoblotting for size and ELISAs for concentration. Likewise, lysates were obtained by scraping the cells. The wild-type and mutant constructs of factor B were added to factor B-depleted serum in certain experiments (hemolysis and glomerular endothelial cell stimulation).

**Analysis of complement proteins**

Complement proteins in human samples were assayed by hospital routines. Factor B and FHR5 were measured by ELISAs. Complement by-products formed during activation, including C3a, Ba, C5a and soluble C5b9, were quantified using commercially available ELISA kits. The size of complement proteins factor B and FHR5 was assayed by immunoblotting and compared with the purified proteins, plasma-purified for factor B or recombinant human FHR5.

**Hemolytic assays**

Functional activity of the complement system via the alternative pathway was analysed with a hemolytic assay using rabbit red blood cells (RBCs) in Mg-ethylene glycol-bis(β-aminoethyl ether (EGTA) buffer (allowing activation of the alternative pathway, as EGTA chelates Ca++, which is necessary for activation of the classical/lectin pathways) (215). Human RBCs are protected against lysis by soluble and membrane-bound complement regulators. Sheep and rabbit RBCs do not possess sufficient membrane-bound regulator's (DAF (CD55), protectin (CD59), and CR1(CD35)) for protection against human complement-mediated lysis (216, 217). Rabbit RBCs lyse easily as they in addition possess minimal amounts of sialic acid needed for factor H surface regulation (218, 219). In Papers I and II sheep RBCs were used in a two-step assay originally developed for the detection of C3 nephritic factors (211) in which human serum was first incubated with the RBCs for 10 min at 30°C, the reaction was stopped by the addition of EDTA followed by addition of rat serum in EDTA (as source of the terminal complement pathway components) to induce hemolysis. In Papers I and IV rabbit RBCs were also used. Released hemoglobin was quantified by absorbance at 405 nm.

**Complement activation on endothelial cells**

Complement activation on glomerular endothelial cells was studied by incubating sera with primary glomerular endothelial cells. Serum was from patients or controls or factor B-depleted serum with wild-type and mutant constructs in the presence/absence of a factor D inhibitor. The cells were preactivated with ADP (in Paper I), and incubated with 25% serum for 2 h at 37°C. The supernatants were collected for analyses of...
complement activation products C3a, C5a, sC5b9 and Ba. The cells were further stained for C3c or C5b-9 and analysed by microscopy.

*Factor B binding to C3 assayed by surface plasmon resonance*
The binding of wild-type and mutant constructs of factor B to C3b and the formation of the C3 convertase were studied by surface plasmon resonance. C3b was immobilized on a carboxymethylated sensor chip, and factor B with factor D were injected over the surface, to study their capacity to form the convertase. In a second set-up binding between factor B and C3b was assayed, and the affinity/dissociation constant was calculated.

*Factor B cleavage in the presence of a factor D inhibitor*
C3 convertases were constructed on a plate coated with C3b and incubated with 20% sera, or factor B constructs, with or without a factor D inhibitor, for 1 hour at 37°C. Samples were collected and after separation transferred to PVDF membranes, incubated with anti-factor B and immunoblotted to detect the Bb fragment and the effect of factor D inhibition on the cleavage of factor B.

*Analysis of CFHR5 dimerization*
An assay was performed to detect dimers of CFHR5. Monomers in serum would be eluted in the flow-through using 100 kDa concentration tubes. Proteins larger than 100 kDa (such as dimers of CFHR5 at approximately 130 kDa) would be retained in the concentrate. The content of the flow-through and concentrates was subject to SDS-PAGE and immunoblotting. Dimers of CFHR5 separate to monomers under these conditions and detection is performed using a polyclonal anti-human FHR5 antibody.

*Statistics*
GraphPad Prism software was used to analyse statistical differences. Comparison between the two groups was assessed by the two-tailed Mann Whitney U test, or, when paired, by Wilcoxon signed rank test. Kruskal-Wallis multiple-comparison test followed by Dunn’s procedure was applied to evaluate differences between more than two groups. A P value ≤ 0.05 was considered significant.
Results

Paper I

This study aimed to investigate the phenotype of \textit{CFB} variants in patients with aHUS and IC-MPGN and the effect of a factor D inhibitor on factor B in the presence of wild-type and mutated factor B. The \textit{CFB} variants investigated were D371G and E601K associated with aHUS and I242L associated with IC-MPGN.

The results using patient sera indicated that the presence of the \textit{CFB} D371G variant was associated with increased complement activation as primarily demonstrated by the induction of sheep RBC hemolysis. This was also true for the \textit{CFB} I242L variant. Using the mutated constructs in factor B-depleted serum, in the presence of rabbit RBCs, similar results were obtained for the D371G construct. The results were compared to a positive control construct, \textit{CFB} D279G. The D371G construct formed a stronger C3 convertase and bound more C3 than the wild-type construct while the other constructs, I242L and E601K, did not. Likewise, the D371G construct, in factor B-depleted serum, induced more sC5b-9 release from glomerular endothelial cells than the wild-type. Importantly, the enhanced hemolysis and C5b-9 release caused by the D371G and D279G variants was inhibited by factor D inhibition. The inhibitor also effectively blocked factor B degradation in patient and normal serum and in the presence of constructs in factor B-depleted serum. The results clearly showed that the \textit{CFB} D371G variant induced a gain-of-function which could be inhibited by danicopan.

Paper II

This paper continued the observations made in Paper I, as the \textit{CFB} D371G variant was detected in a large pedigree in which seven family members were carriers of the variant and three of these were affected by aHUS. No other mutations in complement genes were found in the affected carriers. The aim was to describe the pedigree and investigate the founder effect within a pedigree of 210 individuals. The origin of the mutation could be traced to an ancestor most probably born in the late 1800s. In the process, an interesting observation was made as two family members were monozygotic twins with a discordant phenotype for aHUS. The phenotype of complement activation in the twin sera was investigated but no differences between them were found regarding the induction of sheep RBC hemolysis as well as levels of C3a, C5a, and C5b-9. One sample exhibited elevated Ba in the affected twin. Furthermore, no differences were found regarding the release of Ba, C3a, C5a and C5b-9 into supernatants when twin sera were separately incubated with glomerular endothelial cells. Likewise, no differences were found in C3c and C5b-9 deposition on the cells when incubated with sera. To our knowledge, this is the first report of monozygotic twins with a family history of aHUS and a discordant phenotype indicating that the genotype, the \textit{CFB} gain-of-function variant, is an important risk factor but does not solely predict phenotype.
Paper III

This study aimed to describe genetic variants in a cohort of Nordic patients (n=141) with aHUS, C3G and IC-MPGN with a focus on 16 genes including complement-encoding genes. Of altogether 141 patients, 94 patients had aHUS and one of these also developed IC-MPGN (124), 40 had C3G and 7 had IC-MPGN. A large majority of patients (85/141) were found to have rare genetic variants, with a MAF <1%, or variants that were previously found to be disease-associated. In aHUS patients 68 different genetic variants or deletions were identified and 30 patients had more than one variant, variants in CFH were the most common. Most variants were heterozygous, but six variants were homozygous. In C3G patients 30 genetic variants, deletions or duplications, were identified, and eight patients had more than one variant, C3 variants were the most common. Likewise, in the patients with IC-MPGN five genetic variants were identified. Altogether 26 novel variants were identified. This study did not carry out functional studies and phenotypic correlations are referred to if previously described. The study also correlated genetic findings to the presence of kidney failure. One of the most interesting findings here was that the same genetic variant could, in separate individuals, present as either aHUS or C3G. This was described for 18 different variants in CFH, C3, CFI, MCP/CD46, CFHR3/1 (deletion), CFHR5, THBD, and PLG.

Paper IV:

This project aimed to describe the complement-associated phenotype of a CFHR5 variant in a pediatric patient with aHUS and autoantibodies to FH. Genotyping by Sanger and whole exome sequencing showed that the patient had a homozygous deletion of CFHR3/CFHR1, previously shown to be associated with autoantibodies to factor H (220), and a heterozygous variant in CFHR5 c.1541T>G; p.M514R. The serum level of FHR5 was low. The parents were unaffected by aHUS and did not have antibodies to factor H. The father was a heterozygous carrier of the CFHR5 variant M514R and the deletion of CFHR3/CFHR1. Serum levels of FHR5 were also low in the father’s sample. FHR5 in the serum from the patient and the father formed dimers. The mother was a heterozygous carrier of the CFHR3/CFHR1 deletion but did not carry the CFHR5 variant M514R.

The CFHR5 M514R variant was previously reported in C3 glomerulopathy (221) and age-related macular degeneration (222), but its phenotype had not been described. Our goal was to evaluate if the CFHR5 variant, in addition to antibodies to factor H, could contribute to complement activation, or if it, instead, had a protective role. Serum from the patient, her father or controls induced hemolysis of rabbit RBCs. Adding recombinant human FHR5 to the patient’s serum, or her father’s, to achieve physiological concentrations, reduced hemolysis. In control sera, in which physiological concentrations of FHR5 were presumably present, the addition of FHR5 at physiological concentrations did not significantly alter the degree of hemolysis. However, the addition of FHR5 at higher than physiological concentrations
significantly decreased hemolysis of rabbit RBCs even in control sera suggesting a regulatory role on RBCs.

The mutant variant was expressed by mutagenesis and transfection of HEK cells. The cells secreted minute amounts of the protein compared to the wild-type and the mutant variant was retained intracellularly as cell lysates had higher amounts of the mutant variant compared to the wild-type. The low levels of secreted variants could explain low serum levels in carriers of the variant.
Discussion

Patients seeking healthcare for possible aHUS, C3G or IC-MPGN are diagnosed based on their clinical history, signs and symptoms during the clinical presentation, physical exam, laboratory findings, and, in C3G or IC-MPGN, specific indicative findings in light microscopy, immunofluorescence and ultra morphologic examination of their kidney biopsies. Once a diagnosis is determined, patients are further investigated comprehensively by the measurement of complement levels, thereby determining which complement pathway is activated, if possible, and assays of auto-antibodies (anti-factor H in aHUS and nephritic factors in C3G or IC-MPGN). Genetic testing comprises a panel of disease-associated genes. From the original observations that complement is activated in HUS (even before subclassification to aHUS was achieved) (223) and MPGN (224, 225), and comprehensive studies showing how complement is activated in these diseases, an enhanced understanding of how the complement system works has emerged, and what happens when complement is dysregulated. To add to the complexity of these conditions, not all rare genetic variants lead to complement dysfunction and not all bearers of disease-associated variants develop the disease. It is essential to investigate patients comprehensively to be able to advise as to the appropriate choice of therapy (anti-complement or immunosuppressive), risk of recurrence, risk of discontinuation of therapy, suitability of kidney transplantation and choice of donor, risk of disease development in family members, including family members considered as kidney donors.

This thesis addressed some of these pertinent issues. In Paper III most patients living in Sweden and Norway with aHUS, C3G, and some with IC-MPGN, underwent genetic investigation, and the findings were correlated with disease phenotype. Next generation sequencing included a panel of 16 genes encoding factor H, C3, factor I, MCP, factor B, properdin, clusterin, factor H-related proteins 1-5, thrombomodulin, C5, DGKE and plasminogen. The panel also included genetic variants in ADAMTS13, encoding the von Willebrand cleaving protease (226), but as these are associated with thrombotic thrombocytopenic purpura, and not with the diseases investigated herein, they were not included in Paper III. We describe many novel variants mostly in genes encoding complement proteins. Assessment of their phenotype can be achieved by mutagenesis. Variants in PLG and DGKE are not associated with complement dysfunction. We found 4 rare variants in PLG and 3 in DGKE of which 2 in DGKE and one in PLG were novel. The mechanism by which variants in PLG and DGKE contribute to the development of aHUS is not necessarily related to complement activation and possibly associated with the promotion of thrombosis. Patients with variants in DGKE usually present early in life with proteinuria and hypertension (227). The mechanism by which DGKE, present
in the endothelium and platelets, prevents thrombosis by inactivating diacylglycerol signalling. Loss-of-function variants can therefore induce thrombosis (227). Plasminogen has a known role in the fibrinolytic system, and as such prevents excess thrombosis. Thus, one could assume that mutant variants may have less of a fibrinolytic effect. Plasminogen was, however, shown to bind C3, C3b and C5 and enhance factor I-mediated inactivation of C3b (228) as well as the assembly of C5b-9 (229). Plasmin also inhibited complement-mediated hemolysis and cleaved C3 (228) and iC3b (230). Mutated PLG may therefore play a role in promoting thrombosis and activating complement and both mechanisms could contribute to the development of aHUS.

Patient DNA was assayed by Sanger sequencing until 2016 and from 2017 by next generation sequencing. Sanger sequencing is more time-consuming and expensive and was carried out for genes encoding five proteins: factor H, C3, factor I, MCP, and factor B. For this reason, certain other variants could have been missed in patients sequenced by this method.

The finding of a genetic variant in one or more of the 16 genes investigated in the panel included here could explain the propensity for disease. Particularly in families in which all affected individuals carry the same variant or set of variants, the risk for the development of disease in carriers can be assessed by this evaluation. The investigation of unaffected carriers can, however, have consequences, such as living with the prospect of developing disease or passing the disease on to descendants, in the ambiguous setting of incomplete penetrance.

In Paper I we described a factor B variant, D371G, that clearly led to overactivation of complement, as demonstrated by the low C3 levels in carriers (affected and unaffected), induction of sheep red blood cell hemolysis, enhanced binding to C3 and formation of the C3 convertase, and induction of C5b-9 release from glomerular endothelial cells. And yet, even though we could demonstrate that this genetic variant was hyper functional and strongly associated with disease, we show in Paper II that monozygotic twins bearing the same genetic background, and this rare variant, differed in disease expression. This finding, although only described in one set of twins, is of importance as it demonstrates that genetic composition is a risk factor for the development of aHUS but not the sole disease-causing factor. Other environmental and epigenetic factors can drive disease as well. Thus, in providing genetic counselling to individuals with a family history of aHUS practitioners should exert excess caution as the presence of a genetic variant, regardless of how dysfunctional it is in testing, does not necessarily indicate that the individual will develop disease.

An interesting finding in Paper I and Paper III was that the same genetic variants could be found in aHUS, C3G or IC-MPGN. This finding, as well as the study of monozygotic twins, strengthens the assumption that genetic make-up is just one, albeit important, aspect, but does not fully determine the disease phenotype.

Along the same lines, prediction models, such as CADD, attempt to predict the phenotype of certain variants based on a combination of conservation and functional prediction data (231). We applied prediction models to variants described in Paper III as these can differ from actual phenotypic studies of mutated recombinant proteins.
expressed in transfected cells (232). Furthermore, as healthy carriers of disease-associated variants can be informed of the predictions one should exercise caution in over-interpretation of these prediction tools.

In Papers I and II patient sera were incubated with glomerular endothelial cells for detection of complement activation by the release of complement activation products or deposition of C3c and C5b-9. In Paper II these assays were used to detect differences between the monozygotic twins bearing the same CFB gain-of-function variant CFB D371G. In Paper I the assays aimed to compare patient sera with normal sera. Deposition of C3 or C5b-9 on endothelial cells has been recently suggested to be a useful method for the detection of gain-of-function mutant variants in patient sera as well as in their unaffected relatives (233, 234). In our hands control sera also exhibited C3 deposition on glomerular endothelial cells (Paper I) and we found measurement of C5b-9 release into the cell supernatant to be a more reliable method for the detection of complement activation.

FHR5 has been suggested to be both a complement activator or regulator, in different studies (83). Its function as a complement activator can be due to direct interaction with C3b (235), or by interfering with the interaction between factor H and glycosaminoglycans on host cells (236) as well as its binding to pentraxin 3, extracellular matrix (94) and apoptotic cells (95). An earlier study demonstrated complement regulatory functions including cofactor activity for factor I-mediated C3b degradation and inhibition of C3 convertase activity (89) using both physiological (3-6 μg/mL) and super-physiological FHR-5 concentrations. The use of higher concentrations has brought those results into question.

Genetic variants in CFHR5 have been detected in aHUS (120, 140, 141), C3G (191) and IC-MPGN (237). A subtype of C3G is termed FHR5 nephropathy (191). In C3G glomerular FHR5 is highly prevalent in glomeruli with C3 deposits (238). The contribution of variants in FHR5 to these diseases could be due to gain-of-function if FHR5 is an activator, or loss-of-function if it is a regulator. With these conflicting data in mind, we carried out a study on a patient with aHUS and the CFHR5 variant M514R (Paper IV). This patient also had a homozygous deletion of CFHR3/1 and antibodies to factor H. The latter could suffice in explaining the development of disease. We wondered whether the presence of a variant in CFHR5 was protective or promoted disease. The M514R variant was not secreted and the patient, as well as her father, carrying the CFHR5 variant without antibodies to factor H, had low levels of circulating FHR5, most probably representing the normal allele that formed dimers. Patient serum induced rabbit RBC hemolysis. Adding FHR5 to the patient's serum, her father’s serum, or even normal serum (at higher concentrations), reduced the degree of hemolysis. These results suggest that FHR5 may have a regulatory role in complement activation on RBCs and that the low levels of FHR5 in the patient could contribute to the development of disease.

To date, there are no specific treatments available for C3G while aHUS can be treated with eculizumab or, in patients with antibodies to factor H, with immunosuppressive therapies. Eculizumab is exceedingly expensive (239). In Paper I we used Danicopan,
a factor D inhibitor that can be administered orally. Danicopan blocked factor B cleavage to Bb, hemolysis of rabbit RBCs and the assembly of C5b-9 and its release from glomerular endothelial cells in the presence of gain-of-function CFB mutations. We suggest that Danicopan could have a beneficial add-on effect in C3G or in aHUS patients in which eculizumab is insufficient. Danicopan will only block the proximal effects of the alternative pathway and leave the classical and lectin pathways, as well as the terminal complement pathway, intact with possibly less risk of infection with encapsulated bacteria. Another benefit could be its cost and that it is administered orally.
Conclusions

- Genetic assessment of patients with complement-mediated kidney diseases is essential for diagnostics, choice of therapy and donor, and genetic counselling.
- Genetic variants in complement-mediated diseases, even if proven to exhibit gain-of-function, are a predisposing factor and not the sole cause of disease.
- Factor D inhibition can effectively block complement activation initiated by $CFB$ variants.
- Factor H-related protein 5 may have a regulatory role regarding complement activation on RBCs.
Populärvetenskaplig sammanfattning


Komplementsystemet spelar en viktig roll i immunförsvaret. Det består av en grupp proteiner i blodet som interagerar med varandra när de stöter på "alarmsignal" och stimulerar snabba skyddsåtgärder. Dessa bidrar till eliminering av bakterier och virus, aktivering av andra delar av immunsystemet samt omhändertagandet av döende celler och immunkomplex. C3, faktor B och faktor D är centrala komplementproteiner. Förändringar i gener som kodar för komplementproteiner och antikroppar mot komplementproteiner kan leda till överdriven och ohämmad komplementmedierad attack.


Vid atypiskt hemolytiskt syndrom angriper komplementsystemet celler på insidan av blodkärl, blodet levrar sig och det kan resultera i att kärlen blockeras vilket kan leda till syrebrist i vävnaden och celldöd.

Vid C3 glomerulopati är komplementsystemet överaktivt i cirkulationen, C3 förbruks samtidigt som nedbrytningsprodukter inte renas bort utan istället inlagras. Detta kan leda till kronisk inflammation i njuren. Vid immunkomplexmedierad membranoproliferativ glomerulonefrit inlagras immunkomplex istället.


Avhandlingens syfte är att beskriva genetiska varianter hos patienter med komplementmedierade njursjukdomar, sammanställa betydelsen av dessa, samt att undersöka effekten av en alternativ komplementämmande behandling hos patienter med komplementfaktor B variant.

Avhandlingen består av följande fyra delarbeten:
**Arbete I:**


Tre faktor B varianter detekterades bland patienter med atypiskt hemolytiskt uremiskt syndrom och membranoproliferativ glomerulonefrit. Enbart en faktor B genvariant bevisades leda till överaktivering av komplement. Tillägg av en ny komplementfaktor D hämmare normaliserade komplement-aktiviteten både i blodprov samt i experiment med rena proteiner.

**Arbete II:**


**Arbete III:**


Genetiska varianter i komplementgener associerade med atypiskt hemolytiskt uremiskt syndrom, C3 glomerulopati eller immunkomplex medierat membranoproliferativ glomerulonefrit beskrevs i en stor nordisk kohort. Genetisk utredning är av betydelse när det kommer till behandlingsalternativ (komplementhämmande handling eller immunhämmande behandling), för att förutse sjukdomsförlopp, samt planera strategier i samband med njurtransplantation. Sammanlagt 141 patienter från Sverige och Norge undersöktes i en retrospektiv studie och resultaten visar vilka varianter som detekterades. Tjugosex nya varianter beskrevs. Arton gemensamma varianter detekterades hos två av patientgrupperna, vilket är bevis på att en och samma komplementgenvariant kan vara bidragande faktor i utvecklingen av två olika sjukdomar.
Arbete IV:


Oenighet råder bland forskare avseende ”factor H-related 5” proteinets funktion, om det aktiverar eller hämmar komplementsystemet. I detta arbete beskrivs konsekvensen av en genetisk variant i komplementproteinet ”factor H-relaterad 5” i atypiskt hemolytiskt uremiskt syndrom. Patienten, samt anhörig som var bärare av samma variant, hade lägre halt av cirkulerande ”factor H-related 5” protein i blodbanan. Vid framställning av proteinet uttrycktes enbart små mängder av ”factor H-relaterad 5” protein. Vår tolkning är att genförändringen leder till ett ”factor H-related” protein som inte frisätts ifrån cellerna. I denna studie hade ”factor H-relaterad 5” hämmande effekt på komplementfunktion på röda blodkroppar. Om proteinets funktion är att kontrollera komplementfunktion, så skulle en låg halt i blodbanan kunna göra patienten mer utsatt för komplementattack och komplementmedierad njursjukdom.

Slutsatserna av avhandlingen är:

• Genetisk utredning är väsentlig för diagnostik, val av behandlingsalternativ, strategi vid transplantation och för genetisk rådgivning till anhöriga.
• Även om det bevisas att genvarianter leder till ett protein som resulterar i ohämmad komplementaktivering, så fungerar de närmast som risk och inte sjukdomsorsakande faktor.
• Komplement faktor D inhibitor är effektiv mot överaktivering av komplementsystemet orsakat av faktor B-varianter.
• Faktor H relaterad 5 proteinet kan ha en reglerande roll av komplementaktivering på röda blodkroppar.
Íslensk samantekt


Magnakerfið (e. complement system) er hluti af varnarkerfi mannsins og samanstendur af kerfi próteina í blóðinu sem hvarfast hvert við annað í návist „hættumerkja“. Próteinin C3, faktor B og faktor D spila lykillhlutverk. Kerfið magnar ónæmissvör t.a.m aðferðir sem leiða til eyðingar sýkils svo sem áothúðun, beinu frumurof, bólgusvörun ásamt því að virkja aðra hluta ónæmiskerfisins. Meinvalandi breytingar í genum próteina magnakerfisins og/eða sjálfsmótefni geti valdið hömlulausri árás magnakerfisins.

Ódæmigert blóðsundrunar- og þvageitrunarheilkenni (e. atypical hemolytic uremic syndrome) og C3 gauklavilli (e. C3 glomerulopathy) eru dæmi um magnakerfis miðlaða nýrnasjúkdóma. Þessir langvinnu sjúkdómar geta leitt til nýrnaskemmda og lokastigs nýrnabilunar, krefst meðferðar meðnýrnaskilun og nýrnaígræðslu. Oft eru það börn og ungir einstaklingar sem veikjast.

Ódæmigert blóðsundrunar- og þvageitrunarheilkenni er blóðsegasmáæðakvilli (e. thrombotic microangiopathy) sem einkennist af ofvirkni magnakerfisins á innraborði smára æða í gaukulum (e.glomeruli), sem leiðir til blóðtappamyndunar og æðateppu í starfseiningu nýrans.

C3 gauklavilli einkennist af því að virkni magnakerfisins er óhamin í blóðrásinni, C3 mælist oftast lágt í blóði, en safnast fyrir innan á æðaveggi í gaukulum nýrans. Sjúkdómmum fylgir langvinn gauklabólga. Mótefnafléttutengd himnu- og frumufjöl-gunargauklabólga (e. membranoproliferative glomerulonephritis) er svipaður sjúkdómmur þar sem mótefnafléttur og í vissum tilfellum C3 safnast fyrir og trufla starfsemi nýrans.

Sértæk meðferð með magnakerfis hemli (Soliris, eculizumab) hefur stórbætt horfur sjúklinga með ódæmigert blóðsundrunar- og þvageitrunarheilkenni. Meðferðin er mjög kostnaðarsöm og krefst sjúkrahúsvaldar. Meðferðin hefur ekki gefið jafn góda raun fyrir sjúklinga sem þjást af C3 gauklavilla eða mótefnafléttutengdri himnu- og
frumufjölgunargauklabólgu. Það er gríðarleg þörf á þróun ódýrari og betri meðferðarvalkosta.

Markmið doktorsverkefnisins var að lýsa erfðabreytileikum hjá sjúklingum með magnakerfismiðlaða nýrjasjúkdóma, að skýra þýdingu þessara breytinga fyrir framvindu sjúkdóms, nýrnaígræðslukosti og til að geta veitit erfðafráðilega ráðgjöf til aðstandenda. Einnig eru skoðuð áhrif annars meðferðarvalkosts við blóðsundrunar- og þvageitrunarheilkenni. Að lokum lýsi ég mögulegu hlutverki einstaks magna-kerfispróteins. Doktorsverkefníð byggir á fjórum vörðum:

Fyrsta grein:

Lýst er þremur erfðabreytingum í faktor B sem fundust í sjúklingum með blóðsundrunar- og þvageitrunarheilkenni og frumufjölgunargauklabólgu. Einvörðungu stök faktor B erfðabreyting leiddi til stjórnlausar ræsnings magnakerfisins. Lýst er áhrifum meðferðarvalkostsins faktor D-hemils á tvö mismunandi faktor b protein með aukna virkni. Faktor D-hemill gat stýrt magnakerfinu í blóði sjúklinga og einnig þegar áhrifin voru skoðuð með hreinum próteinum.

Önnur grein:


Þriðja grein:

Fjórða grein:

Arbete IV:


Niðurstöður doktorsritgerðarinnar eru eftirfarandi:
• Uppvinnsla á erfðabreytileika er afar mikilvæg fyrir greiningu, meðferðarval, í tengslum við aðgerðaáætlu fyrir nyrnasgræðslu og einnig í tengslum við erfðafraedeilega ráðgjöf til aðstandanda sjúklinga.
• Þratt fyrir að unnt sé að sanna að erfðabreytileiki leiði til próteins sem valdi hómlulausri virkjun magnakerfisins, það er það ekki eitt og sér orsök sjúkdóms heldur áhættaleáltur.
• Magnakerfis-hemillinn, faktor D, hindrar ofvirkni magnakerfisins hjá sjúklingum með faktor B erfðabreytileika.
• Hlutverk „faktor H-related 5“ próteinsins er mögulega að vera hemill á magnakerfismiðlaðu blóðrauðalosi.
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References

1. Schorn BE. 'How can his word be trusted?': speaker and authority in Old Norse wisdom poetry (doctoral thesis). University of Cambridge; 2012. https://doi.org/10.17863/CAM.15907


   https://doi.org/10.1111/j.1365-2249.1995.tb02289.x

   https://doi.org/10.1016/j.molimm.2018.05.023

   https://doi.org/10.1111/imr.13131

   https://doi.org/10.3389/fimmu.2021.660194

   https://doi.org/10.1111/imr.13166

   https://doi.org/10.1016/0167-5699(94)90155-4

   https://doi.org/10.1016/j.molimm.2013.06.001

   https://doi.org/10.3389/fimmu.2017.01328

   https://doi.org/10.1073/pnas.1219260110

71
https://doi.org/10.1016/C2015-0-06595-9

https://doi.org/10.4049/jimmunol.174.10.6250

https://doi.org/10.1016/j.asn.2015020212

https://doi.org/10.3389/fimmu.2018.01691

https://doi.org/10.4049/jimmunol.1403121

https://doi.org/10.3389/fimmu.2022.845953

https://doi.org/10.4049/jimmunol.1403121

https://doi.org/10.3389/fimmu.2020.01297

https://doi.org/10.1111/joim.12546

https://doi.org/10.2215/CJN.00620117

https://doi.org/10.2215/CJN.04760512


https://doi.org/10.1681/asn.2013070796

https://doi.org/10.1182/blood-2014-06-585430

https://doi.org/10.1016/0161-5890(94)00157-v

https://doi.org/10.1016/j.molimm.2011.04.004

https://doi.org/10.1073/pnas.2135497100

https://doi.org/10.1196/annals.1352.032

https://doi.org/10.1016/j.blood.2020010069

https://doi.org/10.1038/jhg.2012.57

https://doi.org/10.1016/j.molimm.2006.08.004

https://doi.org/10.1016/j.molimm.2015.06.021

https://doi.org/10.1016/j.molimm.2011.11.003


https://doi.org/10.1681/ASN.2017030258

https://doi.org/10.1371/journal.pone.0241989

https://doi.org/10.1007/s00281-017-0655-8

https://doi.org/10.1038/s41467-022-33003-7

https://doi.org/10.1172/JCI136094

https://doi.org/10.1016/j.molimm.2019.07.030

https://doi.org/10.1007/s40259-022-00567-1

https://doi.org/10.1038/nrneph.2017.156

https://doi.org/10.1111/imr.13164

https://doi.org/10.1002/ajh.26875

https://doi.org/10.1007/978-1-4939-8949-2_13

https://doi.org/10.1016/0022-1759(82)90386-6


Factor D Inhibition Blocks Complement Activation Induced by Mutant Factor B Associated With Atypical Hemolytic Uremic Syndrome and Membranoproliferative Glomerulonephritis

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Complement factor B (FB) mutant variants are associated with excessive complement activation in kidney diseases such as atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy and membranoproliferative glomerulonephritis (MPGN). Patients with aHUS are currently treated with eculizumab while there is no specific treatment for other complement-mediated renal diseases. In this study the phenotype of three FB missense variants, detected in patients with aHUS (D371G and E601K) and MPGN (I242L), was investigated. Patient sera with the D371G and I242L mutations induced hemolysis of sheep erythrocytes. Mutagenesis was performed to study the effect of factor D (FD) inhibition on C3 convertase-associated FB cleavage, complement-mediated hemolysis, and the release of soluble C5b-9 from glomerular endothelial cells. The FD inhibitor danicopan abrogated C3 convertase-induced FB cleavage, complement-mediated hemolysis, and the release of soluble C5b-9 from glomerular endothelial cells. The FD inhibitor danicopan abrogated C3 convertase-associated FB cleavage to the Bb fragment in patient serum, and of the FB constructs, D371G, E601K, I242L, the gain-of-function mutation D279G, and the wild-type construct, in FB-depleted serum. Furthermore, the FD-inhibitor blocked hemolysis induced by the D371G and D279G gain-of-function mutants. In FB-depleted serum the D371G and D279G mutants induced release of C5b-9 from glomerular endothelial cells that was reduced by the FD-inhibitor. These results suggest that FD inhibition can effectively block complement overactivation induced by FB gain-of-function mutations.

Keywords: complement, factor B, factor D, danicopan, atypical hemolytic uremic syndrome, C3 glomerulopathy
INTRODUCTION

The innate immune system is a first line of defense against pathogens and contributes to removal of apoptotic host cells. One of the mainstays of protection is the complement system responding to non-self molecules and eliminating them or neutralizing their effects by opsonization or lysis as well as induction of leukocyte chemoattraction, inflammation and phagocytosis (1). The main enzymatic activity of the alternative complement pathway is mediated by the C3 convertase. For C3 convertase formation to occur C3b binds to factor B (FB) which is cleaved by factor D (FD) to the Ba and Bb fragments, the latter possessing catalytic activity. Bb remains bound to C3b and forms the C3bBb convertase that exponentially cleaves more C3 into C3a and C3b. Binding of additional C3b molecules generates the C5 convertase. The complement system is kept in balance by multiple cellular and soluble regulators (1).

FB is essential for defense against encapsulated bacteria, and thus individuals with FB deficiency are prone to infection with Neisseria meningitidis and Streptococcus pneumoniae (2). Conversely, an overactive FB can lead to excessive complement activation via the alternative pathway resulting in kidney diseases such as atypical hemolytic uremic syndrome (aHUS) or C3 glomerulopathy. In both of these rare conditions, patients may exhibit complement activation but there are distinct differences in clinical presentation and renal pathology. While aHUS is characterized by hemolytic anemia, thrombocytopenia and renal failure with lesions indicative of thrombotic microangiopathy (3), C3 glomerulopathy is a form of chronic glomerulonephritis presenting with hematuria and proteinuria leading to renal failure (4). These conditions can arise due to mutant variants in complement factors, including CFB mutations, or autoantibodies against factor H (5). Autoantibodies against FB have been described in C3 glomerulopathy (6).

In aHUS the complement system is overactive due to loss-of-function mutations in complement regulators or gain-of-function mutations in C3 or CFB (7). Gain-of-function variants in CFB are rare and have in certain cases been associated with low C3 levels in patient sera (8–11) indicating complement activation in vivo. Mutations have been shown to increase FB binding affinity to C3b thereby stabilizing the C3bBb convertase (12) and enhancing resistance to factor H mediated decay acceleration (9, 13). This was particularly demonstrated for mutations located in close proximity to the C3b binding region, i.e. the Mg$^{2+}$-binding site in the von Willebrand factor type A domain of FB (14). Of note, not all CFB mutations have been shown to induce complement activation and not all individuals carrying CFB mutations associated with aHUS develop disease (11, 12, 14), even if circulating C3 levels are low. In addition to aHUS, CFB mutations and rare variants have also been demonstrated in C3 glomerulopathy and immune complex-associated membranoproliferative glomerulonephritides (MPGN) (15–17).

Binding of C3b to FB elicits a conformational change exposing the scissile bond at position Arg$^{234}$-Lys$^{235}$ enabling cleavage by FD (18). Small molecule FD inhibitors have been developed as potential treatments for complement-mediated diseases (19) and efficiently inhibited activation of the alternative pathway in vitro as well as in animal models (19, 20). FD inhibitors present the advantage of blocking complement activation at the level of the C3 convertase, while leaving the classical and lectin pathways intact. A phase 2 trial has been completed and a phase 3 trial with an oral FD inhibitor as an add-on therapy to C5 inhibition is ongoing in patients with paroxysmal nocturnal hemoglobinuria (PNH) (21, 22).

The aim of this study was to investigate if FD inhibition impacted complement overactivation induced by CFB mutations. To this end we investigated four CFB mutations associated with aHUS or MPGN, two of which mediate a gain-of-function phenotype. We studied the effect of FD inhibition in the presence of the FB mutations on C3 convertase-induced FB cleavage, complement-mediated hemolysis, and release of soluble C5b-9 from glomerular endothelial cells.

MATERIALS AND METHODS

Subjects

Patients from Iceland, Sweden and Norway with complement-mediated renal diseases are referred to the laboratory at the Dept of Pediatrics in Lund for genetic diagnostics. Three patients were found to have CFB mutations. The patients and their laboratory data are presented in Table 1. Samples were obtained from apparently healthy adult controls (n=12, 6 female) who were not using any medications. The study of patients and healthy controls was performed with the approval of the Ethics Review Board at Lund University. Approval included genetic analysis of Nordic patients and phenotypic studies of complement mutations. The study was also approved by the National Bioethics Committee of Iceland and the Data Protection Officer at Oslo University Hospital, Oslo Norway. Informed written consent was obtained from the patients or the parents of Patient 3 and the healthy controls.

Blood Samples

Whole blood in EDTA tubes was used for DNA purification. Serum samples were taken during chronic disease in Patients 1-3 and from healthy controls, centrifuged after one hour at room temperature and stored at -80°C until assayed.

Genetic Analysis and Mutation Screening

Next generation sequencing was performed focusing on a panel of genes encoding the following 17 proteins: complement C3, CFB, factor H (CFH), factor H-related proteins-1, -2, -3, -4, -5, C5, factor I, properdin, CD46 (membrane co-factor protein), a disintegrin and metalloprotease with a thrombospondin type 1 motif 13 (ADAMTS13), diacylglycerol kinase epsilon (DGKE), plasminogen, thrombomodulin and clusterin.

Whole-exome sequencing was performed at the Center for Molecular Diagnostics, Skåne University Hospital and Clinical Genomics Lund, SciLifeLab. In brief, genomic DNA was subject to tagmentation-based library preparation and hybrid capture
TABLE 1 | Clinical characteristics of patients included in this study.

<table>
<thead>
<tr>
<th>Pat.</th>
<th>Sex</th>
<th>Age at presentation (yrs)</th>
<th>Diagnosis</th>
<th>Clinical presentation</th>
<th>Biopsy findings</th>
<th>Disease course</th>
<th>Complement levels</th>
<th>Genetic assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>1</td>
<td>aHUS</td>
<td>Uremia</td>
<td>TMA C3 deposition</td>
<td>CKD stage 5</td>
<td>0.4 100</td>
<td>D371G – –</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>54</td>
<td>aHUS</td>
<td>Uremia</td>
<td>NA</td>
<td>CKD stage 3</td>
<td>0.9 66</td>
<td>E601K Q580R –</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>6</td>
<td>MPGN</td>
<td>Nephrotic syndrome</td>
<td>MPGN C3, C1q, C5b-9 and IgM deposition</td>
<td>CKD stage 5</td>
<td>0.75 79</td>
<td>I242L N1050YV P457L</td>
</tr>
</tbody>
</table>

*All genetic variants shown are heterozygous. **Two kidney transplants were performed before the eculizumab era, the first functioned for 15 years and the second for 7 years. Three years after the second transplant, treatment with eculizumab was initiated due to HUS recurrence and was continued until the patient returned to dialysis. Eculizumab therapy was restarted at the time of the third kidney transplant without evidence of recurrence. \(^{a}\) The patient underwent two biopsies within 2 months. C3 deposits in capillary walls and mesangium increased in the second biopsy. The patient did not have circulating C3 nephritic factor. DGKE, diacylglycerol kinase epsilon; aHUS, atypical hemolytic uremic syndrome; TMA, thrombotic microangiopathy; CKD, chronic kidney disease; MPGN, membranoproliferative glomerulonephritis; NA, not available/not performed. Normal reference values for C3: 0.5-0.95 g/L (Patient 1), 0.77-1.38 g/L (Patients 2 and 3). Reference values vary between different clinical laboratories, Factor B: 75-125% (Patient 1), 59-154% (Patients 2 and 3). Patients 1 and 2 did not have antibodies against factor H.

Temporary Transfusion

Transfusion was performed as previously described (27). Briefly, human embryonic kidney 293 cells (HEK) cells (ATCC, Teddington, Middlesex, UK) were seeded and grown in DMEM/high glucose Hyclone medium (GE Healthcare Life Sciences, South Logan, UT), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum to approximately 95% confluence before transfection. Plasmid DNA (2 μg) was added to each well and transfection performed with Lipofectamine (Invitrogen, Life Technologies, Waltham, MA) according to the manufacturer’s instructions. Twenty-four h after transfection, the medium was changed to Optimem (Thermo Fisher Scientific) and cells were cultured for an additional 72 h. The media were collected and supplemented with protease inhibitors Complete Mini without EDTA (Roche Diagnostic, Mannheim, Germany) and centrifuged to remove cell debris.

Determination of Factor B Size by Immunoblotting

FB size in sera and cell media was determined by immunoblotting. Sera was diluted 1:2000, samples were reduced with mercapto-ethanol and incubated at 100°C for 5 minutes. Proteins were separated by SDS electrophoresis and transferred to a PVDF membrane. Plasma purified FB (1 mg/mL, Complement Technology, Tyler, Texas) was used as the control. Membranes were blocked overnight. Polyclonal goat anti-human FB antibody (1:1000, Complement Technology) was used as the primary antibody followed by rabbit anti-goat horse-radish-peroxidase (1:1000, DAKO, Glostrup, Denmark). Detection was performed by chemiluminescence (Pierce ECL2, Western Blotting Substrate, Rockford, IL) and detected using ChemiDoc™ Touch, Bio-Rad (Hercules, CA).
Measurement of Factor B Protein Levels
FB concentration of constructs was quantified by ELISA using mouse anti-human factor Ba (Quidel, San Diego, CA) for capture and goat anti-human FB polyclonal antibody (Complement Technology) for detection, followed by rabbit anti-goat horse-radish-peroxidase (HRP, 1:1000, DAKO, Glostrup, Denmark), alternatively an ELISA kit for detection of human FB (Abcam, Cambridge UK) showing comparable FB levels. Plasma purifed FB was used as the standard. Absorbance was measured at 450 nm using Glomax Discover (Promega, Madison, WI).

Complement Activation on Primary Glomerular Endothelial Cells
Primary glomerular endothelial cells (Cell Systems, Kirkland, WA) were plated on cell culture slides (Thermo Fisher Scientific) in endothelial growth medium (EGM-2, Lonza, Walkersville, MD), approximately 75000 cells per well and cultured to monolayer confluency. Cells were activated with adenosine diphosphate (ADP, 1 mM, Sigma-Aldrich) in serum-free EGM-2 for 10 min and washed with PBS with Mg/Ca (GE Life Sciences). Serum samples were diluted 1:4 in serum-free EGM-2 and magnesium-ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (Mg-EGTA, Complement Technology) 0.1M 1:10, incubated with the cells for 2 h at 37°C. Cells were washed, fixed in paraformaldehyde 4% for 30 min, washed thrice and blocked in 1% BSA for 30 min. C3c deposition on the cells was detected using rabbit anti-human C3c antibody:FITC (DAKO, Glostrup, Denmark) diluted 1:50 in 1% BSA for 1 h. Cells were stained with 4’,6-diamidino-2-phenylindole (DAPI, Thermo Fisher, Eugene, OR). Fluorescence was detected using a Ti-E inverted illumination microscopy module (Nikon Instruments, Tokyo, Japan). Image stacks at 10 x magnification were converted to maximal intensity images. Stained cells were outlined with a threshold above the background to select the area occupied by cells (DAPI-positive). Quantification was performed using ImageJ Fiji Software (Version 1.53b, NIH, Bethesda, MD).

In certain experiments the cells were incubated with FB constructs (50 µg/mL) in FB-depleted serum diluted 1:4 in serum-free EGM-2. The FB constructs were preincubated with and without the FD inhibitor danicopan ACH-4471 (MedChemtronica AB, Monmouth Junction, NJ) 10 µM for 15 min before a 2-hour incubation with the cells. Cell supernatants were collected and kept at -20°C until assayed using the sC5b-9 ELISA described below.

Hemolytic Assays Using Human Sera and Factor B Constructs
Complement activation in serum was assayed by incubation of the serum with sheep erythrocytes (5x10⁹/mL, Håtunalab, Bro, Sweden). Serum (20%) was added to gelatin veronal buffer (GVB) with Mg-EGTA 0.1M 1:10 to which normal human serum (20%) was added, as a source of normal C3, for 10 min at 30°C (28). Ethylenediaminetetraacetic acid (EDTA) 10 mM was added, and samples were centrifuged. Absorbance was measured at 405 nm using Glomax Discover (Promega, Madison, WI). Rabbit erythrocytes (5x10⁹/mL, Håtunalab) were used in GVB-Mg-EGTA buffer, as above, and incubated with FB constructs 5 µg/mL in FB-depleted serum (Complement Technology). Samples were incubated for 1 h on a shaker at 37°C, after which complement activation was terminated by addition of EDTA (Complement Technology). In certain experiments the FD inhibitor was incubated with erythrocytes in buffer to which FB-depleted serum was added before addition of the constructs. Absorbance was measured at 405 nm using Glomax Discover.

Binding of Factor B Constructs to C3 Measured by Surface Plasmon Resonance
Purified C3b (33 µg/mL in 10 mM sodium acetate (GE Healthcare Bio-Sciences), pH 5.0, Complement Technology) was amine-coupled to a CM5 sensor chip (GE Healthcare) corresponding to 5517.3 response units. The surface of the sensor chip was activated with a mixture of N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide. After covalent binding of C3b to the dextran matrix the surface was blocked with ethanolamine. Running buffer (10 mM Hepes (pH 7.4), 50 mM NaCl, 10 mM MgCl₂) was injected over the flow cells at a flow rate of 10 µL/min and 25°C. The C3 convertase was generated as previously described (29) by serial injections of FB 0.28 µg (60 nM) together with FD 0.02 µg in 50 µl running buffer followed by C3/H2O 2 µg. After the last step (FB+FD) the surface was extensively washed with 3 M NaCl in acetate buffer (pH 5.2) and 50 mM NaOH to rinse away residual noncovalently bound proteins.

Additional experiments were performed to study the interaction between C3b and FB, C3b was diluted in 10 mM sodium acetate (pH 5.5) at a concentration of 20 µg/mL, and then immobilized as above. The D371G, D279G and wild-type FB constructs were preincubated with and without the FD inhibitor danicopan ACH-4471 (MedChemtronica AB, Monmouth Junction, NJ) 10 µM for 15 min before a 2-hour incubation with the cells. Cell supernatants were collected and kept at -20°C until assayed using the sC5b-9 ELISA described below.

Factor B Cleavage by Factor D in Solid Phase
Microtiter wells were coated with C3b at 3 µg/mL diluted in phosphate-buffered-saline (PBS) overnight and blocked with bovine serum albumin (BSA, Sigma) for 1 h. Serum, 20% in Mg-PBS, or FB constructs 5 µg/mL in assembly buffer (Mg, FD 100 ng/mL in PBS with BSA 1%), were incubated for 30 min at 37°C and an additional 30 min with slow shaking. Samples were washed four times with PBS-Tween 20 0.1%. Twenty µl 10 mM EDTA with sodium dodecyl sulfate (SDS) 1% were added to the empty wells for 1 h on a microplate shaker 1000 rpm at rt.
Protein complexes were detached by scraping as described (30) and samples were stored at -20°C. In certain samples the FD inhibitor at 10 μM (final concentration) was preincubated with the samples for 15 min before addition to the plate.

Samples were reduced and loaded onto a Tris-TGX gel 10% (Bio Rad) and after protein separation transferred to PVDF membranes (Bio Rad) and electroblotted (Transblot Turbo, Bio Rad). Immunoblot was carried out with goat anti-FB and rabbit anti-goat:HRP and detected as described above.

**Soluble C5b-9 Measurement**

Soluble C5b-9 in the supernatant from activated glomerular endothelial cells was quantified using the MicroVue SC5-b9 Plus kit (Quidel, San Diego, CA) according to the manufacturer’s protocol. Absorbance was measured at 450 nm using Glomax Discover.

**Statistics**

Kruskal-Wallis multiple-comparison test followed by Dunn’s procedure was used for evaluating differences between more than two groups. A P value ≤ 0.05 was considered significant. Statistical analysis was performed using GraphPad prism 8 software (version 8.4.3, GraphPad Software, La Jolla, CA).

**RESULTS**

**Factor B Variants**

Three FB mutations were identified in Patients 1-3 (Table 1). The location of the gene products within FB domains is depicted in Figure 1. FB variants D371G (rs1258425617) (31) and I242L (rs1299040443) (7, 12, 32) have been reported before in patients with aHUS. Variant D371G (rs756325732) is located in the von Willebrand factor (VWF) type A domain of the Bb fragment, but far from the C3b binding site (Figure 1). The E601K variant has not been previously reported and is located in the serine protease domain, not near the catalytic site, at the VWF type A domain binding interface in the context of the pro-convertase C3bB (but not in the convertase C3bBb). The Bb fragment undergoes a conformational change upon release of Ba, leading to assembly of the metal ion dependent adhesion site (MIDAS). The mutated residue is far from the MIDAS, which participates in C3b binding, but may affect its assembly by allosteric effects (Figure 1). I242L is located in the linker between Ba and Bb fragments near the R234-K235 scissile bond (33). The D279G variant was used as a positive control as it was previously shown to induce a gain-of-function phenotype (34) and is located in proximity to the MIDAS in the von Willebrand factor A domain. The patients had normal serum FB levels (Table 1) and normal FB size (Supplementary Figure 1) and did not have autoantibodies to FB (data not shown).

**Phenotypic Assays of the FB Mutations**

Assays were performed to investigate the factor B phenotype using patient sera and mutant constructs, as outlined in Table 2.

**C3 Deposition on Glomerular Endothelial Cells**

Serum from Patients 1-3 and normal sera (n=10) were incubated with primary glomerular endothelial cells. Patient sera induced C3 deposition on the cells which was also detected for 3 normal...
sera incubated with the cells (Figure 2). C5b-9 deposition on the cells could not be assayed because the patients were treated with the anti-C5 antibody eculizumab (Table 1).

**Patient Sera Induced Hemolysis of Sheep Red Blood Cells**
Sera from Patients 1-3 and normal serum from two healthy controls were incubated with sheep erythrocytes. Sera from Patients 1 (FB: D371G) and 3 (FB: I242L, CFH: V62I, N1050Y and ADAMTS13: P457L) induced hemolysis whereas samples from Patient 2 (FB: E601K, DGKE: Q560R) and the normal controls (n=2) did not (Figure 3).

**Binding of Factor B Constructs to C3b and Formation of the C3 Convertase Determined by Surface Plasmon Resonance**
In binding assays, we first examined FB binding to C3b. C3b was immobilized on a Biacore sensor surface. The purified FB constructs, D279G (positive control, gain-of-function mutation in aHUS) (13), D371G, I242L or E601K were injected together with FD. Sensograms were aligned at t = 0 for comparison and showed that the FB construct D371G bound most, followed by D279G. The I242L, E601K and wild-type constructs demonstrated similar binding capacity (see arrow in Figure 4).

The C3 convertase was assembled on the sensor chip by serial injections of purified FB and FD followed by C3 (Figure 4) and showed that the factor B variant D279G yielded the highest binding, indicating C3 convertase formation, followed by D371G, the wild-type, E601K and I242L (see arrowhead in Figure 4).

Binding experiments showed that the FB mutant constructs D279G and D371G exhibited stronger binding to C3 than the wild-type construct (Figure 4B). Using a concentration range of the D371G and wild-type constructs (Figure 4C) the Ka, Kd and KD constant were calculated showing that the D371G construct had a higher affinity for C3 than the wild-type construct.

**Effects of Factor D Inhibition**
**Factor B Cleavage by the C3 Convertase**
A functional C3bBb(Mg2+) complex was formed in a microtiter plate by incubating C3b-coated wells with serum. In the presence

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**TABLE 2** | Complement functional assays performed in this study using patient sera and mutant constructs.

<table>
<thead>
<tr>
<th>Complement assays</th>
<th>Patient 1 D371G</th>
<th>Patient 2 E601K</th>
<th>Patient 3 I242L</th>
<th>Positive control D279G</th>
<th>Normal controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mutant construct</td>
<td>Serum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mutant construct</td>
<td>Serum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mutant construct</td>
</tr>
<tr>
<td>C3 deposition on glomerular endothelial cells</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysis sheep RBCs</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Factor B binding to C3b (surface plasmon resonance)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>Factor B degradation by factor D</td>
<td>Degr</td>
<td>Degr</td>
<td>Degr</td>
<td>Degr</td>
<td>Degr</td>
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<tr>
<td>Hemolysis of rabbit RBCs</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Soluble C5b-9 release from glomerular endothelial cells</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Serum from Patients 1 and 2 was taken during eculizumab treatment. Serum from Patient 3 was taken before the start of eculizumab treatment. +, complement activation detected; –, complement activation was not detected; ND, Not done; RBCs, red blood cells; Degr, degradation detected.

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**FIGURE 2** | C3 deposition on glomerular endothelial cells in the presence of serum from patients and controls. Sera from Patients 1-3 and controls (n = 10) were incubated with glomerular endothelial cells for 2 h and C3 detected by immunofluorescence. (A) Serum from Patients 1-3 induced excessive C3 deposition on the cells. The scale bar represents 100 µm. (B) Quantification of C3 deposition fluorescence showing that even 3/10 sera from apparently healthy adult controls exhibited C3 deposition. The bar represents the median.
of normal serum, as well as serum from Patients 1-3, the C3 convertase was formed and cleavage of FB to the Bb fragment was detected (Figure 5A). In the presence of the FD inhibitor, danicopan ACH-4471, FB cleavage was inhibited in normal serum, as well as in the serum of Patients 2 and 3, and partially inhibited in the serum of Patient 1 in which a weak Bb band was still visible.

Similarly, the C3b-coated plates were incubated with purified FB constructs together with FD showing that all constructs, wild-type, D279G, D371G, E601K and I242L, exhibited FB cleavage to the Bb fragment, albeit weaker for the I242L variant, and that cleavage was entirely inhibited in the presence of the FD inhibitor (Figure 5B).

Complement-Mediated Hemolysis of Rabbit Red Blood Cells

Rabbit red blood cells were incubated with FB constructs in FB-depleted serum and underwent hemolysis in the presence of FB variants D279G and D371G whereas the wild-type, as well as E601K and I242L constructs did not induce hemolysis. The FD inhibitor inhibited D279G- and D371G-induced hemolysis (Figure 6).

Release of Soluble C5b-9 From Primary Glomerular Endothelial Cells

Soluble C5b-9 was detected in supernatants from primary glomerular endothelial cells that were incubated with FB constructs D279G and D371G in FB-depleted serum. The FB construct D371G induced increased C5b-9 release compared to the wild-type construct (Figure 7). In the presence of the FD inhibitor the soluble C5b-9 levels were comparable with those released in the presence of the wild-type construct (Figure 7). Factor B mutant constructs E601K and I242L did not induce C5b-9 release compared to the wild-type.

DISCUSSION

Complement activation is a hallmark of aHUS, C3 glomerulopathy and MPGN. Here we explored three patients with CFB variants. One of these variants, D371G, was shown to be a gain-of-function mutation, as indicated by enhanced binding to C3b, formation of the C3 convertase, increased hemolysis of rabbit erythrocytes and release of soluble C5b-9 from glomerular endothelial cells. Additionally, the D279G variant, also found in aHUS (13), was used as a positive control and exhibited similar properties. Both CFB variants, D371G and D279G, could be effectively controlled by the FD small molecule-inhibitor danicopan (ACH-4471). The remaining two variants did not show gain-of-function but also did not perturb the inhibitory activity of danicopan. This suggests that FD inhibition should effectively inhibit complement activation in these patients.

CFB variants have been described in patients with aHUS (7–14, 31, 35, 36), in C3 glomerulopathy (15, 17) and in a few patients with immune-complex associated MPGN (16) but not all of them exhibit gain-of-function (12). Here we show that the mutant variant D371G, found in Patient 1, and reported previously (31), induces a clear-cut gain-of-function. These functional consequences can explain why the serum from Patient 1, without other complement mutations, induced C3 deposition on glomerular endothelial cells and hemolysis of sheep erythrocytes. Increased hemolysis in serum from this patient, treated with eculizumab at the time of sampling, is explained by the addition of rat serum, as a source of C5b-9, at which point the effect of eculizumab is eliminated by a washing step.

A novel CFB mutation E601K, in the serine protease domain of the protein, was found in Patient 2. This variant did not exhibit gain-of-function in the tests performed. Therefore, the increased complement deposition on endothelial cells cannot be explained by this genetic variant. The patient also had a mutation in the diacylglycerol kinase epsilon (DGKE) gene. DGKE mutations associated with aHUS do not directly cause complement activation and usually present during the first year of life (37), however this patient first presented with aHUS at mid-life. Thus, we assume that the DGKE variant was not associated with the patient’s disease.

The FB mutant variant I242L was detected in Patient 3, a child with what initially appeared to be immune complex-mediated MPGN. However, a second biopsy within 2 months showed more C3 deposition and suggested that the child might develop C3 glomerulopathy over time. This mutation was previously described in patients with aHUS and did not induce a clear gain-of-function (12). Serum from the patient induced C3 deposition on glomerular endothelial cells and enhanced hemolysis of sheep erythrocytes. The child also has previously reported genetic variants in CFH, V62I and N1050Y (38), suggesting that complement activation on cells may be a
combined effect of CFB and CFH variants, although functional data regarding the CFH variant N1050Y are lacking.

One limitation of this study was that only three patients were investigated and only one of the three (Patient 1) was found to have a gain-of-function mutation in factor B (D371G). In Patients 2 and 3 we could not determine a link between the patients’ clinical disease and the factor B mutations. The absence of functional consequences of the two CFB mutant variants, E601K and I242L, is in apparent contradiction with the complement activation observed on endothelial cells, incubated with patient sera. Ex vivo complement activation on endothelial cells has been previously reported as positive in aHUS patients without identified genetic abnormalities (39). Moreover, it is positive in patients with sickle cell disease (40), preeclampsia and HELLP syndrome (41) and as shown herein, even in some apparently healthy controls. In sickle cell disease, the complement overactivation was mediated, at least in part, by heme (40, 42). Heme or other pro-inflammatory factors may be present in the patient sera, activating the endothelial cells, rendering them susceptible to complement activation. Furthermore, although serum from Patient 2 induced C3 deposition on endothelial cells the serum did not induce hemolysis (Table 2) which is in line with the presence of a pro-inflammatory factor inducing changes on the surface of endothelial cells which did not fully activate the terminal complement complex.

FD inhibition has been previously assessed in samples from patients with the complement-mediated diseases PNH and aHUS. Low concentrations of FD inhibitors were shown to reduce C3 fragment deposition on PNH erythrocytes as well as complement-mediated hemolysis (19, 20). Likewise, serum from aHUS patients induced complement-mediated cell death in PIGA-null PNH-like cells which was abrogated by the FD-inhibitor (20). The results of the current study focused on FB mutations utilizing both patient sera and recombinant mutants, showing that the FD inhibitor prevented FB cleavage to Bb, hemolysis and the formation of C5b-9 in the presence of gain-of-function mutations, thereby blocking excessive complement activation.

Danicopan was found to be effective in preventing complement-mediated hemolysis in a phase 2 trial in patients with PNH (43). A phase 3 trial is ongoing in which Danicopan is being investigated as add-on therapy to C5 inhibitor for patients with PNH with extravascular hemolysis (22). For patients with aHUS current consensus recommends treatment with intravenous eculizumab.

**FIGURE 4** | Binding of factor B variants to C3b and formation of the C3 convertase. (A) Purified C3b was coupled to a CM5 sensor chip. Factor B (FB) variants and factor D (FD) were injected over the surface and binding curves visualized. The FB D371G mutant exhibited the strongest binding to the C3b-coated surface (see arrow) followed by D279G, I242L, the wild-type (WT) and E601K. This was followed by serial injections of C3 alternating with FB+FD to form the C3 convertase on the chip. The strongest C3 convertase generation was demonstrated for the D279G mutant (see arrowhead), followed by D371G, the wild-type, I242L and E601K. Baseline values were adjusted at t = 0 in each cycle for comparison. (B) Binding between C3b and FB alone was assessed using the wild-type construct, D371G and D279G showing that both mutant constructs, at 50 nM, exhibited stronger binding than the wild-type construct. (C) The coefficient of dissociation was evaluated using a range of FB concentrations comparing construct D371G to the wild-type.

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**Aradottir et al. FD-Inhibitor Blocks aHUS/MPGN FB Mutants**
FIGURE 6 | The effect of factor D inhibition on hemolysis of rabbit erythrocytes. Factor B constructs were incubated in factor B-depleted serum with rabbit erythrocytes. The mutant variants D279G (positive control) and D371G (corresponding to Patient 1) induced hemolysis. The other mutant constructs (E601K, I242L) and wild-type (wt) did not induce hemolysis. The factor D inhibitor (FD-inh) inhibited hemolysis induced by factor B mutants D279G and D371G. Eight separate experiments are shown. ***P < 0.001, ****P < 0.0001.

FIGURE 5 | The effect of factor D inhibition on C3 convertase formation using human serum or factor B mutants. An immunoblot assay was used to detect the Bb fragment of the alternative pathway C3bBb: (A) C3bBb(Mg2+) complexes were formed by incubating C3b-coated wells with normal serum (NS) or patient serum (Patients 1–3). The C3 convertase formed in the presence of all sera effectively cleaved factor B to the Bb component and this reaction was inhibited by the factor D inhibitor. The factor D inhibitor only partially blocked the C3 convertase in the presence of serum from Patient 1 (D371G mutation) as a weak Bb band was still visible. (B) The same assay was performed with the wild-type (WT) and mutant factor B constructs (D371G, E601K, I242L, and D279G) showing cleavage to the Bb fragments and effective inhibition by the factor D inhibitor. FB, factor B; Bb, the Bb fragment of factor B; FD, factor D; FD-inh, factor D inhibitor.
or ravulizumab (5, 44) thereby blocking C5. FD inhibitors should also be evaluated in clinical trials either as add-on therapy for C3 glomerulopathy patients, or for aHUS patients in whom C5 inhibition is insufficient, or as an alternative therapy. FD inhibitors present certain advantages over eculizumab for the treatment of aHUS. In addition to the extremely high price of eculizumab (45) and the oral mode of administration of danicopan, FD blockade will selectively inhibit the alternative pathway and allow activity of the classic and lectin pathways. Patients treated with eculizumab are at risk of meningococcal infection due to blockade of C3b-9 mediated bacterial killing, a risk that is considerably less with FD inhibitors (46). However, C3 degradation fragments physiologically promote opsonization and phagocytosis (47) which are also important for defense against meningococcal infections. Inhibition of these proximal effects would not occur in the presence of eculizumab, while they would be impeded in the presence of an FD inhibitor. In line with this upstream inhibition, the spike protein of SARS-CoV-2 was shown to activate the alternative pathway of complement, and a small molecule FD inhibitor prevented the cellular deposition of C3 fragments and the generation of C5b-9 (48). Similarly, we could show that danicopan inhibited formation of the C3 convertase and FB cleavage as well as release of soluble C5b-9 from cells exposed to the gain-of-function CFB D371G mutation.

In summary, we describe at the molecular level, the response of FB mutations to FD inhibition and that FB mutations do not impact the effective response to FD inhibition. The data suggest that FD inhibition should be further studied in clinical trials as a possible treatment for complement-mediated kidney diseases aHUS, MPGN and C3 glomerulopathy.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

**ETHICS STATEMENT**

The study of patients and healthy controls was performed with the approval of the Ethics Review Board at Lund University. The study was also approved by the National Bioethics Committee of Iceland and the Data Protection Officer at Oslo University Hospital, Oslo Norway. Informed written consent was obtained from the patients or the parents of Patient 3 and the healthy controls.
AUTHOR CONTRIBUTIONS

SA conceived and designed the analysis, analyzed the data; performed experiments and wrote the paper. A-CK performed experiments and wrote the paper. LR contributed conceptually, contributed plasmids and wrote the paper. AB contributed patient data and write-up. PK contributed patient data and write-up. RP contributed patient data, design and write-up. DK conceived and designed the analysis and wrote the paper. All authors contributed to the article and approved the submitted version.

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REFERENCES

9. Zhang Y, Kremsdorf R, Sperati CJ, Henrikson KJ, Mori M, Goodfellow R, et al. Mutation of Complement Factor B Causing Massive Fluid-Phase Dysregulation of the Alternative Complement Pathway can Result in acknowledged for their help with the surface plasmon resonance assays. The authors thank Drs Markus Heidenblad, Sofia Saal and Björn Hallström of the Center for Molecular Diagnostics, Region Skåne and Clinical Genomics Lund, SciLifeLab, Lund University for next-generation sequencing. Dr Henning Gong carried out part of the mutagenesis study as part of his master’s thesis. The kidney biopsies of Patient 3 were assessed by Dr. Melinda Raki, Department of Pathology, Oslo University Hospital, Oslo Norway. Dr. Sabine Leh, Department of Pathology, Haukeland University Hospital Bergen, Norway, Professor Sanjeev Sethi and Professor Fernando Fervenza of the Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota. This work was presented in preliminary poster form at the 17th Congress of the International Pediatric Nephrology Association, Igacua Brazil, September 20-24, 2016, at the 6th International Conference “HUS & related diseases”, Innsbruck, Austria, June 11-13, 2017, the 16th European Meeting of Complement in Human Disease, Copenhagen, Denmark, September 8-12, 2017, the 18th Congress of the International Pediatric Nephrology Association, Venice, Italy October 17-21, 2019.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.690821/full#supplementary-material

17. Osborne AJ, Breno M, Borsa NG, Bu F, Fremeaux-Bacchi V, Gale DP, et al. Statistical Validation of Rare Complement Variants Provides Insights Into the


21. Wiles JA, Galvan MD, Podos SD, Geffner M, Huang M. Discovery and Reproduction is permitted which does not comply with these terms. copyright owner(s) are credited and that the original publication in this journal is reprinted in other forums is permitted, provided the original author(s) and the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in any commercial or financial relationships that could be construed as a potential conflict of interest. Copyright © 2021 Aardottir, Kristoffersson, Roumenina, Bjerre, Kashoulus, Palsson and Karpman. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms. Confidential Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Supplementary Figure 1: Immunoblot of factor B size in sera from Patients 1-3
Immunoblot showing factor B from normal serum and from the serum of Patient 1 (factor B mutation D371G), Patient 2 (E601K) and Patient 3 (I242L). All patients had normal factor B size.
Paper II
Factor B Mutation in Monozygotic Twins Discordant for Atypical Hemolytic Uremic Syndrome

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KEYWORDS: atypical hemolytic uremic syndrome; complement; Factor B; genetics; monozygotic twins

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INTRODUCTION

Atypical hemolytic uremic syndrome (aHUS) is a life-threatening condition characterized by dysregulation of the alternative pathway of the complement system and manifesting with microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury. If left untreated aHUS often leads to kidney failure.¹ The disease is associated with heterozygous variants in genes encoding complement proteins and regulators including complement factor H, factor I, membrane cofactor protein, (CD46), C3, factor B, and hybrid genes or deletions in factor H-related proteins, or autoantibodies to factor H, leading to uncontrolled alternative complement pathway activation on endothelial cells and thrombotic microangiopathy.² Patients with aHUS may have a family history of the disease³ but not all carriers of genetic variants develop disease, even if the phenotype of the mutant variant leads to complement activation. A triggering complement-amplifying event occurring in an individual at risk, possessing one or more genetic variants in alternative complement pathway genes may thereby precipitate aHUS.

In this study, the gain-of-function factor B (CFB) variant we previously described, D371G,⁴ was examined in a large Icelandic family with aHUS. The family included monozygotic twins, both of whom are carriers of the rare variant but only one is affected by aHUS. The study traced the ancestral origin of this variant and investigated the phenotype of complement activation in the monozygotic twins discordant for disease expression to further understand the importance of genotype for the development of disease.

RESULTS

A Family With Hereditary aHUS

Three members of one family (see Supplementary Methods) were affected by aHUS. The pedigree is presented in Figure 1 in which individuals III-8, IV-9, and IV-10 are affected. The clinical characteristics and biopsy findings of the affected family members and their treatments are described in Supplementary Table S1. The affected individuals presented with manifestations of aHUS at different ages and experienced a variable disease course.

At presentation, complement levels were available from two of the subjects, III-8 and IV-10, both exhibiting low to low-normal C3 levels (Supplementary Table S2). During remission all three aHUS patients exhibited low C3 levels and normal factor B levels.

Factor B Variant in the Icelandic Family

The three affected family members were found to have the CFB mutation D371G mutation in exon 8 (c.1112 A>G; p.Asp371Gly, ACMG classification: pathogenic). In addition, four family members, unaffected by aHUS, were mutation carriers. Two unaffected carriers (II-5 and III-7, Figure 1) had low C3 levels (data not shown). Two related non-carriers of the CFB mutation (III-3 and III-5) had normal C3 levels.

Family members in the extended family (N = 203) were screened for the CFB variant and were found to
be non-carriers. The mutation occurred in the late nineteenth century (in the founder couple of this pedigree or their parents). The mutation was only observed in this family and is absent in 43,445 Icelanders who had whole-genome sequencing data available in the deCODE genetics database.

Discordant Phenotype in Monozygotic Twins

Individuals III-7 and III-8 are monozygotic twins. The zygosity of the twins was determined by examining common variants (minor allele frequency >5% in GnomAD) in the whole-genome sequencing data. The twins were concordant for all examined variants with high quality calls in both individuals and therefore deemed monozygous.

One of the twins (III-8) developed aHUS while his twin brother is an unaffected carrier of the mutation (III-7). The affected twin experienced a flu-like illness three weeks before developing aHUS. Two decades have passed since twin III-8 presented with aHUS and to-date his twin brother III-7 has not developed any signs or symptoms of aHUS. The twins have a similar body mass index. Further genetic investigation did not reveal de novo variants or genomic rearrangements in the affected twin. Samples were not available from the descendants of the unaffected twin to assess their carrier status, and they remain unaffected by disease.

Sheep Red Blood Cell Hemolysis Induced by Serum From the Monozygotic Twins

Serum from both the twins increased lysis of sheep erythrocytes and hemolysis was significantly increased using serum from the affected twin (III-8) compared with normal serum. A similar, albeit nonsignificant, trend was noted using serum from the unaffected twin.

Results from all three different timepoints of sampling (2014, 2016, and 2022, see Supplementary Methods) are shown in Figure 2a.

Complement Activation Products in the Serum of the Monozygotic Twins

Serum levels of the complement activation products C3a, C5a, and sC5b−9 were elevated in both twins compared with normal sera (n = 6, Figure 2b). Ba levels were not elevated when compared with normal controls except in one sample from the affected twin, III-8, obtained shortly after an infection. A significant difference was not detected when twin sera were compared by multivariate analysis.

Complement Activation on Glomerular Endothelial Cells Induced by Serum From the Monozygotic Twins

Serum from the twins (from two timepoints) was incubated with primary glomerular endothelial cells and analyzed for complement activation and deposition. Sera from both twins led to detection of complement activation products Ba, C3a, C5a and C5b−9 in the cell supernatants as shown in Figure 2c. There was no significant difference between the complement activation induced by serum from the affected twin compared with serum from the unaffected twin.

Glomerular endothelial cells were analyzed for C3c and C5b−9 deposition (Figure 2d). Serum from both twins induced C3c and C5b−9 deposition on cells but there was no visible difference between the twin sera regarding complement deposition (Figure 2e). These comparisons were not evaluated for statistical differences because serum from only one time-point was used.
Figure 2. Complement activation mediated by twin sera. (a) Hemolysis of unsensitized sheep erythrocytes incubated with twin (III-7 and III-8, Figure 1) sera obtained at three separate timepoints (2014, 2016, and 2022). Hemolysis is presented as percentage of 100% lysis induced by water. *: $P < 0.05$. ns: nonsignificant. Kruskal-Wallis multiple comparisons test followed by Dunn’s procedure. The bar represents the median. (b) Complement degradation products Ba, C3a, C5a, and C5b–9 were analyzed in serum samples from two different timepoints (2016 and 2022). Serum Ba was elevated in twin III-8 in a single sample taken shortly after an episode of infection. Complement degradation products C3a, C5a, C5b–9 were found to be at the same level in the sera from both twins and were elevated compared with normal controls ($n = 6$). The bar represents the median. (c) Complement activation was investigated by incubating serum with endothelial cells. Complement degradation...
DISCUSSION

aHUS has been associated with mutations and/or autoantibodies leading to excessive complement activation. aHUS may occur in families, but penetrance is incomplete.113 Harboring a disease-associated mutation does not necessarily lead to development of disease. This study investigated a gain-of-function CFB mutation, D371G,14 in a large Icelandic family in which three family members were affected by aHUS. Within this family, monozygotic twins carrying the CFB mutant variant exhibited discordant phenotypes, with one twin remaining unaffected while the other developed aHUS. Furthermore, serum from both twins induced similar complement activation on endothelial cells. These findings suggest that carrying the mutated variant, as such, does not fully account for disease expression but should be considered an important risk factor.

aHUS is not necessarily a monogenic disease because patients may have more than one disease-associated mutation or a risk-associated haplotype.1,4 Conversely, aHUS is not a polygenic disease either because a single mutation may be sufficient for developing disease.6 aHUS has been previously described in monozygotic twins, but not with a discordant phenotype. As monozygotic twins share most genetic variants, the discordance would most probably result from epigenetic variations which may also be linked to environmental exposures such as prenatal and postnatal events, infections, inflammations, vaccinations, as well as lifestyle variations, including body mass index discordance, that may contribute to complement activation and differences between monozygotic twins.9

By investigating hundreds of individuals in the extended family, comprising 5 generations, the origin of the mutation could be traced to an ancestral couple born in the late 1800s, or their parents. No other persons were found to carry the mutation in a sample representing close to one-seventh of the entire Icelandic population. Within this family, seven individuals were mutation carriers but only three were affected by aHUS. The aHUS disease phenotype may vary considerably between carriers of the same mutation even in the same family9 and the affected individual’s genotype cannot solely explain his or her phenotype, as described herein.

In summary, we describe a large family with a gain-of-function CFB mutation and individuals affected by aHUS in 2 generations. In this family, monozygotic twins exhibited a discordant presentation of aHUS despite similar levels of complement activation. We therefore conclude that other nongenetic factors are likely to affect the expression of disease in these individuals.

ACKNOWLEDGMENTS

The authors acknowledge scientific discussions with Professor Nancy Segal, California State University Fullerton. We are grateful for bioinformatic expertise provided by Dr. Petter Storm, Lund University. We thank Dr. Ida Arvidsson, Dr. Annie Wellysson, and Dr. Ashmita Tontanahal of the Department of Pediatrics, Lund University for their help with microscopy and quantification. This paper was presented in poster form at the 18th European Meeting on Complement in Human Disease 26-29/8, 2022 in Bern Switzerland. The authors are grateful to the patients and their family members for participating in the study.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)
Supplementary Methods.
Table S1. Clinical characteristics of the aHUS patients included in this study.
Table S2. Laboratory data of aHUS patients at presentation.

Figure 2. (continued) Product levels were measured in the supernatant from glomerular endothelial cells incubated with sera from the twins. Samples from two timepoints (2016: unfilled circles and 2022: filled circles) were analyzed. For each serum sample, six replicates incubated with cells are presented. Levels of activation products in diluted sera incubated in parallel without cells were subtracted from the presented values. ns: nonsignificant. Mann-Whitney U test. (d) C3c and C5b–9 deposition on glomerular endothelial cells incubated with serum from twins III-7 and III-8. A slight amount of C3c and more C5b–9 were visualized (green labeling). Scale bar: 100 μm. (e) Three replicates of serum incubated with cells were quantified. Fluorescence levels presented in panel B were calculated by measuring mean fluorescence intensity per cell showing an increase in C3c deposition in twin III-7 compared with twin III-8 but no differences regarding formation of C5b–9. Background staining (EGM-2 buffer incubated with cells) was deducted. Statistical comparison was not performed because only samples from 2016 were included. The bar represents the median. MFI, mean fluorescence intensity.
REFERENCES


Aradottir SS, et al.

Factor B mutation in monozygotic twins discordant for atypical hemolytic uremic syndrome

Supplementary methods

Subjects

Three patients with aHUS were diagnosed in the same family (Figure 1 in the main text). One of the family members carrying the gain-of-function factor B variant D371G was previously described.\(^1\) Samples for complement analysis were collected from the three affected family members and from four unaffected family members. Control samples were obtained from apparently healthy adults (n=13, 10 females).

The genotype of an additional 203 relatives was available in the deCODE database. DNA from 210 family members underwent Sanger sequencing. In addition to Sanger sequencing, whole-genome sequencing was performed in 78 of these individuals. Of these, seven individuals are descendants of II-1 and 18 individuals are descendants of II-3 (Figure 1 in the main text). Eleven individuals are second-degree (e.g. niece and nephew) and 61 are third-degree (e.g. grandniece and grandnephew) relatives of a founder male (I-1). Seven individuals are second-degree and 35 are third-degree relatives of the founder female (I-2). Fifty-one individuals are more distant relatives of the founder couple. The database contains whole-genome sequencing data from 45,000 Icelanders. The process of collecting samples and the subsequent imputation of data into the database has been previously described.\(^2\)

The study was conducted with the approval of the Regional Ethics Review Board of Lund University (approval number 2006/323), The Swedish Ethical Review Authority (2021-
04438), the National Bioethics Committee of Iceland and the Icelandic Data Protection Authority. Written informed consent was obtained from all individuals included in the study, patients, healthy controls as well as individuals included in the deCODE database.

**Blood samples**

Whole blood was drawn into vacutainer tubes according to standard procedure. Samples were centrifuged after one hour to separate the serum, stored at -80°C and, if necessary, transported in dry ice, thawed, aliquoted and frozen at -80°C until assayed. Whole blood in EDTA was transported at room temperature for DNA purification. Samples from two individuals (III-7 and III-8 in Figure 1) were obtained at three separate time-points (in the years 2014, 2016 and 2022).

**Genetic analysis and variant screening**

Next generation sequencing was performed using Next Seq 500 (Illumina, San Diego, CA) in collaboration with the Center for Molecular Diagnostics, Skåne University Hospital, Lund. The following gene panel was analyzed: CFH, CFHR1-5, CFI, MCP, CFB, C3, C5, CFP, ADAMTS13, DGKE, PLG, THBD and CLU. Whole-genome sequencing was performed on Novaseq (2x150 bp) with mean target depth of 30x. Bioinformatic analysis of variants was performed using Scout software (Similarities from COntinUous Traits [https://clinical-genomics.github.io/scout](https://clinical-genomics.github.io/scout)).

Whole-genome sequencing was also performed by deCODE genetics. Paired-end libraries for sequencing were prepared from DNA samples using Illumina preparation kits (TruSeq DNA, TruSeq Nano or TruSeq PCR-Free) according to the manufacturer’s instructions. Paired-end sequencing-by-synthesis (SBS) was performed on Illumina sequencers (GAIIx, HiSeq
2000/2500, HiSeq X or NovaSeq) to a target depth of 30x. Read lengths varied from \(2 \times 76\) to \(2 \times 150\) bp, depending on the instrument and/or sequencing kit used. Reads were aligned to the human genome assembly GRCh38 using the Burrows–Wheeler Aligner version 0.7.10. Alignments were merged into a single BAM file and marked for duplicates using Picard 1.117. Only non-duplicate reads were used for the downstream analyses. Variants were called using version 2014.4-2-g9ad6aa8 of the Genome Analysis Toolkit (GATK). The effects of sequence variants were annotated using release 80 of the Variant Effect Predictor (VEP-Ensembl), with RefSeq gene annotations. Sanger sequencing was performed using BigDye Terminator chemistry on a 3730 system (Applied Biosystems, Thermo Fisher Scientific), with primers designed using the Primer 3 software. Data from the large-scale whole-genome sequencing of 43,445 Icelanders was available at deCODE genetics.

**Complement biomarkers**

C3 and factor B levels were assayed according to hospital routines at the Department of Clinical Immunology and Transfusion Medicine, Skåne University Hospital Lund, Sweden. C3 concentration was assayed by nephelometry and factor B by rocket electrophoresis.

**Hemolytic assay**

C3 convertase activity leading to hemolysis was assayed by incubation of serum with sheep erythrocytes as previously described. Briefly, serum samples (20%) were combined with equal volumes of normal serum (20%), diluted in gelatin veronal buffer (GVB) containing Mg-EGTA (Complement Technology, Tyler, Texas, USA) and incubated with sheep erythrocytes (Håtunalab, Bro, Sweden). Ethylenediaminetetraacetic acid 10 mM (EDTA, Complement Technology) was added and the samples were centrifuged. The pellet was incubated with rat serum (1:5 in EDTA), as a source of terminal complement pathway proteins, for 1 h at 37°C.
After centrifugation absorbance in the supernatant was measured at 405 nm using Glomax Discover (Promega, Madison, WI).

**Complement activation on endothelial cells**

Primary glomerular endothelial cells (Cell Systems, Kirkland, WA) were plated on cell culture slides (Thermo Fisher Scientific, Eugene, OR) and grown to confluence. Serum was diluted 1:4 in endothelial growth medium-2 (EGM-2, Lonza, Walkersville, MD). Diluted serum was incubated with (or without) the cells for two h at 37°C. The supernatant was removed and stored at -80°C until analyzed. C3a, C5a, sC5b9 and Ba were measured in the cell supernatant, and in the diluted serum incubated without cells, using commercially available ELISA kits (Quidel, San Diego, CA) according to the manufacturer’s instructions.

After removal of the supernatant, cells were washed, fixed with paraformaldehyde 4% and stained for C3c and C5b-9 as previously described with minor modifications. Briefly, after discarding the supernatant, the cells were washed with phosphate buffered saline (PBS) with Mg²⁺/Ca²⁺ (GE Life Sciences, Logan) and blocked with 1% Bovine Serum Albumin (BSA, Sigma, St Louis, Missouri) for 1 h at room temperature and fixed. C3c deposition was detected with rabbit anti-human C3c:FITC 1:50 (DAKO, Glostrup, Denmark) in 1% BSA, and sC5b-9 deposition using rabbit anti-human complement C5b-9 1:1000 (Calbiochem, San Diego, CA) followed by Alexa 488-labeled anti-rabbit IgG (green) (Invitrogen #A11070). Nuclear counterstaining was performed with HCD Nuclear mask blue stain (Thermo Fisher Scientific). Slides were stored at -4°C until visualized. Fluorescence was detected using a Ti-E inverted fluorescence microscope equipped with a Nikon structured illumination microscopy module (Nikon Instruments Inc., Tokyo, Japan) and imaged using a Hamamatsu Flash 4 camera and Nikon NIS-elements AR software v.5.11.01. Quantification was performed by Image J Fiji.
v2.1.0/1.53c (NIH, Bethesda). Whole well images (10x magnification) were acquired. Triple replicates were performed for each serum sample. Double replicates were performed for the control, i.e., EGM-2 buffer incubated with cells without serum. As the distribution of cells was not uniform in the wells, areas without cells were deducted by choosing the region of interest (ROI) containing confluent cells. The ROI coordinates were marked in the FITC image before quantification. The threshold for fluorescence and area occupied by cells, was set above the background. The mean fluorescent area in each image was quantified as mean fluorescence intensity by multiplying the area with the intensity and dividing by the number of cells. Nonspecific labelling was estimated by quantification of fluorescent labelling in control samples in which the cells were incubated with EGM-2 buffer. This value was subtracted from values calculated for the cells incubated with sera.

Statistics

Comparison between two groups was assessed by two-tailed Mann Whitney U test. For multiple comparisons the Kruskal-Wallis test was performed followed by Dunn’s procedure between specific groups. Statistical analysis was performed using Prism software version 9.2.0 (GraphPad, La Jolla, CA).

References


Table S1: Clinical characteristics of the aHUS patients included in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age&lt;sup&gt;a&lt;/sup&gt; (yrs)</th>
<th>Clinical manifestations at presentation</th>
<th>Renal biopsy findings</th>
<th>Recurrences</th>
<th>Treatments</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma exchange</td>
</tr>
<tr>
<td>III-8</td>
<td>M</td>
<td>41</td>
<td>Malaise, headache, hypertensive crisis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Severe TMA C3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IV-9</td>
<td>M</td>
<td>24</td>
<td>Dizziness, headache, nausea</td>
<td>NA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IV-10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>M</td>
<td>1</td>
<td>Failure to thrive and irritability</td>
<td>Severe TMA C3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>, Age at presentation. <sup>b</sup>, Three weeks before presentation this patient experienced a flu-like illness. <sup>c</sup>, This patient was previously described. M: Male. TMA: Thrombotic microangiopathy. NA: Not available. Patient III-8 suffered a single episode of aHUS with severe renal failure that improved markedly following treatment with plasma exchange. One of his two sons (Patient IV-10) developed aHUS with renal failure in early childhood and has since undergone three kidney transplantations<sup>1</sup> while the other son experienced aHUS with severe renal failure requiring transient dialysis, followed by good recovery of kidney function that has since remained stable.

Table S2: Laboratory data of aHUS patients at presentation

<table>
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<tr>
<th>Patient</th>
<th>Hemoglobin (g/L)</th>
<th>Platelets x10^9/L</th>
<th>LD (U/L)</th>
<th>Haptoglobin (g/L)</th>
<th>Creatinine (µmol/L)</th>
<th>C3 (g/L)</th>
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<tr>
<td>III-8</td>
<td>83 (130-175)b</td>
<td>76 (150-400)b</td>
<td>1877 (105-205)b</td>
<td>&lt;0.06 (0.35-2.05)b</td>
<td>527 (60-100)b</td>
<td>0.74 (0.74-0.95)b</td>
</tr>
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<td>IV-9</td>
<td>85 (134-171)</td>
<td>77 (150-400)</td>
<td>662 (105-205)</td>
<td>&lt;0.06 (0.35-2.05)</td>
<td>298 (60-100)</td>
<td>NA</td>
</tr>
<tr>
<td>IV-10</td>
<td>75 (110-150)</td>
<td>52 (150-400)</td>
<td>1119 (120-600)</td>
<td>&lt;0.06 (0-2.05)</td>
<td>719 (20-40)</td>
<td>0.4 (0.5-0.95)</td>
</tr>
</tbody>
</table>

a, Patient numbering according to Figure 1.
b, Reference values at the clinical laboratory according to the sex and age of the patient at the time of sampling.
LD: Lactate dehydrogenase. NA: Not available.
Genetic investigation of Nordic patients with complement-mediated kidney diseases

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Department of Pediatrics, Clinical Sciences Lund, Lund University, Lund, Sweden

Background: Complement activation in atypical hemolytic uremic syndrome (aHUS), C3 glomerulonephropathy (C3G) and immune complex-mediated membranoproliferative glomerulonephritis (IC-MPGN) may be associated with rare genetic variants. Here we describe gene variants in the Swedish and Norwegian populations.

Methods: Patients with these diagnoses (N=141) were referred for genetic screening. Sanger or next-generation sequencing were performed to identify genetic variants in 16 genes associated with these conditions. Nonsynonymous genetic variants are described when they have a minor allele frequency of <1% or were previously reported as being disease-associated.

Results: In patients with aHUS (n=94, one also had IC-MPGN) 68 different genetic variants or deletions were identified in 60 patients, of which 18 were novel. Thirty-two patients had more than one genetic variant. In patients with C3G (n=40) 29 genetic variants, deletions or duplications were identified in 15 patients, of which 9 were novel. Eight patients had more than one variant. In patients with IC-MPGN (n=7) five genetic variants were identified in five patients. Factor H variants were the most frequent in aHUS and C3 variants in C3G. Seventeen variants occurred in more than one condition.

Conclusion: Genetic screening of patients with aHUS, C3G and IC-MPGN is of paramount importance for diagnostics and treatment. In this study, we describe genetic assessment of Nordic patients in which 26 novel variants were found.

KEYWORDS complement, atypical hemolytic uremic syndrome, C3 glomerulopathy, membranoproliferative glomerulonephritis, genes

Introduction

Rare genetic variants have been associated with the ultra-rare complement-mediated kidney diseases atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy...
IC-MPGN is also a chronic form of glomerulonephritis that can progress to end-stage renal disease (26). It cannot be differentiated from C3GN based on renal symptoms and ultramorphology but immunofluorescence exhibits deposits of immunoglobulins as well as complement (23, 26). Although IC-MPGN can be associated with infections, such as viral hepatitis, or monoclonal gammapathy (23), cluster analysis revealed that some patients have excessive complement activation via the alternative pathway (27), properdin-dependent C3 nephritic factors (28) as well as genetic variants in complement (26). This suggests an overlapping spectrum of disease between C3G and IC-MPGN with regard to etiology and clinical course (26, 29).

Genetic variants have been found in these complement-mediated kidney diseases in several population studies (24, 30–37). The aim of this paper is to describe genetic variants associated with aHUS, C3G and IC-MPGN in patients from Sweden and Norway. Sequencing encompassed the genes encoding complement factor H, C3, factor I, factor B, MCP, C5, factor H-related proteins 1-5, clusterin, DGKE, thrombomodulin, plasminogen, and properdin. Twenty-six novel genetic variants were found, and certain variants were found in more than one condition.

Methods

Patients

Patients with suspected complement-mediated renal diseases or thrombotic thrombocytopenic purpura (TTP) are referred to the Dept of Pediatrics, Lund University, for genetic analysis. In this study patients with suspected TTP were not included. A total of 141 patients, both children and adults, living in Sweden or Norway, were included, 94 patients with a clinical diagnosis of aHUS, 40 patients with C3G and 8 patients with IC-MPGN. One patient had both aHUS and IC-MPGN. The diagnosis of aHUS was made by the referring physician based on the simultaneous presentation of non-immune hemolytic anemia, thrombocytopenia, and acute kidney injury as well as negative testing for enterohemorrhagic E. coli. Patients with documented defects in cobalamin metabolism were excluded. The diagnosis of C3G and IC-MPGN was based on kidney biopsy results. Patient data consisted of age of disease debut, laboratory data (such as complement levels and the presence of auto-antibodies), course of disease including kidney failure and familial cases. Data regarding treatment was not uniformly available as most samples were collected upon initial diagnosis. Patient data are presented in Table 1. Informed consent was obtained for diagnostic genetic analysis and the project was approved by the Swedish Ethical Review Authority, approval no. 2021-04438. The Swedish Ethical Review Authority waived the requirement for written consent from patients included retrospectively in this study. All patients included after October 2021 gave informed written consent.
Genomic analysis

Genomic DNA was extracted and analyzed by Sanger sequencing until the end of 2016. These samples were assayed for variants in genes encoding factor H, factor I, membrane cofactor protein CD46, C3 and factor B. From 2017 samples were assayed by next generation sequencing first using whole exome sequencing and from 2020 by whole genome sequencing. A panel of 17 genes was assayed including complement factor H (CFH), factor I (CFI), membrane cofactor protein (MCP, CD46), C3, factor B (CFB), properdin (CFP), clusterin (CLU), factor H-related proteins 1-5 (CFHR1-CFHR5), ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), thrombomodulin (THBD), DGKE, C5 and plasminogen (PLG). In this study variants in ADAMTS13 were not included.

For Sanger sequencing genomic DNA was combined with DNA polymerase, primers and nucleotides. The PCR product was sequenced bidirectionally using fluorescent chain-terminating nucleotides (38) from Big dye terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems DNA Analyzer, model 3730.

Next generation sequencing was performed in collaboration with the Center for Molecular Diagnostics, Skåne University Hospital, Lund, using either whole exome or whole genome sequencing. Whole-exome sequencing (WES) libraries were generated using Agilent SureSelect Clinical Research Exome v2. Whole-genome sequencing libraries were prepared using Illumina TruSeq PCR-Free. Sequencing was done on either Next Seq 500 (WES) or Novaseq (WES and NGS), with 2 x 150 bp paired end reads and a target depth of at least 30x. Resulting reads were analyzed using the Broad Institute best practices (https://www.broadinstitute.org/gatk/guide/best-practices) as implemented in the Sentieon software suite (https://www.sentieon.com/). Briefly, reads were mapped to the human genome (build hg19) with BWA MEM and variants were identified using DNASequence as implemented in Sentieon. Structural variants were detected with CNVnator and Manta for WGS, and with CNVkit for WES. Variants were annotated using Ensemble Variant Effect Predictor (VEP, https://www.ensembl.org/info/docs/tools/vep/) and filtered for the following genes: CFH, CFHR1-5, CFI, MCP, CFB, C3, C5, CFP, DGKE, PLG, THBD and CLU. All relevant variants were verified in Integrative Genomics Viewer (IGV, https://software.broadinstitute.org/software/igv). Interpretation of variants was performed using Scout software (Similarities from COntinUous Traits https://clinical-genomics.github.io/scout) and prediction was performed using Mastermind (mastermind.genomenon.com).

Certain DNA samples were also sequenced at Centogene, Rostock Germany.

Data analysis

Sequencing data included nucleotide shift and amino acid alterations as well as zygosity. This information was analyzed using databases describing mutations and polymorphisms in the included genes such as www.complement-db.org, https://gnomad.broadinstitute.org/ and www.ncbi.nlm.nih.gov/snp for variant calling. Minor allele frequency was defined by the frequency of the second most common allele. Minor allele frequency less than 1% was defined as a possible mutation. Variants that were previously described as associated with disease were included even if the minor allele frequency was > 1%. Variants were considered novel if not previously published in the medical literature. Variants that were previously reported in the ClinVar database in association with complement-mediated diseases such as age-related macular degeneration, aHUS or C3G are mentioned.

Assay of complement and auto-antibodies

C3 was analyzed by nephelometry and C3d by double-decker rocket immunoelectrophoresis according to hospital routines. Low and high levels were defined as below or above the laboratory reference values. Antibodies against factor H and C3 or C4 nephritic factor were detected at the Department of Clinical Immunology, Skåne University Hospital in Lund as per hospital routines. Factor H antibodies were detected as previously described with minor modifications (3). C3 nephritic factor was detected using three methods, by ELISA (39), by hemolytic assay (40) and by crossed immunoelectrophoresis (41) and (42). If any of these assays were positive the patient was defined as having C3 nephritic factor. C4 nephritic factor was detected as previously described (43) with minor modifications.
Results

Genetic variants associated with disease

Of all 141 patients with aHUS, C3G and IC-MPGN 80 patients were found to have genetic variants. Of 94 aHUS patients, 60 patients had genetic variants. Of these 32 patients had more than one variant and a total of 68 different aHUS-related variants were identified, considering deletions in CFHR1 and CFHR3 as one variant, as these genes are adjacent to each other. The genetic variants in aHUS patients are summarized in Table 2 and Supplementary Table 1. Deletions in CFHR1 and CFHR3 are only reported when homozygous except for one patient with antibodies to factor H (patient 17). Eighteen variants were novel to this study and have not been described before. Two of these was previously reported in ClinVar in association with complement-mediated disease. The association between the genetic variants and kidney function, if known, is summarized in Supplementary Table 1.

In 40 C3G patients 15 patients were found to have 29 genetic variants which could be associated with disease (considering homozygous deletions in CFHR1 and CFHR3 as one variant). Of these 8 patients had more than one variant. Nine variants in C3G were novel to this study and have not been described before (two of these were previously reported in ClinVar in association with complement-mediated diseases). The genetic variants are summarized in Table 3. The association between the genetic

<table>
<thead>
<tr>
<th>Variant</th>
<th>Nucleotide shift</th>
<th>Type of variant</th>
<th>dbSNP</th>
<th>Domain</th>
<th>Minor Allele frequency</th>
<th>Functional studies</th>
<th>ACMG classification</th>
<th>Reference</th>
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<td>CFH</td>
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<td>VUS</td>
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<td>–</td>
<td>SCR10</td>
<td>0.000007969</td>
<td>LeF</td>
<td>LP</td>
<td>(47)</td>
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<td>0.00011592</td>
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<td>–</td>
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### TABLE 2 Continued

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variants and kidney function, if known, is summarized in Supplementary Table 2.

Eight patients with IC-MPGN were investigated but one of these also had aHUS (patient 254 in Supplementary Tables 1, 3). In the remaining seven patients, four patients had five genetic variants. The genetic variants are summarized in Table 4. The association between the genetic variants and kidney function, if known, is summarized in Supplementary Table 3.

The number of genetic variants detected in patients with aHUS and C3G is summarized in Tables 5, 6, respectively. For patients with IC-MPGN only five variants were detected. In aHUS most variants were detected in the gene encoding CFH followed by C3 and in C3G the reverse was found.

The location of all genetic variants detected is presented in Figure 1 which also depicts which variants are novel and which were found in more than one condition. Most variants were heterozygous, however, homozygous variants were found in CFH (P621T), CFI (S326P), CD46 (c.97 + 1G>A), CFHR5 (V110A).

### TABLE 2 Continued

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<tr>
<th>Variant</th>
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<th>Type of variant</th>
<th>dbSNP</th>
<th>Domain</th>
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### CLU

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### DGKE

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### THBD

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### PLG

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Variants in aHUS and C3G

The number of genetic variants detected in patients with aHUS and C3G is summarized in Tables 5, 6, respectively. For patients with IC-MPGN only five variants were detected. In aHUS most variants were detected in the gene encoding CFH followed by C3 and in C3G the reverse was found.

The location of all genetic variants detected is presented in Figure 1 which also depicts which variants are novel and which were found in more than one condition. Most variants were heterozygous, however, homozygous variants were found in CFH (P621T), CFI (S326P), CD46 (c.97 + 1G>A), CFHR5 (V110A),...
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<td>(48)</td>
</tr>
<tr>
<td>C3</td>
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<td>(58, 59)</td>
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<td>V326Mf</td>
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<td>VUS</td>
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<td>This study</td>
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<td>rs1019532370</td>
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<td>0.00001193</td>
<td>-</td>
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<td>This study</td>
</tr>
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<td>W1631*</td>
<td>c.4893G&gt;A</td>
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<td>c.4030-4C&gt;G</td>
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<td>-</td>
<td>P</td>
<td>(55)</td>
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</tr>
<tr>
<td>CFI</td>
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<td></td>
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</tr>
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<td>c.1534+5G&gt;T</td>
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<td>rs114013791</td>
<td>Intron 12</td>
<td>0.00866</td>
<td>-</td>
<td>-</td>
<td>(33)</td>
<td></td>
</tr>
<tr>
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<td>Missense</td>
<td>rs144164794</td>
<td>Linke2</td>
<td>-</td>
<td>LoF</td>
<td>LP</td>
<td>(35, 65)</td>
</tr>
<tr>
<td>CD46</td>
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<td></td>
<td></td>
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<td>A355Vab</td>
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<td>Missense</td>
<td>rs53566573</td>
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<td>0.01541</td>
<td>LoF, NFE</td>
<td>Conflicting</td>
<td>(33, 73)</td>
</tr>
<tr>
<td>C5</td>
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<tr>
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<td>G385R</td>
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<td>Missense</td>
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<td>MG4</td>
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<td>-</td>
<td>This study</td>
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<td>Deletion</td>
<td>Deletion</td>
<td>–</td>
<td>–</td>
<td>LB</td>
<td></td>
<td></td>
<td>(76)</td>
</tr>
<tr>
<td>Exon 6 duplication</td>
<td>Duplication</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>LB</td>
<td>This study. Other duplications reported in (83)</td>
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</tr>
<tr>
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<td>c.423G&gt;A</td>
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<td>Deletion</td>
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<td>–</td>
<td>–</td>
<td>(76)</td>
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<td></td>
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<tr>
<td>CFHR4</td>
<td>Y43F</td>
<td>c.128A&gt;T</td>
<td>Missense</td>
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<td>SCR1</td>
<td>0.001747</td>
<td>-</td>
<td>LB</td>
</tr>
<tr>
<td>c.799+3A&gt;C</td>
<td>Intron splice</td>
<td>Rs196876631</td>
<td>–</td>
<td>0.001286</td>
<td>-</td>
<td>LB</td>
<td>(82)</td>
<td></td>
</tr>
<tr>
<td>CFHR5</td>
<td>E163Kfs*10</td>
<td>c.485_486dup</td>
<td>Frameshift (insertion)</td>
<td>rs565437964</td>
<td>SCR3</td>
<td>0.006750</td>
<td>NPE</td>
<td>-</td>
</tr>
<tr>
<td>E226Dfs*7</td>
<td>c.678del</td>
<td>Deletion</td>
<td>rs1438537910</td>
<td>SCR4</td>
<td>0.000007964</td>
<td>-</td>
<td>P</td>
<td>This study</td>
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</table>

(Continued)
DGKE (Y326* and S485F), in patients with aHUS (the patient with the homozygous CFH variant P621T also had IC-MPGN). In both aHUS and C3G homozygous deletions in CFHR1 and CFHR3 were detected. Certain variants were found in both aHUS and C3G or IC-MPGN patients. These included CFH (P621T in the same patient, D693N, Q950H, N1050Y (the latter with MAF>1%) and S1209T), C3 (K155Q and W1631*), CFI (G328R, c.1534 + 5G>T), CD46 (A353V, MAF >1%), CFHR5 (E163Kfs*10, E226DFS*7, Y279N), THBD (P501L), PLG (R89K and R261H) and homozygous deletions of CFHR1 and CFHR3. Of these CFHR5 E226DFS*7 was novel and found in both aHUS and C3G patients.

Genetic variants and the course of disease

Upon referral for genetic testing, we had access to clinical information describing the course of disease in most patients. When these data were available, we correlated the genetic findings to the presence of kidney failure, as presented in

### TABLE 3 Continued

<table>
<thead>
<tr>
<th>Variant or deletion</th>
<th>Nucleotide-shift</th>
<th>Type of variant</th>
<th>dbSNP</th>
<th>Domain</th>
<th>Minor Allele frequency</th>
<th>Functional studies</th>
<th>ACMG classification</th>
<th>Reference</th>
</tr>
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<tbody>
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<td>Y279N</td>
<td>c.835T&gt;G</td>
<td>Missense</td>
<td>rs143240067</td>
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<td>0.0001274</td>
<td>-</td>
<td>Conflicting</td>
<td>(78)</td>
</tr>
<tr>
<td>R356H</td>
<td>c.1067G&gt;A</td>
<td>Missense</td>
<td>rs35662416</td>
<td>SCR6</td>
<td>0.01633</td>
<td>NPE</td>
<td>LB</td>
<td>(77, 84)</td>
</tr>
<tr>
<td>CFP</td>
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<td></td>
</tr>
<tr>
<td>D299N</td>
<td>c.895G&gt;A</td>
<td>Missense</td>
<td>rs61737993</td>
<td>TSP t1 5</td>
<td>0.001472</td>
<td>-</td>
<td>B</td>
<td>(85)</td>
</tr>
<tr>
<td>CLU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>K444Q</td>
<td>c.1339A&gt;C</td>
<td>Missense</td>
<td>rs2612311022</td>
<td>β-chain</td>
<td>0.0001026</td>
<td>-</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td>PLG</td>
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<td>R89K</td>
<td>c.266G&gt;A</td>
<td>Missense</td>
<td>rs143079629</td>
<td>PAN</td>
<td>0.006191</td>
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<td>B</td>
<td>(48)</td>
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<td>R261H</td>
<td>c.782G&gt;A</td>
<td>Missense</td>
<td>rs4252187</td>
<td>Kringle 2</td>
<td>0.002501</td>
<td>-</td>
<td>Conflicting</td>
<td>(80)</td>
</tr>
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</table>

a, Mentioned in the complement database (www.complement-db.org) with reference to (48). b, Minor allele frequency > 1% but this variant was previously associated with aHUS. c, Previously reported in the ClinVar database in association with age-related macular degeneration and aHUS. d, Previously reported in the ClinVar database in association with aHUS. CFH: Complement factor H; C3: Complement C3; CFB: Complement factor B; CFI: Complement factor I; CD46: CD46/Membrane cofactor protein; C5: Complement C5; CFHR1-5: Complement factor H related 1-5; CFP: Complement factor properdin; PLG: Plasminogen; Domains, SCR: Short consensus repeats; MG1-8: Macroglobulin domain 1-8; TSP: Thrombospondin type-1 1-5; PAN: Plasminogen-Apple-Nematode; NPE: No phenotypic effect; GoF: Gain of function; LOF: Loss of function (including low plasma concentration); VUS: Variant of unknown significance; LP: Likely pathogenic; LB: Likely benign; P: Pathogenic.

### TABLE 4

<table>
<thead>
<tr>
<th>Variant or deletion</th>
<th>Nucleotide-shift</th>
<th>Type of variant</th>
<th>dbSNP</th>
<th>Domain</th>
<th>Minor Allele frequency</th>
<th>Functional studies</th>
<th>ACMG classification</th>
<th>Reference</th>
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<td>rs762422305</td>
<td>SCR10</td>
<td>0.000007969</td>
<td>LoF</td>
<td>LP</td>
<td>(47)</td>
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<td>N1050Y</td>
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<td>0.01469</td>
<td>NPE</td>
<td>LB</td>
<td>(45, 54)</td>
</tr>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>A353V</td>
<td>c.1013C&gt;T</td>
<td>Missense</td>
<td>rs35366573</td>
<td>TM</td>
<td>0.01541</td>
<td>LoF, NFE</td>
<td>Conflicting</td>
<td>(33, 73)</td>
</tr>
<tr>
<td>CFB</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>I242L</td>
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<td>rs144812066</td>
<td>Linker</td>
<td>0.001060</td>
<td>NFE</td>
<td>VUS</td>
<td>(30, 69)</td>
</tr>
<tr>
<td>THBD</td>
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<td></td>
<td></td>
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<td></td>
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<td>Linker</td>
<td>0.001785</td>
<td>-</td>
<td>VUS</td>
<td>(11)</td>
</tr>
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<tr>
<td>R356H</td>
<td>c.1067G&gt;A</td>
<td>Missense</td>
<td>rs35662416</td>
<td>SCR6</td>
<td>0.01633</td>
<td>NPE</td>
<td>LB</td>
<td>(77, 84)</td>
</tr>
</tbody>
</table>

a, Minor allele frequency > 1% but this variant was previously associated with aHUS. CFH, Complement factor H Hom, homozygous; CFB, Complement factor B; THBD, Thrombomodulin; Domains, SCR, Short consensus repeats; TM, Transmembrane protein; LoF, Loss of function; NPE, No phenotypic effect; GoF, Gain of function; LP, Likely pathogenic; LB, Likely benign; VUS, Variant of unknown significance.
Supplementary Tables 1-3. The genetic variants were correlated to levels of C3, C5d and the presence of antibodies to factor H in aHUS (Supplementary Table 1) or nephritic factors in C3G or IC-MPGN (Supplementary Tables 2, 3).

Discussion

In complement-mediated kidney diseases aHUS, C3G, and IC-MPGN understanding a patient’s genotype and its correlation to disease phenotype is of paramount importance for diagnostics and choice of treatment. Genetic investigation is also crucial for determining the risk of disease recurrence, the suitability of kidney transplantation and the choice of donor, as well as evaluation of the risk of disease development in family members bearing the same variant, including family members considered as kidney donors. This study investigated the genotype of a large cohort of patients with these diseases using a panel of disease-associated genes. In aHUS 68 different variants were identified and in C3G 29 different variants were identified, most variants were heterozygous and 26 were novel. Importantly, 40 patients with aHUS and C3G had more than one genetic variant, exemplifying the complexity of interpreting disease heredity.

Seventeen genetic variants, novel as well as previously reported, had a dual disease phenotype occurring in both patients with aHUS and C3G. The presence of a disease-associated genetic variant is not tantamount to the development of symptoms, as it is known that disease penetrance is incomplete (67, 86) and we show here that identical genetic alterations may be associated with different disease phenotypes. This suggests that disease expression may be related to additional genetic factors (complement genes as well as others), the exposome such as triggering factors and, as yet, undefined environmental, lifestyle or epigenetic factors.

In aHUS and C3G genetic variants were previously identified in complement components and complement regulatory proteins (24, 30–37). The variants may cause a loss-of-function in regulators or a gain-of-function in complement factors. Variants in CFH have been shown to lead to a loss-of-function by reducing cofactor activity, as shown for Q950H (52) and V1168E (50), impairing C3b binding, demonstrated for S1191L (87), or by deficiency associated with decreased protein secretion, as shown for P621T (47). Additionally, variant S1191L exhibited impaired ability to regulate complement activation on cell surfaces (87). Genetic variants in factor I can lead to quantitative deficiency or functional defects in protease activity rendering the enzyme incapable of inactivating C3b (59). This was, however, not specifically demonstrated for the variants presented herein, as, for example, the aHUS-associated variant G261D did not exhibit complement dysregulation (64). CD46 variants may also cause loss-of-function by reduced expression on cell surfaces, as shown for c.286 + 2T>G and S201L (70), decreased cofactor activity, or affect C3b/C4b binding capacity (72, 86). Likewise, thrombomodulin inhibits complement activation by promoting C3b inactivation and mutated variants have exhibited less C3b inactivation to iC3b on cell surfaces, as shown for the P501L variant (11).

### TABLE 5 Prevalence of variants in aHUS patients included in this study (n=94).

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Number of novel variants</th>
</tr>
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<tbody>
<tr>
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<td>7</td>
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<td>CFI</td>
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<td>C5</td>
<td>4</td>
<td>3</td>
</tr>
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</tr>
<tr>
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<td>CFHR4</td>
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</tr>
<tr>
<td>CLU</td>
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<td>1</td>
</tr>
<tr>
<td>DGKE</td>
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<td>2</td>
</tr>
<tr>
<td>THBD</td>
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<td></td>
</tr>
<tr>
<td>PLG</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>68</strong></td>
<td><strong>18</strong></td>
</tr>
</tbody>
</table>

* A deletion in CFHR1 and CFHR3 was considered one variant as these are neighboring genes and deleted together. CFH: Complement factor H. C3: Complement C3. CFB: Complement factor B. CFI: Complement factor I. CD46: CD46/Membrane cofactor protein. C5: Complement C5. CFHR1-5: Complement factor H related 1-5. PLG: Plasminogen. THBD: Thrombomodulin. DGKE: Diacylglycerol kinase epsilon.

### TABLE 6 Prevalence of variants in C3G patients included in this study (n=40).

<table>
<thead>
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<th>Gene</th>
<th>Number of variants</th>
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</tr>
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<tbody>
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<td>C3</td>
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<td>3</td>
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<td>CFI</td>
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<td>3</td>
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<tr>
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</tr>
<tr>
<td>CLU</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PLG</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CFP</td>
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<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>29</strong></td>
<td><strong>9</strong></td>
</tr>
</tbody>
</table>

* A deletion in CFHR1 and CFHR3 was considered one variant as these are neighboring genes and deleted together. CFH: Complement factor H. C3: Complement C3. CF: Complement factor B. CFI: Complement factor I. CD46: CD46/Membrane cofactor protein. C5: Complement C5. CFHR1-5: Complement factor H related 1-5. PLG: Plasminogen. CFP: Complement factor properdin.
C3 and CFB variants may exhibit gain-of-function properties in the respective encoded proteins. The variant V1658A in C3 results in increased C3 convertase formation (62) and K155Q confers increased hemolytic activity (58). Additionally, the C3 variant K65Q is associated with reduced binding to factor H (57), and variant R592W exhibits impaired binding to the complement regulator CD46/MCP (4).

CFB variants may increase the affinity to C3b, thus stabilizing the convertase, as shown for variant D371G (69) and by increased resistance to factor H-mediated decay (32, 88). However, not all variants show dysfunction in in vitro models, as reported for L433S, I242L and E601K (67, 69).

Variants in DGKE cause the development of aHUS in young children, often in combination with proteinuria. It has been suggested that homozygous DGKE variants lead to loss-of-function and consequently a prothrombotic state (12), however the mechanism by which the variants Y326* and S485F lead to
disease has yet to be deciphered. Likewise, plasminogen deficiency or dysfunction is associated with reduced proteolytic activity in growing thrombi which may contribute to a thrombosis (89).

Many patients had several variants in more than one gene. In these cases, the individual variants themselves may not lead to disease development, but when combined may result in complement over-activation (67, 90). As previously described in an anephric aHUS patient, with a CFI disease-associated haplotype as well as CFI and CFB variants, the patient had evidence of complement activation, developed carotid artery stenosis, and was successfully treated with eculizumab (67). Some genetic variants are distinctly pathological, even without the presence of other variants, such as the C3 mutation V1636A (62). On the contrary, some variants that in vitro exhibit a dysfunctional protein, may be present in unaffected carriers that remain disease-free, such as the CFI variant V1168E (50) and the CFB variant D371G (91).

For the variants presented herein we provide prediction as to their possible pathogenicity, when available. Prediction models can efficiently assess if a genetic alteration is benign or pathogenic (92), however, they are not always accurate and may require combining various prediction scores (93), thus interpretation can be challenging. Functional tests, using mutant compared to wild-type proteins, can more accurately demonstrate protein dysfunction. Considering the rarity and complexity of disease expression, we recommend performing mutagenesis to predict the effect of genetic variants on complement activation.

This study found that 72% of aHUS and 38% of C3G patients had genetic variants, therefore not all patients who develop disease carry variants in the screened genes. In aHUS this may, in part, be due to the presence of antibodies to factor H and in C3G to the presence of nephritic factors which may cause disease. Additionally, 47 patients were sequenced using only the Sanger method. In these patients, all the known disease-related genes were not sequenced and the percentage of patients with genetic variants may be higher. Even though the frequency of variants in this cohort may be underestimated, other cohorts have identified patients with no known cause for both aHUS and C3G. Previous studies show that about 45-60% of aHUS patients (13, 30, 94) and 30-40% of C3G patients carry mutations (24, 35, 36). Additionally, in C3G and IC-MPGN 50% of patients included herein had C3NeF (or C4NeF) which is comparable to previous reports regarding C3NeF (35, 36).

Clearly, all disease-related mechanisms for aHUS and C3G have not been fully elucidated. Some patients had multiple variants in genes encoding complement proteins. Bearing a genetic variant does not necessarily lead to occurrence of disease as there is incomplete penetrance of the disease phenotype. Furthermore, certain variants were found in both aHUS and C3G suggesting that factors additional to genetic composition can dictate the phenotype. Further investigations are required to better understand the impact variants have on protein functionality, and how they lead to disease expression.

Data availability statement

All data are available within the manuscript and its supplements. Datasets were deposited in Zenodo, doi: 10.5281/zenodo.8124309.

Ethics statement

Informed consent was obtained for diagnostic genetic analysis and the project was approved by the Swedish Ethical Review Authority, approval no. 2021-04438. The Swedish Ethical Review Authority waived the requirement for written consent from patients included retrospectively in this study. All patients included after October 2021 gave informed written consent. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants or their legal guardians/next of kin.

Author contributions

VR: Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. SA: Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. A-CK: Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. NS: Investigation, Writing – original draft, Writing – review & editing.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1254759/full#supplementary-material

References

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Supplementary Materials Genetic investigation of Nordic patients with complement-mediated renal diseases

Viktor Rydberg V, Sigríður Sunna Aradóttir, Ann-Charlotte Kristoffersson, Naila Svitacheva and Diana Karpman.

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Supplementary table 1: Genetic variants in patients with atypical hemolytic uremic syndrome included in this study

Supplementary table 2: Genetic variants in patients with C3 glomerulopathy included in this study

Supplementary table 3: Genetic variants in patients with immune complex-membranoproliferative glomerulonephritis included in this study

The supplementary materials are found in the electronic version of the paper: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1254759/full#supplementary-material
Complement dysregulation associated with a genetic variant in factor H-related protein 5 in atypical hemolytic uremic syndrome

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Abstract

Background: Atypical hemolytic uremic syndrome (aHUS) can be associated with mutations, deletions, or hybrid genes in factor H-related (FHR) proteins.

Methods: A child with aHUS was investigated. Genetics was assessed by Sanger and next generation sequencing. Serum FHR5 was evaluated by immunoblotting, ELISA and by induction of rabbit red blood cell hemolysis in the presence/absence of recombinant human rFHR5. Mutagenesis was performed in HEK cells.

Results: A heterozygous genetic variant in factor H-related protein 5 (CFHR5), M514R, was found in the child, who also had a homozygous deletion of CFHR3/CFHR1 and factor H antibodies, as well as low levels of C3. Patient serum exhibited low levels of FHR5. In the presence of rabbit red blood cells, patient serum induced hemolysis which decreased when rFHR5 was added at physiological concentrations. Similar results were obtained using serum from the father, bearing the CFHR5 variant without factor H antibodies. Patient FHR5 formed normal dimers. The CFHR5 M514R variant was expressed in HEK cells and minimal secretion was detected whereas the protein level was elevated in cell lysates.

Conclusions: Decreased secretion of the product of the mutant allele could explain the low FHR5 levels in patient serum. Reduced hemolysis when rFHR5 was added to serum suggests a regulatory role regarding complement activation on red blood cells. As such, low levels of FHR5, as demonstrated in the patient, may contribute to complement activation.

Keywords: atypical hemolytic uremic syndrome, complement, factor H-related protein 5, hemolysis, sequencing
Introduction

Atypical hemolytic uremic syndrome (aHUS) is a complement-mediated kidney disease characterized by the development of non-immune hemolytic anemia, thrombocytopenia, and kidney failure [1]. The disease is ultra-rare and may have a recurring and chronic nature. aHUS can be associated with heterozygous genetic variants in genes encoding complement factors or regulators [2]. These include gain-of-function variants in C3 [3] or factor B [4] which can result in the formation of a stable C3 convertase resistant to factor H-mediated dissociation, or loss-of-function variants in complement regulators including factor H [5], membrane-cofactor-protein/CD46 [6] and factor I [7], allowing excess complement activation via the alternative pathway.

Mutations in factor H have been localized to the C terminal of the protein. The C terminal has properties associated with host cell recognition thereby directing complement to be activated on foreign surfaces but not on host cells [8]. Genetic variants in this region of the protein can disturb the differentiation between self and non-self and thereby allow complement to become activated on host cells. Similarly, some patients with aHUS have been shown to have antibodies to factor H, mostly directed to the C terminal [9], and these are associated with deletions, genomic rearrangements, and hybrid genes involving factor H-related (FHR) proteins [10, 11]. Homozygous deletions of CFHR3 and CFHR1 have been described in patients with antibodies to factor H. Whereas factor H consists of 20 short consensus repeats (SCRs), with the surface recognition C terminal domain at SCRs 19-20, factor H-related proteins consist of 4-9 SCRs with a certain degree of homology to factor H, and with dimerization motifs [12, 13].

FHR5 is a 65 kDa protein consisting of nine SCRs and capable of binding C3 and heparin. Its exact function in the complement pathway is still a matter of investigation. Studies using higher
than physiological concentrations have shown it to regulate complement activation [14], while other studies have demonstrated a role in promoting complement activation by competing with factor H [15] and by contributing to formation of the C3 convertase [16]. Genetic variants in CFHR5 have been mostly associated with C3 glomerulopathy and one subtype of the disease is termed CFHR5-nephropathy [17]. However, genetic variants have also been identified in aHUS [18-20].

In this study, a rare CFHR5 variant in aHUS, M514R, was investigated. This variant was previously reported in C3 glomerulopathy [21] and age-related macular degeneration [22], without a description of its phenotype. The aim of the study was to investigate the CFHR5 M514R variant regarding complement activation or regulation on red blood cells.
Methods

Case study

A five-year-old girl presented in 2012 with hemolytic anemia (hemoglobin 58, reference value 100-150 g/L; lactate dehydrogenase 25, reference value 2.2-5.3 μkat/L; bilirubin 31, reference value 5-25 μmol/L, direct antiglobulin test negative), thrombocytopenia (platelet count 22, reference value 125-340 x10⁹/L), kidney failure (creatinine 124, reference value 14-42 μmol/L) without diarrhea. A fecal sample for enterohemorrhagic *Escherichia coli* was negative. Initial assessment of complement values showed C3 0.63, reference value 0.77-1.38 g/L and C3d 14, reference value < 5 mg/L. ADAMTS13 was normal.

At the initial presentation antibodies to factor H were detected in the child’s serum. Initial anti-factor H antibody levels were 16460 E/mL. The levels did not correspond to international units as a standard assay had not yet been developed [9]. The presumptive diagnosis was aHUS and the child was initially treated with daily plasma infusions followed by mycophenolate mofetil (MMF) and plasma treatment at prolonged intervals up to every other week. Eighteen months after the initial episode, the patient had a recurrence during a febrile infection and one month later an additional recurrence. These recurrences were treated with plasma exchanges, altogether 9 exchanges three times a week. During this period an additional mild recurrence occurred. Treatment with cyclophosphamide pulses every 3rd week was given during a 5-month period followed by MMF. In 2014 the Clinical Immunology Laboratory in Lund in collaboration with other European laboratories developed a standardized assay and the patient’s anti-factor H value was 2540 E/mL. Levels decreased continuously over the years, in 2015: 840, 2019: 710, 2022: 570 and in 2023: 260 E/mL.
The girl did not experience any recurrences after this and MMF was slowly tapered and discontinued when she was 15 years old. Kidney function was assessed by glomerular filtration rate measured with iohexol clearance was 97 ml/min/1.73m$^2$ at 9 years and 79 ml/min/1.73m$^2$ at 16 years.

The child’s mother had, during childhood, an unclear transient episode of thrombocytopenia. The child’s father has not had any symptoms associated with HUS.

**Controls**

Apparently healthy adults (n=9) were controls, four of these were female.

The study of the patient, her parents and healthy controls was performed with the approval of the Swedish Ethical Review Authority, approval no. 2021-04438. Informed written consent was obtained from the patient, her parents and the healthy controls.

**Blood samples**

Whole blood in EDTA tubes was used for DNA extraction as previously described. Serum samples were centrifuged after one-hour incubation at room temperature, aliquoted, and stored at -80°C until used. Samples from the patient were collected during remission.

**Genetic analysis and mutation screening**

DNA samples were subject to Sanger sequencing (Eurofins Genomics, Konstanz, Germany) and analyzed using BioEdit (Ibis Biosciences, Carlsbad, CA). In addition, whole exome sequencing and whole genome sequencing were carried out at the Center for Molecular Diagnostics, Skåne University Hospital as previously described [23]. The gene panel included
CFH, CFI, CD46, C3, C5, CFB, CFP, CLU, CFHR1-5, ADAMTS13, THBD, DGKE and PLG. 

Allele frequencies in the normal population were obtained from (Genome Aggregation Database, gnomAD, http://gnomad.broadinstitute.org/).

**Determination of FHR5 levels**

FHR5 concentration was assayed by an ELISA developed in-house. A MaxiSorp® plate (Thermo Fisher Scientific, Roskilde, Denmark) was coated overnight at 4°C with the polyclonal goat anti-hFHR5 for capture, the antibody was diluted in 0.1 M carbonate buffer (pH 9.6) to a final concentration of 1 µg/mL. The wells were blocked for 1 h with bovine serum albumin (Sigma, St. Louis, MO). Recombinant hFHR5 was used as the standard (catalog number: 3845-F5, R&D Systems, Minneapolis, MN). The subsequent 1 h incubations were performed at room temperature, and washed with PBS-Tween (Medicago, Uppsala, Sweden). Monoclonal mouse anti-hFHR5-antibody (1 µg/mL, R&D Systems) was followed by goat anti-mouse IgG: HRP (1:1000 Dako, Glostrup Denmark) and tetramethylbenzidine (TMB) (Dako, Carpinteria, CA). The reaction was terminated with sulfuric acid (H₂SO₄, Sigma-Aldrich) and absorbance measured at 450 nm using GloMax Discover (Promega, Madison, WI).

**Determination of FHR5 size**

FHR5 size was determined by immunoblotting under non-reducing conditions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%, Mini-PROTEAN®, Bio-Rad Laboratories, Hercules, CA) and transferred by electroblotting to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). Recombinant hFHR5 was used as the control. Polyclonal goat anti-hFHR5-antibody (catalog number: MAB3845, R&D Systems) diluted 1:2000 was used for detection, followed by rabbit anti-goat IgG: HRP antibody (Dako)
at 1:2000. Detection was performed using chemiluminescence (Pierce ECL2, Rockford, IL) and imaged using ChemiDoc™ Touch, Bio-Rad.

Hemolysis of rabbit red blood cells

Rabbit red blood cells (RBCs, 5x10⁸/mL, Håtunalab) in gelatin veronal buffer (GVB) with Mg-ethylene glycol-bis(β-aminoethyl ether (EGTA) 0.1M were incubated with serum diluted 1:10. The total volume of the samples was 100 µl including 20 µl rabbit RBCs, 70 µl Mg-EGTA buffer with or without added recombinant hFHR5 and 10 µl serum (hFHR5 added in the final step or first combined with serum, preincubated for 10 min, and then added to the tube). Added hFHR5 was 50 or 500 ng in the total volume of 100 µl, in which serum was diluted 1:10, as such the lower amount corresponds to physiological serum concentrations of 3-6 µg/mL.

Samples were incubated for 1 h on a shaker at 37°C, with additional manual shaking every 10 min. GVB-EDTA was added (100 µl, Complement Technology, Tyler, Texas) followed by a centrifugation step. The supernatant was collected, and absorbance was measured at 405nm using GloMax Discover.

Analysis of CFHR5 dimerization

Serum samples were diluted 1:5 in PBS (GE Life Sciences, Uppsala, Sweden) and added to centrifugal tubes (Amicon® Ultra-4 Centrifugal Filter Unit, Sigma- Aldrich) with 100 kDa cutoff. Samples were washed with PBS twice. The concentrate as well as the filtrate, were analyzed by SDS-PAGE and immunoblotting. Recombinant hFHR5 was used as the control.

Mutagenesis

The pcDNA3.1(-)Myc/His A plasmid (ampicillin-resistant, Invitrogen, Life Technologies, Waltham, MA) containing wild-type CFHR5 gene was kindly provided by M. Pickering.
A vector sequence containing the mutation was separately obtained (Eurofins Medigenomix). The plasmid containing the wild-type sequence and the vector containing the mutated sequence were cleaved at two sites by AflIII (New England Biolabs, Ipswich, MA). The resulting cleaved DNA fragment was 822 bp. The fragments and inserts were separated on an agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Dephosphorylation of the vector solution was performed with the rAPid Alkaline Phosphatase kit (Roche Diagnostics, Mannheim, Germany) to prevent auto-ligation. The two fragments (mutant insert and vector without the wild-type sequence) were ligated using a T4 DNA Ligase kit (Roche Diagnostics, Indianapolis, IN).

The transformation was performed in *E. coli* (XL-Gold Ultracompetent cells, Agilent Technologies, Santa Clara, CA) and the plasmid was purified (QIAprep® Spin Miniprep Kit, QIAGEN). Correct insertion and the sequence were verified by Sanger sequencing performed at Eurofins Genomics and interpreted.

**Cell transfection**

HEK 293 T cells (Human embryonic kidney cells, ATCC, Teddington, Middlesex, UK) were seeded and grown in DMEM/high glucose Hyclone medium (GE Healthcare Life Sciences, South Logan, UT), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum to approximately 95% confluence before transfection. Transient transfection was performed with Lipofectamine (Invitrogen, Life Technologies, Waltham, MA) according to the manufacturer’s instructions. A control well without added DNA was included. Twenty-four hours after transfection, the medium was changed to Optimem (Thermo Fisher Scientific) and cells were cultured for an additional 72 h. The media were collected, supplemented with protease inhibitors cOmplete Mini without EDTA (Roche Diagnostic,
Mannheim, Germany) and centrifuged to separate supernatant and cell debris. The supernatant was concentrated 25x to 200 µl using a 10 kDa filter (Ultra-4 Centrifugal Filter Units, Merck Millipore, Billerica, MA). The cells were diluted in RIPA buffer for protein extraction (Sigma) with cOmplete Mini without EDTA, scraped from the wells and the dilution underwent three freeze-thaw cycles to enhance lysis.

Statistics

Non-parametric Wilcoxon signed-rank test was used to compare paired sera from the same individual with and without added FHR5. Statistical analysis was performed using GraphPad Prism 8 software (version 8.4.3, GraphPad Software, La Jolla, CA).
Results

Patient work-up

Genetic workup showed that the patient was homozygous for a deletion of CFHR3/CFHR1 and heterozygous for a rare variant in CFHR5 c.1541T>G; p.M514R. The father was shown to be a heterozygous carrier of the CFHR5 variant M514R and for the deletion of CFHR3/CFHR1. The mother did not carry the CFHR5 M514R variant but was a heterozygous for the CFHR3/CFHR1. Neither one of the parents had antibodies to factor H.

FHR5 levels in the patient’s and the father’s serum measure by ELISA were low, in the patient: 0.76 and 0.8 µg/mL (two separate samples), in the father: 0.8 µg/mL, compared to controls (n=5) ranging between 1.49 – 3.1 µg/mL. Although the size was normal only a very weak band was detected by immunoblotting, corresponding to FHR5. The antibody to FHR5 also detects FHR1 but no bands corresponding to this protein were detected in the patient’s serum (Figure 1).

An experiment was designed to determine if FHR5 in patient serum could form dimers. Using a cut-off filter of 100 kDa FHR5 dimers (approximately 130 kDa) would be collected in the concentrate and be separated by SDS-PAGE to the 65 kDa subunits, as shown in Figure 1. FHR5 in patient serum formed dimers.

FHR5 decreases hemolysis of rabbit RBCs

Combining rabbit red blood cells (RBCs) with patient serum induced hemolysis which decreased when recombinant hFHR5 was added at physiological concentrations (Figure 2a). Similar results were obtained using serum from the father, bearing the CFHR5 M514R variant, without antibodies to factor H. The father’s serum was also combined with hFHR5 at higher
concentrations, also showing a decrease in hemolysis (Figure 2b). Using control sera, the
degree of hemolysis decreased when adding high concentrations of hFHR5 but did not change
significantly when adding physiological concentrations (Figure 2). Adding hFHR5 to the cells
before adding the serum, or combined with serum, gave similar results.

Studies of the transfected CFHR5 M514R variant
In order to characterize the phenotype of the mutated protein the CFHR5 M514R variant was
expressed in HEK cells. Levels of the wild-type FHR5 protein in the cell supernatants were
assayed by ELISA and detected at a median of 4176 ng/mL (three separate transfections),
whereas the mutant variant was secreted at 0.21-fold of the wild-type (median of two separate
transfections). Cell lysates from the transfected cells containing the mutant variant showed 8.5-
fold higher levels than the wild-type (median of the wild-type in lysates 118 ng/mL). These
results were confirmed by immunoblotting showing a strong FHR5 band in the supernatant
from HEK cells expressing the wild-type protein, but a very weak band in cells expressing the
mutant variant M514R (Figure 3). In contrast, the cell lysates from HEK cells expressing the
wild-type protein showed a weak band and the lysates from cells expressing the mutant variant
showed a stronger FHR5 band. Taken together, these results indicate that the mutant variant
accumulated intracellularly. The non-transfected control supernatants or cell lysates did not
contain FHR5 protein (Figure 3).
Discussion

Genetic variants in CFHR5 have been detected in patients with complement-mediated kidney disease such as aHUS [19, 20], C3 glomerulopathy and immune complex-associated membranoproliferative glomerulonephritis [17, 24]. The detection of a rare variant does not, however, indicate its involvement in the disease process. Here we describe the phenotype of the CFHR5 variant M514R showing that it is minimally secreted from cells. Negligible amounts of the product of the mutant allele would therefore be present in the circulation. FHR5 has been suggested to be either an activator or a regulator of complement [25]. The patient described herein had a homozygous deletion of CFHR3/CFHR1 and initially high levels of antibodies to factor H. The latter could explain her predisposition to develop aHUS. Low levels of FHR5 were demonstrated in the patient’s serum, most probably representing translation of the normal allele, which could form FHR5 dimers. Addition of physiological concentrations of recombinant human FHR5 to patient serum decreased hemolysis of rabbit RBCs. This was also demonstrated using the father’s serum (carrying the same CFHR5 variant without antibodies to factor H). The results suggest FHR5 has a regulatory role on the surface of RBCs and its deficiency could thereby contribute to complement activation leading to hemolysis.

FHR5 colocalizes with glomerular C3 deposits in the kidney [26] and binds to C3b in vitro [27]. These properties suggest a role in interacting with C3. A previous study showed that FHR5 could inhibit complement activation by functioning as a cofactor for factor I-induced C3b degradation to iC3b and by inhibiting C3 convertase activity, both assays performed in the fluid phase and with higher than physiological FHR5 concentrations [14]. For this reason, the relevance could be questioned. In the current study, we show that FHR5 has a complement regulatory role on RBCs using physiological concentrations and sera with the M514R variant. Similarly, a study of patients with CFHR5 nephropathy with duplication of exons 2 and 3 in
CFHR5 demonstrated that the mutant variant exhibited decreased binding to chicken RBCs [17]. If FHR5 is a complement regulator on RBCs then its deficiency due to lack of cellular secretion (as presented herein) or decreased binding (as shown in [17]) could allow excess complement-mediated RBC lysis to proceed.

In contrast, other studies have suggested that FHR5 could be a complement activator showing that it interferes with factor H binding to C3b and that this interaction is influenced by the presence of surface glycosaminoglycans [15]. The discrepancies may be related to the assay used and the presence of other serum proteins. There is also the possibility that FHR5 has dual roles, both an activating and a regulatory role in the complement system depending on the microenvironment and its activity in the fluid phase versus the cell surface.

The mutant variant of CFHR5, M514R, studied here, led to low circulating serum levels. The addition of recombinant human FHR5 seemed to have a protective role on rabbit RBCs, when higher than physiological concentrations were used, or at physiological concentrations in the patient’s serum. Thus, low serum levels could contribute to complement-mediated hemolysis. We do not suggest, however, that the CFHR5 variant plays a major role in the disease phenotype in this patient. It seems that levels of anti-factor H antibodies play a more important role in the induction of disease. However, the CFHR5 variant may have an additional role in promoting complement activation once the disease has been triggered.
**Statements and declarations**

**Competing interests**

The authors have no competing interests to declare.

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**Data availability statement**

All data are available within the manuscript and its supplement. Genetic data was also deposited in Zenodo, doi: 10.5281/zenodo.8124309.

**Author contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Sigridur Sunna Aradottir, Ann-Charlotte Kristoffersson, Erik Linnér and Diana Karpman. The first draft of the manuscript was written by Sigridur Sunna Aradottir and Diana Karpman and all authors commented All authors read and approved the final manuscript.

Conceptualization: Diana Karpman, Methodology: all authors; Formal analysis and investigation: all authors, Writing - original draft preparation: Sigridur Sunna Aradottir and Diana Karpman; Funding acquisition: Diana Karpman; Supervision: Diana Karpman.
Figure legends

Figure 1: Immunoblot of factor H-related protein 5 in serum

Lanes 1-4 show the patient’s serum. Lane 1: Patient serum exhibiting a weak band corresponding to factor H-related protein 5 (FHR5) but no bands corresponding to FHR1. Lanes 2-3: Serum filtrate < 100 kDa lacking bands indicating that all circulating FHR5 was dimerized. Lane 4: Serum concentrate > 100 kDa, after electrophoresis separation to 65 kDa subunits, indicating that FHR5 was initially dimerized (at 130 kDa). Lanes 5-8 show control serum. Lane 5: Control serum showing bands corresponding to FHR5 and FHR1. Lanes 6-7: Filtrate of control serum lacking bands indicating that all circulating FHR5 was dimerized. Lane 8: Serum concentrate > 100 kDa, after electrophoresis separation to 65 kDa subunits, indicating that FHR5 was dimerized. Lane 9: Recombinant human FHR5. The band is somewhat higher than serum FHR5 presumably due to differences in glycosylation.

Figure 2: Hemolysis induced by patient and normal serum in the presence of factor H-related protein 5

a) Serum from the patient (pink) and father (green), both bearing the CFHR5 variant M514R, induced hemolysis of rabbit red blood cells, inhibited by the addition of physiological concentrations of recombinant human factor H related 5 (hFHR5). Control sera (n=6) did not exhibit a significant change in hemolysis when FHR5 was added. Ns: not significant.

b) Serum from the father bearing the CFHR5 variant M514R induced hemolysis that was reduced when FHR5 was added at higher than physiological concentrations. Similar results were obtained using control sera (n=7). *: P, < 0.01.

Figure 3: Factor H-related protein 5 wild-type and mutant proteins in cell supernatants and lysates

Factor H-related protein 5 was assayed by immunoblotting in transfected cell supernatants and lysates containing the wild-type sequence and the mutant M514R variant. Wild-type supernatant exhibited a strong band corresponding to FHR5. The supernatant from cells transfected with the CFHR5 M514R variant exhibited a very weak band. Control supernatants from HEK cells that were not transfected did not exhibit a band corresponding to FHR5. The FHR5 band was stronger in the lysates of the cells containing the M514R mutant variant.
compared to the wild-type, as the mutant variant was minimally secreted. hFHR5: recombinant
human factor H-related protein 5, WT: wild-type, M514R: transfected mutant variant.
Figure 1

- Serum
- Filtrate 1
- Filtrate 2
- Concentrate
- Serum
- Filtrate 1
- Filtrate 2
- Concentrate

FHR5

FHR1

1  2  3  4  5  6  7  8  9

Patient (M514R)  Control  hFHR5
Figure 2
Figure 3