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

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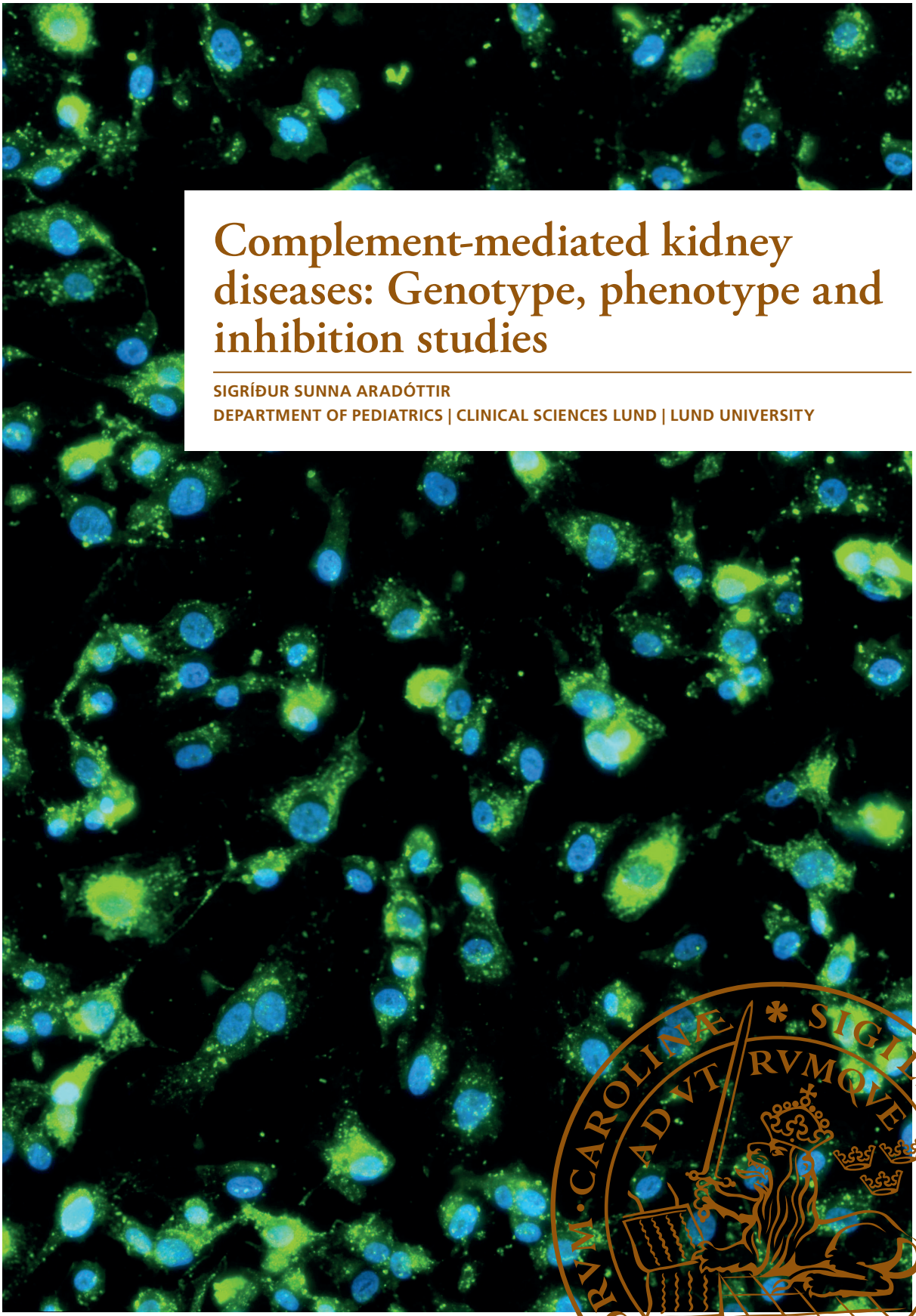

Complement-mediated kidney diseases: Genotype, phenotype and inhibition studies

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

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Faculty opponent:
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Abstract:

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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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MADE IN SWEDEN

Meðalsnotr
skyli manna hverr,
æva til snotr sé;
örlög sín
viti engi fyrir,
þeim er sorgalausastr sefi.

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

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I. **Aradóttir SS**, Kristoffersson A-C, Roumenina LT, Bjerre A, Kashioulis P, Palsson R, Karpman D. Factor D inhibition blocks complement activation induced by mutant factor B associated with atypical hemolytic uremic syndrome and membranoproliferative glomerulonephritis. *Frontiers in Immunology.* 2021;12:690821.

II. **Aradóttir SS**, Kristoffersson, A-C, Jensson BO, Sulem P, Gong, H, Palsson R, Karpman D. Factor B mutation in monozygotic twins discordant for atypical hemolytic uremic syndrome. *Kidney International Reports.* 2023;8:1097-101.

III. Rydberg V, **Aradóttir SS**, Kristoffersson A-C, Svitacheva N, Karpman D. Genetic investigation of Nordic patients with complement-mediated kidney diseases. *Frontiers in Immunology* 2023; 14:1254759.

IV. **Aradóttir SS**, Kristoffersson A-C, Linnér E, Karpman, D. Complement dysregulation associated with a genetic variant in factor H-related 5 in atypical hemolytic uremic syndrome. Under review.

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 specialities. 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.*

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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.

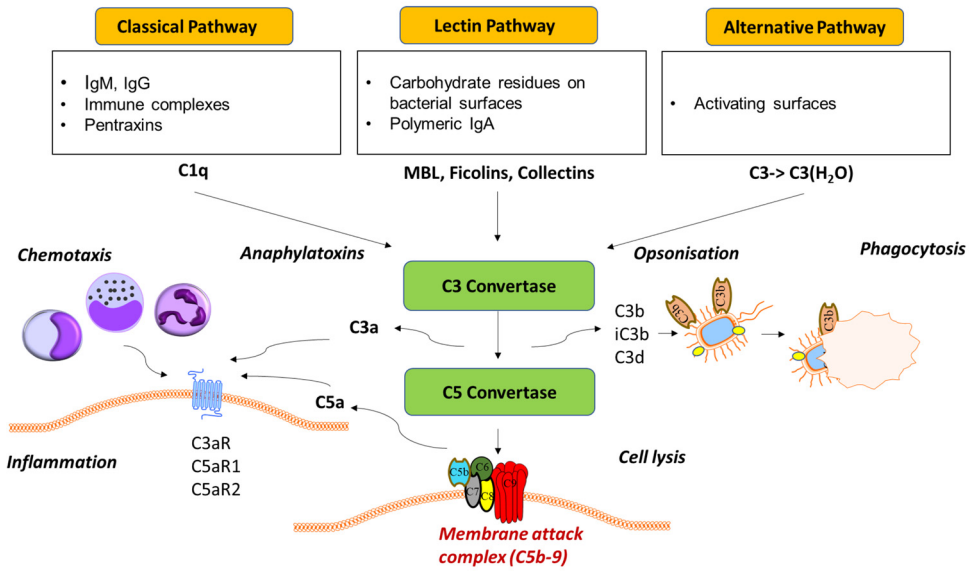

Figure 1: Overview of the complement system
Complement is initiated through three pathways the classical, lectin and alternative pathways that converge at the level of C3 in the cleavage of C3 to its fragments C3b and C3a. The C3b fragment can attach to surfaces via a thioester bond, and the soluble 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. Mannan binding lectin (MBL). C3a receptor (C3aR), C5a receptor 1 (C5aR1), C5a receptor 2 (C5aR2). Created with BioRender.com

Overview of complement physiology

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.

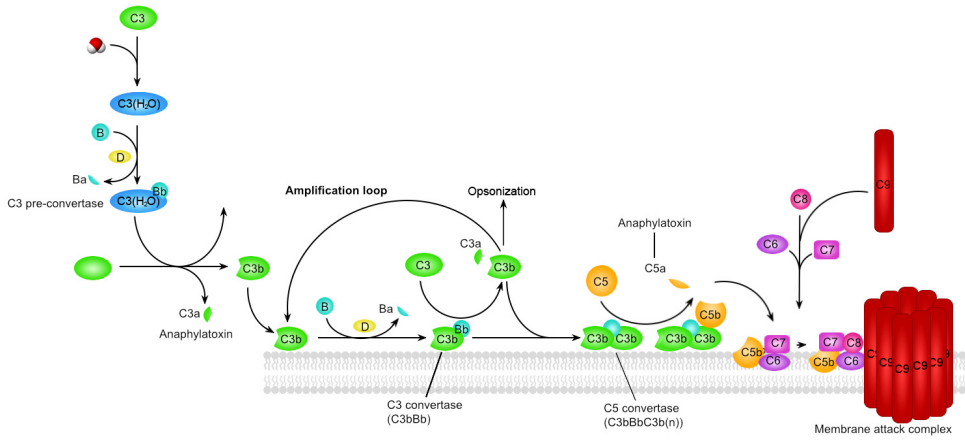

Figure 2: Alternative pathway activation and the amplification loop
(Adapted from a figure by Erik Linnér with permission)

Terminal pathway activation

C3bBb or C4b2a convertases possess the ability to attach extra C3b molecules, resulting in the formation of C5 convertases $C3bBb3b_n$ or $C4b2a3b_n$ (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 200µg/mL, compared to C3 which circulates at a concentration of approximately 1200 µg/mL (58).

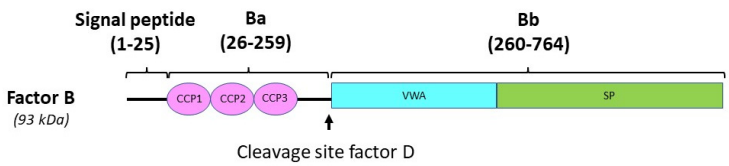

Figure 3: Structure of complement factor B
Factor B is composed of two main fragments Ba 30 kDa and Bb 73 kDa and a linker. Numbering refers to what nucleotides comprise the fragments. The Ba fragment is composed of three CCPs. The Bb fragment is composed of a von Willebrand type A domain (VWA) that contains the metal-ion dependent adhesion site (MIDAS) and a serine protease (SP). The factor D cleavage site is marked by an arrow.

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.

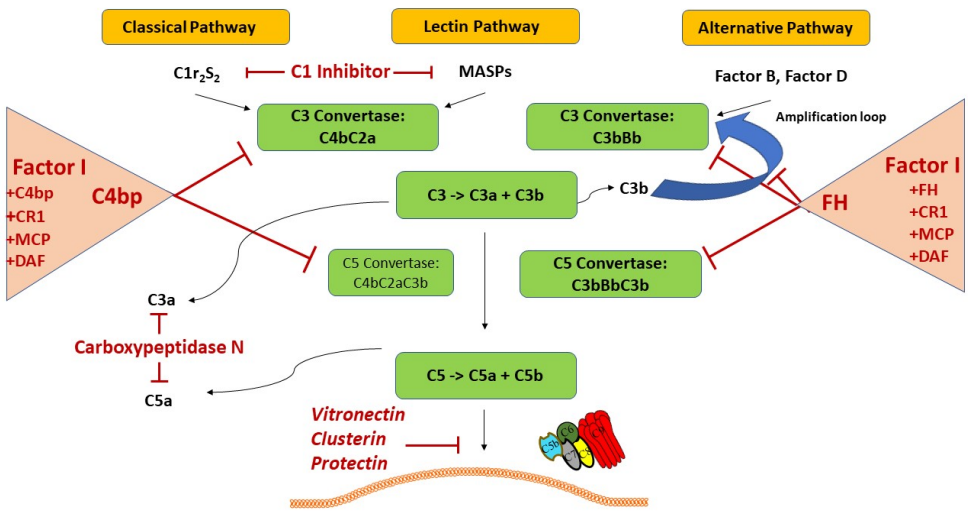

Figure 4: Overview of complement regulation
Complement regulation is performed at all steps of the cascade and in all pathways as depicted. The main regulators of the alternative pathway are factor H and factor I. The main regulators of the classical and lectin pathway are C1 inhibitor, C4bp and factor I. The carboxypeptidase N inactivates C3a and C5a. Vitronectin, Clusterin, and Protectin 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 *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).

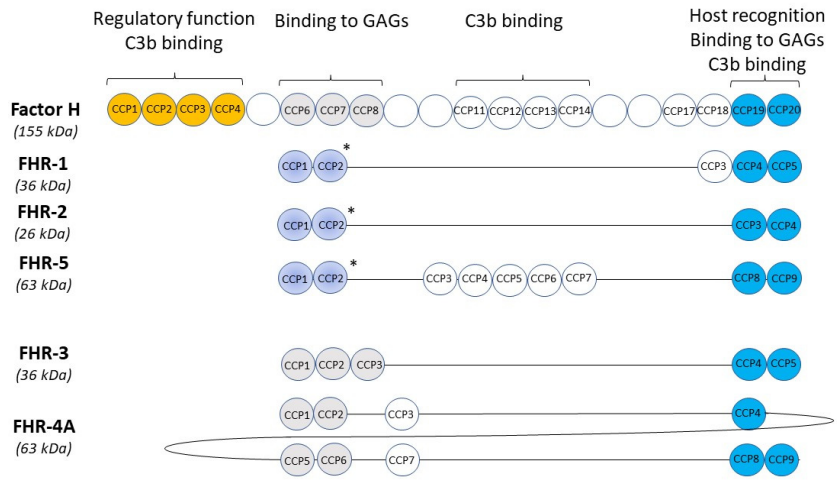

Figure 5: Homologous CCPs of factor H and factor H-related proteins
FHRs bear variable homology to corresponding CCPs of factor H. The N-terminal FHRs resemble CCP 6-8 of factor H. The C-terminal part shares homology to CCPs 19–20 of factor H. FHR-5 also shares certain homology to factor H CCPs 10–14 (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 1: Work-up of a patient suspected of aHUS

First evaluation of the patient with kidney injury and clinically suspected HUS:
History, physical examination, and medical review. History penetrated with regards to triggers: infections, vaccinations, drugs, pregnancy and family history. 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. Urine for dipstick, microscopy.
If clinical suspicion of aHUS:
Complement C3, C3dg. Anti-factor H antibodies. ADAMTS13.Homocysteine, methylmalonic acid (plasma and urine). Screening for rheumatologic disease. Blood culture. Stool: Swab/culture or PCR for EHEC genes. Genetic aHUS panel

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 failur is 10-15% (112).

The pathology

aHUS is a form of thrombotic microangiopathy (TMA). The TMA lesion ischaracterized 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 aHUS 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 life-long treatment for patients carrying variants associated with a more severe phenotype while others suggest discontinuation is possible (155, 156).

Treatment of aHUS 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) intra-venous 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 disases 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 2: Complement inhibitors approved or in clinical studies

Target	Generic name	Type	Disease	Status
C1s, C1r, MASPs, FXIIa, kallikrein	C1 inhibitor (human)	Plasma protein rC1-inhibitor	Hereditary angioedema	Approved
C1s	Sutimlimab	Antibody	Cold agglutinin disease ITP*	Approved
C1q	ANX005, ANX007, ANX009	Antibody	Huntingtons disease, Guillain-Barré syndrome, GA/AMD, lupus nephropathy, ALS, AIHA	III/II/I
	RLS0071	Peptide	Hypoxic ischemic encephalopathy, asthma	II/I
MASP2	Narsoplimab	Antibody	**aHUS**, **C3G**, IgAN, HSCT-TMA, Covid-19 membranous nephropathy, lupus nephritis	III/II
MASP-3	OMS906	Antibody	PNH	I
C2	ARGX117	Antibody	Multifocal motor neuropathy, dermatomyositis, delayed graft function	II/I
C3	ALXN2030	SiRNA	**C3G**, Chronic antibody-mediated rejection	I
	Pegcetacoplan	Pegylated peptide	PNH **C3G**, GA/AMD, cold agglutinin disease, ALS	Approved II
	AMY-101	Compstatin peptide	**C3G**, COVID19	II
C3 convertase	GT005	AVV Factor I plasmid	AMD	II
C3 convertase	rFH (Gem103)	rFH	AMD	II
FB	Iptacopan (LNP-023)	Small molecule	**aHUS**, **C3G**, PNH, IgAN, cold agglutinin disaese, AMD, membranous nephropathy, lupus nephropathy	II/III
	IONIS-FB-L_{Rx}	ASO	GA, AMD, IgAN	II
Bb	SAR443809	Antibody	**C3G**, PNH	II
FD	Danicopan and derivatives	Small molecule	**C3G**, GA/AMD, PNH, Covid-19, lupus nephropathy, IgAN	III/II

FH	FHmoss	Recombinant protein	**aHUS**	Preclinical
Properdin	CLG561 (NOV-7)	Antibody	GA /AMD	II
C5	Eculizumab, Ravalizumab and derivatives	Antibody	**aHUS**, PNH, MG and NMOSD Guillain-Barré* **C3G,** sicle cell disease, CHAPLE, HSCT-TMA, geographic atrophy/AMD	Approved I/II
	Pozelimab, Crovalimab, Tesidolumab	Antibody	PNH, **aHUS**, SCD, CHAPLE, MG, transplant associated-TMA, AMD	III/II
	Zilucoplan	Peptide	MG	III
	Cemdisiran	C5 RNA interfer-ence	**aHUS**, PNH, IgAN and MG	III
	Zimura	Pegylated RNA aptamer	GA/AMD, HSCT-TMA	III
	Nomacopan	Protein	PNH, HSCT-TMA	III
C5a	Vilobelimab (IFX-1))	Antibody	**C3G**, pyoderma gangrenosum, COVID-19, cutaneous squamous cell carcinoma, hidradenitis suppurativa	II/III
C5aR1	Avacopan	Small molecule	ANCA-associated vasculitis, granulomatous polyangiitis, microscopic polyangiitis **C3G*** Under investigation for **aHUS**	Approved
C5b-9	AAVCAGsCD59	Gene therapy	AMD	I

* Orphan drug approval. MASPs: MBL-associated serine proteases. r: Recombinant. INH: Inhibitor. ASO: Antisense oligonucleotide. GA: Geographic atrophy. AMD: Age-related macular degeneration. ALS: Amyotropic lateral sclerosis. AIHA: autoimmune hemolytic anemia aHUS: atypical hemolytic uremic syndrome. C3G: C3 glomerulopathy. IgAN: IgA nephropathy. MN: membranous nephropathy. LN: lupus nephropathy. HSCT-TMA: Hematopoetic stem cell transplant-associated thrombotic microangiopathy. MG: Myasthenia gravis. PNH: Paroxysmal nocturnal hemoglobinuria. NMOSD: neuromyelitis optica spectrum disor-der. CHAPLE: CD55 deficiency with hyper-activation of complement, angiopathic thrombosis, and severe protein-losing enteropathy. SCD: Sickle cell dis-ease. AAV: Adeno-associated viral vector. ASO: Antisense oligonucleotide. Based on references (66, 202-209).

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 3: Methods used in this thesis

	Description	Paper			
		I	II	III	IV
Clinical data	History, laboratory results, disease course and familial cases	+	+	+	+
Genetics	Sanger sequencing		+	+	+
	Whole exon sequencing	+	+	+	+
	Whole genome sequencing		+	+	+
Mutagenesis	*CFB*	+			
	CFHR5				+
ELISA	Quantification	+			+
	Autoantibody detection	+			
	Activation products (Ba, C3a, C5a, sC5b-9)	+	+		+
Immunoblot	Protein size by immunoblot	+			+
	Protein dimer analysis				+
	C3 convertase formation and visualization of the degradation of factor B	+			
Hemolytic assay[1]	Sheep RBCs	+	+		
	Rabbit RBCs	+			+
pGECs[2]	C3 deposition by immunofluorescence microscopy	+	+		
SPR	Factor B binding to C3b	+			
	Assembly of the C3 convertase	+			
FD inhibition	C3 convertase formation factor B degradation products visualized by immunoblotting	+			
Statistics	Two-tailed Mann-Whitney U-test		+		
	Kruskal Wallis multiple comparison tests followed by Dunns procedure	+	+		
	Non-parametric Wilcoxon signed rank test				+

[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. ELISA: Enzyme-linked immunosorbent assay. FB: Factor B. FHR-5: Factor H-related protein 5. RBCs: Red blood cells. MAC: Membrane attack complex. GEC: Primary glomerular endothelial cells. SPR: Surface plasmon resonance. ADP: Adenosine diphosphate. ELISA: Ensyme-linked immunosorbent assay. RBC: Red blood cells. pGECs: Primary Glomerular Endothelial Cells. SPR: Surface Plasmon Resonance; FD: Factor D.

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 auto-antibodies 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 *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 *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 *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 *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, *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 *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 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 *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 physio-logical (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

Vår tillvaro är präglat av snuviga näsor, halsfluss och allergier. Immunsystemet är kroppens försvarsmekanism som oftast avvärjer angreppet vilket gör att symptomen går över snabbt. Försvarsmekanismen måste vara noga reglerad för att inte angripa kroppens egna strukturer. Obalans kan leda till livshotande organskador och sjukdomar.

Komplementsystemet spelar en viktig roll i immunförsvaret. Det består av en grupp proteiner i blodet som interagerar med varandra när de stöter på "alarmsignaler" 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.

Atypiskt hemolytiskt uremiskt syndrom och C3 glomerulopati är exempel på komplementmedierade njursjukdomar. Dessa kroniska sjukdomar kan resultera i njurskador och slutstadiet av njursvikt, där den enda behandlingen är dialys och njurtransplantation. Det är ofta barn och unga vuxna som drabbas.

Vid atypiskt hemolytiskt uremiskt 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 immunkomplexmedierat membranoproliferativ glomerulonefrit inlagras immunkomplex istället.

Det finns en effektiv komplementhämmande behandling för atypiskt hemolytiskt uremiskt syndrom bestående av en antikropp mot ett protein i terminala delen av komplementkaskaden. Behandlingen är endast framgångsrik för en andel patienter med C3 glomerulopati och är extremt dyr, och behöver ges på sjukhus. Det finns ett starkt behov av att utveckla fler, billigare och bättre behandlingsalternativ.

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

Avhandlingen består av följande fyra delarbeten:

Arbete I:

Aradóttir SS, Kristoffersson A-C, Roumenina LT, Bjerre A, Kashioulis P, Palsson R, Karpman D. Factor D inhibition blocks complement activation induced by mutant factor B associated with atypical hemolytic uremic syndrome and membranoproliferative glomerulonephritis. *Frontiers in Immunology*. 2021;12:690821.

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:

Aradóttir SS, Kristoffersson, A-C, Jensson BO, Sulem P, Gong, H, Palsson R, Karpman D. Factor B mutation in monozygotic twins discordant for atypical hemolytic uremic syndrome. *Kidney International Reports* 2023; 8: 1097–1101.

Släktträdet för patienten som beskrevs i arbete I kartlades. I familjen har tre individer varit sjuka. 203 friska individer gensekvenserades, i syftet att hitta ursprunget för genvarianten. Denna spårades till slutet av 1800 talet. En av patienterna är enäggstvilling och hans tvillingbror är frisk. Tvillingarna analyserades med helgenomsekvensering och där fanns inga signifikanta skillnader mellan deras genom. Vi undersökte komplementaktivering i deras blod samt hur deras komplementsystem påverkade njurceller. Den friska tvillingens komplement-aktivering skilde sig inte ifrån den sjuka tvillingens. Detta är bevis på att fler faktorer än genförändring behövs för att orsaka atypiskt hemolytiskt uremiskt syndrom.

Arbete III:

Rydberg V, **Aradóttir SS**, Kristoffersson A-C, Svitacheva N, Karpman D. Genetic investigation of Nordic patients with complement-mediated kidney diseases. *Frontiers in Immunology*. 2023; 14:1254759

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:

Aradóttir SS, Kristoffersson A-C, Linnér E, Karpman, D. Complement dys-relation associated with a genetic variant in factor H-related 5 in atypical hemolytic uremic syndrome. Under review.

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

Hversdagslegar birtingamyndir árása sem líkaminn verður fyrir eru nefrennsli, hálsbólga og ofnæmi. Ónæmiskerfið sinnir verndandi hlutverki og vinnur oftast bæði hratt og vel. Ofnæmi þróast þegar ónæmiskerfið misles vaka og varnaraðgerðir verða yfirdrifnar. Við líffeðlisfræðilegar aðstæður eru hemlar sem óvirkja ensímvirka hluta í ónæmiskerfinu og koma þannig í veg fyrir árás á hraustar frumur líkamans. Ef stjórnun ónæmiskerfisins fer úr jafnvægi þá getur það valdið lífshættulegum sjúkdómum.

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 lykilhlutverk. Kerfið magnar ónæmissvör t.a.m aðferðir sem leiða til eyðingar sýkils svo sem áthúðun, beinu frumurofi, bólgusvörun ásamt því að virkja aðra hluta ónæmiskerfisins. Meinvaldandi breytingar í genum próteina magnakerfisins og/eða sjálfsmótefni geta valdið hömlulausri árás magnakerfisins.

Ódæmigert blóðsundrunar- og þvageitrunarheilkenni (e. atypical hemolytic uremic syndrome) og C3 gauklakvilli (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 smárra æða í gauklum (e.glomeruli), sem leiðir til blóðtappamyndunar og æðateppu í starfseiningu nýrans.

C3 gauklakvilli einkennist af því að virkni magnakerfisins er óhamin í blóðrásinni, C3 mælist oftast lágt í blóði, en safnast fyrir innan á æðaveggi í gauklum nýrans. Sjúkdómum fylgir langvinn gauklabólga. Mótefnafléttutengd himnu- og frumufjölgunargauklabólga (e. membranoproliferative glomerulonephritis) er svipaður sjúkdómur 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 sjúkrahúsdvalar. Meðferðin hefur ekki gefið jafn góða raun fyrir sjúklinga sem þjást af C3 gauklakvilla 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 nýrnasjúkdóma, að skýra þýðingu þessara breytinga fyrir framvindu sjúkdóms, nýrnaígræðslukosti og til að geta veitt erfðafræðilega ráðgjöf til aðstandenda. Einnig eru skoðuð áhrif annars meðferðarvalkosts við blóðsundrunar- og þvageitrunarheilkenni. Að lokum lýsi ég mögulegu hlutverki einstaks magna- kerfispróteins. Doktorsverkefnið byggir á fjórum vörðum:

Fyrsta grein:
Aradóttir SS, Kristoffersson A-C, Roumenina LT, Bjerre A, Kashioulis P, Palsson R, Karpman D. Factor D inhibition blocks complement activation induced by mutant factor B associated with atypical hemolytic uremic syndrome and membranoproliferative glomerulonephritis. *Frontiers in Immunology.*2021;12:690821.

Lýst er þremur erfðabreytingum í faktor B sem fundust í sjúklingum með blóðsundrunar- og þvageitrunarheilkenni og himnu- og frumufjölgunargauklabólgu. Einvörðungu stök faktor B erfðabreyting leiddi til stjórnlausrar ræsingar 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:
Aradóttir SS, Kristoffersson, A-C, Jensson BO, Sulem P, Gong, H, Palsson R, Karpman D. Factor B mutation in monozygotic twins discordant for atypical hemolytic uremic syndrome. *Kidney International Reports* 2023; 8: 1097–1101.

Ættartré sjúklings með ódæmigert blóðsundrunar- og þvageitrunarheilkenni orsakað af ofvirku faktor B próteini var ritað. Í fjölskyldunni voru þrír einstaklingar greindir með sjúkdóminn og allir voru þeir arfberar á faktor B erfðabreytingunni. Alls 203 hraustir ættingjar voru skimaðir og uppruni erfðabreytileikans rakinn til loka 19.aldar. Meðal sjúklinganna er eineggja tvíburi. Tvíburabróðir hans er hraustur arfberi. Heilu erfðamengi tvíburanna voru raðgreind og fannst enginn mismunur. Við skoðuðum virkjun magnakerfisins í sermi tvíburanna og hvaða áhrif þættir í blóði þeirra höfðu á rauð blóðkorn og frumur sem klæða innra lag æða í starfseiningu nýrans. Það var ekki hægt að finna mun á magnakerfisvirkjun hjá tvíburunum í þessum rannsóknum. Það er sönnun fyrir því að sjúkdómsvaldandi erfðabreyting er ekki nægileg ein og sér til að einstaklingur veikist af ódæmigeru blóðsundrunar- og þvageitrunarheilkenni, aðrir þættir spila hlutverk.

Þriðja grein:
Rydberg V, **Aradóttir SS**, Kristoffersson A-C, Svitacheva N, Karpman D. Genetic investigation of Nordic patients with complement-mediated kidney diseases.
Frontiers in Immunology. 2023; 14:1254759.

Erfðabreytileika í genum magnakerfisins er lýst í mengi sjúklinga frá Noregi og Svíþjóð sem hafa verið greindir með magnakerfis-tengda nýrnasjúkdóma. Í heildina voru 141 sjúklingar skoðaðir í afturskyggnri rannsókn. Niðurstöðurnar sýna hlutfall sjúklinga með erfðabreytingar og/eða sjálfs-mótefni, virkjun magnakerfis í blóðvökva og sjúkdómsframvindu. Tuttugu og sex nýjum stökkbreytingum í genum magnakerfisins er lýst. Átján stökkbreytingar voru sameiginlegar hjá sjúklingum með mismunandi greiningar og er sönnun þess að sami erfðabreytileiki í magnakerfis-próteinum getur valdið tveimur mismunandi sjúkdómum.

Fjórða grein:
Arbete IV:
Aradóttir SS, Kristoffersson A-C, Linnér E, Karpman, D. Complement dysregulation associated with a genetic variant in factor H-related 5 in atypical hemolytic uremic syndrome. Í ritskoðun.

Ekki eru allir sammála um hvaða hlutverki „factor H-related 5“ próteinið gegnir í magnakerfinu, hvort það ræsi eða hemli virkjun. Greinin lýsir afleiðingum erfðabreytileika í “factor H-related 5“ próteini hjá sjúklingi með ódæmigert blóðsundrunar- og þvageitrunarheilkenni. Faðir sjúklingsins var hraustur arfberi. Blóðprufur beggja sýndu lækkaðan styrk „faktor H-related 5“ í blóði. Við túlkum niðurstöðurnar á þann veg að erfðabreytingin valdi breytingum í próteininu sem leiði til framleiðsluvanda. Í þessari rannsókn var „factor H-related 5“ hemill á magnakerfis-miðlað blóðrauðalos. Ef hlutverk „faktor H-related prótein 5“ er að hemja virkni magnakerfisins, þá gæti lágur styrkur í blóði skýrt aukna áhættu á að veikjast af ódæmigerðu blóðsundrunar- og þvageitrunarheilkenni.

Niðurstöður doktorsritgerðarinnar eru eftirfarandi:

- Uppvinnsla á erfðabreytileika er afar mikilvæg fyrir greiningu, meðferðarval, í tengslum við aðgerðaáætlun fyrir nýrnaígræðslu og einnig í tengslum við erfðafræðilega ráðgjöf til aðstandenda sjúklinga.
- Þrátt fyrir að unnt sé að sanna að erfðabreytileiki leiði til próteins sem valdi hömlulausri virkjun magnakerfisins, þá er það ekki eitt og sér orsök sjúkdóms heldur áhættuþáttur.
- 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 magnakerfismiðluðu blóðrauðalosi.

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