Studies of EHEC and the complement system in renal diseases

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To Albert, Bernard and Viktor

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List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Békássy ZD**, Calderon Toledo C, Leoj G, Kristoffersson AC, Leopold SR, Perez M, Karpman D. Intestinal damage in enterohemorrhagic *Escherichia coli* infection. *Pediatr Nephrol* 2011; 26:2059-2071.

II. Karpman D, **Békássy ZD**, Sjögren A-C, Dubois MS, Karmali MA, Mascarenhas M, Jarvis KG, Gansheroff LJ, O'Brien AD, Arbus GS, Kaper JB. Antibodies to intimin and *Escherichia coli* secreted proteins A and B in patients with enterohemorrhagic *Escherichia coli* infections. *Pediatr Nephrol* 2002 17:201-211.

III. **Békássy, ZD**, Kristoffersson AC, Rebetz J, Olin AI, Karpman D. Renin cleavage of C3: a kidney-specific mechanism of complement activation. Submitted.

IV. **Békássy ZD**, Kristoffersson AC, Cronqvist C, Roumenina LT, Rybkine T, Vergoz L, Hue C, Fremeaux-Bacchi V, Karpman D. Eculizumab in an anephric patient with atypical hemolytic uremic syndrome and advanced vascular lesions. *Nephrol Dial Transplant* 2013;28:2899-2907.

Permission to reprint the previously published articles has been granted by the respective publisher.

The following papers have been published but not included in the thesis:

1. Manea M, Tati R, Karlsson J, **Békássy ZD**, Karpman D. Biologically active ADAMTS13 is expressed in renal tubular epithelial cells. *Pediatr Nephrol* 2010; 25: 87-96.
2. Ståhl A-L, Sartz L, Nelsson A, **Békássy ZD**, Karpman D. Shiga toxin and lipopolysaccharide induce platelet-leukocyte aggregates and tissue factor release, a thrombotic mechanism in hemolytic uremic syndrome. *PLoS One* 2009 11;4(9):e6990.

Abbreviations

A/E	Attaching and effacing
aHUS	Atypical hemolytic uremic syndrome
CFB	Complement factor B
CFD	Complement factor D
CHI	Complement factor I
CFH	Complement factor H
CFHR	Complement factor H-related protein
C3GN	C3 glomerulonephritis
C3NeF	C3 nephritic factor
DDD	Dense deposit disease
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Esp	*E. coli* secreted protein
ESRD	End stage renal disease
Gb3	Globotriaosylceramide
GBM	Glomerular basement membrane
HUS	Hemolytic uremic syndrome
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MCP	Membrane cofactor protein
MPGN	Membranoproliferative glomerulonephritis
MMF	Mycophenolate mofetil
RAAS	Renin-angiotensin-aldosterone system
SCR	Short consensus repeats
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
TLR	Toll-like receptor
TMA	Thrombotic microangiopathy
TTP	Thrombotic thrombocytopenic purpura
TTSS	Type three secretion system

Abstract

This thesis addressed the diagnosis, pathogenesis and clinical course of specific renal diseases hemolytic uremic syndrome (HUS) and dense deposit disease (DDD). HUS may be associated with infection caused by Enterohemorrhagic *Escherichia coli* (EHEC) or with complement dysfunction due to complement mutations or auto-antibodies (atypical HUS, aHUS).

EHEC is a non-invasive highly virulent bacterium. In order to gain access to the circulation it first damages the intestinal mucosa. The mechanism of damage and the bacterial factors involved were addressed. Abundant cell death by apoptosis was demonstrated in HUS patients. EHEC virulence factors were correlated to the intestinal damage and symptoms in a mouse model using mutant strains. Intimin and the presence of the type III secretion system effectors were necessary for intestinal colonization and virulence in mice. The presence of Shiga toxin induced intestinal mucosal cell death by apoptosis, which could thus allow EHEC virulence factors to gain access to the circulation and reach target organs. A serotype-independent serodiagnostic assay for detection of EHEC was developed. Patients developed an antibody response to intimin, *E. coli* secreted protein A (EspA) and EspB. Antibodies to EspB were the most specific for detection of recent EHEC infection.

The second part of the thesis addressed complement-mediated kidney disease. We found that renin, a kidney-specific enzyme, activated the alternative pathway of complement by cleaving C3 into C3a and C3b in a manner identical to the C3 convertase. Cleavage was inhibited by the renin inhibitor aliskiren. Aliskiren treatment reduced complement activation and stabilized the clinical course in two DDD patients. Renin cleavage of C3 is a novel kidney-specific mechanism of complement activation, which may explain the renal specificity of complement-mediated renal diseases.

Patients with aHUS develop recurrences as long as there is viable renal tissue that triggers disease activity. We described a patient with multiple complement mutations who developed severe systemic vascular complications in the absence of kidneys and aHUS recurrences. To our knowledge this is the first patient treated

with eculizumab, an anti-C5 antibody blocking the terminal complement cascade, in the absence of kidney tissue.

In summary this thesis defined novel mechanisms of pathogenesis and treatment of severe renal conditions.

Introduction

Thrombotic microangiopathy (TMA) is a pathological process associated with hemolytic anemia, thrombocytopenia and ischemic organ damage due to microvascular thrombosis, predominantly involving the kidney and the central nervous system. TMA is a feature of several disorders including hemolytic uremic syndrome (HUS) associated with Shiga toxin (Stx)-producing bacteria or invasive pneumococcal infection, atypical HUS (aHUS), thrombotic thrombocytopenic purpura (TTP) and malignant hypertension.

HUS occurs as a complication of infection with Stx-producing enterohemorrhagic *Escherichia coli* (EHEC) and is a significant cause of acute renal failure in children worldwide, occurring sporadically or in epidemics. EHEC bacteria have an array of virulence factors enabling the bacterium to colonize the intestine, to translocate Stx across intestinal cells into the bloodstream and to affect target organs possessing the Stx receptor, such as the kidney or the brain. This thesis addressed mechanisms by which EHEC induce intestinal cell injury and developed a serotype-independent serodiagnostic assay for detection of EHEC.

aHUS may be familial and recurrent and is associated with dysfunction of the alternative pathway of complement. These complement alterations include loss-of-function mutations in complement regulators or gain-of-functions mutations in complement proteins as well as circulating autoantibodies against complement regulators or proteins. A link has emerged between aHUS and nephropathies associated with activation of the alternative pathway of complement. These disorders include dense deposit disease (DDD) and C3 glomerulonephritis, which may also be associated with mutations and autoantibodies affecting the alternative pathway of the complement system.

It is as yet unclear why the alternative pathway is activated specifically in the kidney. The hypothesis that renin, an aspartate protease released from the juxtaglomerular cells of the kidney, triggers activation of the alternative pathway of complement, was studied. Patients with viable renal tissue benefit from treatment with the anti-C5 antibody eculizumab to prevent recurrences, and this thesis extended these observations, studying the effect in an anephric patient.

Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *Escherichia coli* (EHEC) is a subset of pathogenic *E. coli* that can, upon infection, cause watery diarrhea, bloody diarrhea (hemorrhagic colitis) and in severe cases lead to the development of HUS[1]. Only strains capable of causing hemorrhagic colitis are termed EHEC. The main virulence factor necessary for induction of HUS is Shiga toxin (Stx) because only strains producing Stx are associated with HUS[2].

Stx-producing *E. coli* (STEC) was discovered in 1977[3] and associated with HUS in 1983[4]. The toxin was originally identified as verotoxin since it was found be to cytotoxic to cultured Vero cells (African green monkey kidney cells)[3]. Later on it was found to be closely related to the toxin discovered in *Shigella dysenteriae* serotype 1 and was renamed Stx[5]. This is why STEC are also called verotoxin-producing *E. coli* (VTEC). Most EHEC strains possess additional virulence factors such as the ability to attach to the intestinal epithelium and cause effacement of the microvilli leading to attaching and effacing (A/E) lesions[6]. This feature is also typical for enteropathogenic *E. coli* (EPEC) strains and explains their ability to induce watery diarrhea due to loss of the absorptive surface. EHEC virulence factors will be described below.

E. coli are serotyped based on their O (lipopolysaccharide) and H (flagellar) antigens (Figure 1). *E. coli* O157:H7 is the serotype most commonly associated with HUS worldwide[7] which may, in part, reflect a detection bias as it is easier to detect in fecal samples than other strains. Other strains are emerging, as non-motile *E. coli* O157:H^- and non-O157 serotypes, presumably due to advances in diagnostics of these strains[8]. Over 100 non-O157 serotypes relevant for human disease have been recognized, particularly *E. coli* serotypes O26, O103, O111, 121 and O145[9].

The main reservoir for EHEC is ruminants, predominantly cattle, and most EHEC outbreaks, irrespective of serotype, have been caused by contaminated food or water. Person-to-person transmission has been reported during outbreaks as well[6]. The potential for EHEC spread is further compounded by globalization of food, which presents an opportunity for EHEC to spread quickly to large populations as exemplified by a large radish-borne outbreak of STEC in Japan in 1996[10].

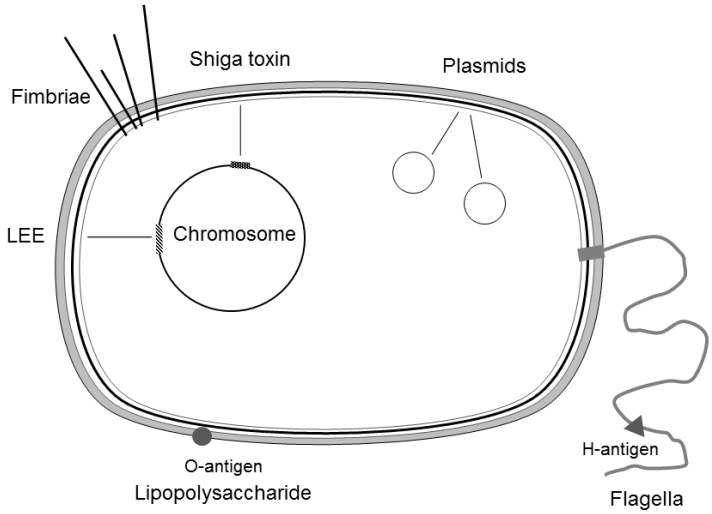

Figure 1. Schematic representation of EHEC. LEE: locus for enterocyte effacement

After an incubation period of 1-8 days EHEC infection typically manifests with watery diarrhea, vomiting and abdominal cramps, which may progress to bloody diarrhea over the coming days[1]. In the majority of infected individuals, the infection resolves within 7 days. The percentage of patients progressing to HUS (within 2-14 days after the onset of diarrhea) depends on the infecting EHEC serotype. For *E. coli* O157:H7 it has been reported to be approximately 15%[11].

HUS is a systemic, sometimes life-threatening, complication characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia and kidney failure. More severely affected patients may develop involvement of other organs, particularly cerebral complications. The estimated mortality from STEC-induced HUS is 2-4%[12, 13]. Although kidney function returns to normal in most patients, permanent nephron loss may occur, which may result in long-term complications (hypertension, proteinuria, decreased renal function)[14].

EHEC in the gastrointestinal tract

Colonization of the intestine

To survive the passage through the gastrointestinal tract EHEC must overcome several impediments including chemical, mechanical and biological barriers[15]. The chemical barrier consists of saliva containing mucins and enzymes, acid stress in the stomach, bile secretion in the small intestine, and antimicrobial peptides through the intestine. The mechanical barrier is formed by a layer of mucus and the biological barrier consists of the normal (commensal) intestinal microflora. These barriers together with the innate and acquired host immune response aim to eliminate the bacteria.

Ingested EHEC survive stomach acidity by expressing acid resistant systems and by promoting enhanced motility and adhesive capacity[16]. Since the infectious dose of EHEC is as low as 50-100 microorganisms, acid tolerance and resistance are important virulence traits[17]. From the stomach bacteria pass to the duodenum where contact with bile may induce protective modifications of the bacterial outer membrane thus enhancing further migration of the pathogen through the small intestine[18].

It is assumed that EHEC binds to follicle-associated epithelium that overlies Peyer's patches and villi of the terminal ileum. This is presumably followed by colonization of the colon[19]. In the intestine EHEC must overcome the mucus layer covering the entire intestinal surface. In the small intestine the mucus consists of one layer, whereas in the large intestine it has two layers. The inner layer is adherent to the epithelial cells and is impermeable to luminal bacteria[20]. Commensal strains in the microflora persist in the outer loose layer and compete with pathogens for space and nutrients.

Quorum sensing

EHEC can communicate with the commensal microflora through hormone-like signals termed autoinducers (AIs)[21]. This chemical communication between bacteria is called quorum sensing. During the initial phase of infection EHEC senses AI-3, derived from the microflora, via a sensor kinase present in the bacterial inner membrane termed QseC, thus promoting intestinal colonization[22]. The same system communicates with host-derived catecholamines whereby EHEC recognizes the host stress hormones adrenaline and noradrenaline via QseC[22].

Activation of sensor kinase QseC further activates genes required for motility (expression of flagella), colonization (type III secretion system) and Stx expression[21]. The importance of AI-3, adrenaline and noradrenaline for in vivo virulence expression was demonstrated using a rabbit infection model[22]. The effects of all three signals (AI-3, adrenaline and noradrenaline) could be specifically blocked by adrenergic antagonists showing that QseC is a bacterial adrenergic receptor[22]. Presumably an increased release of host stress hormones could occur in the local intestinal environment during hemorrhagic colitis thus promoting bacterial colonization and virulence.

Attaching and effacing adherence

Most EHEC strains colonize the intestinal mucosa by the formation of characteristic attaching and effacing lesions[6, 23]. A/E lesions are characterized by effacement of microvilli, intimate attachment of the bacteria to the enterocytes, and accumulation of polymerized actin beneath the site of bacterial attachment to form a pedestal-like structure[6].

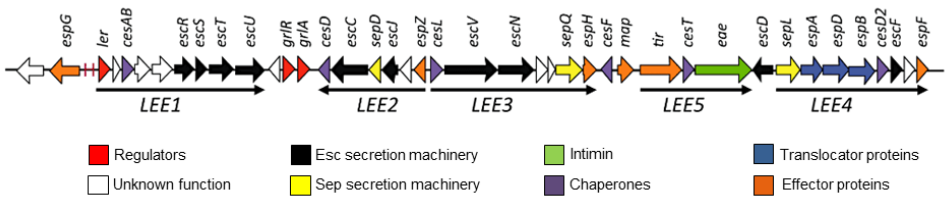

Figure 2. Structure of the LEE (modified from[24])

The genes required for formation of A/E lesions are encoded within the chromosomal pathogenicity island termed the locus for enterocyte effacement (LEE)[25]. The LEE consists of 41 genes, most of which are divided into five major operons (*LEE1-5*) (Figure 2). The first gene in *LEE1* encodes Ler, the main transcription factor of the pathogenicity island, regulating expression of the entire LEE. The majority of the remaining genes in the *LEE1*, *LEE2* and *LEE3* operons encode structural and secondary proteins required for the formation of the type III secretion system (TTSS). *LEE5* contains genes encoding an outer membrane protein intimin and translocated intimin receptor (Tir). *LEE4* encodes several *E. coli* secreted proteins (Esps) that form the translocon portion of the TTSS[26, 27]. There is > 95% LEE sequence homology between EHEC and EPEC strains.

Intimin and its receptors

Intimin is a 94 kDa outer membrane protein encoded by the *eae* gene that enables intimate bacterial adherence to the intestinal epithelium[28]. EHEC injects its own receptor Tir into the host cell using the TTSS as shown in Figure 3. Tir interacts with intimin and this binding induces the rearrangement of the host cytoskeletal structure to form the attaching and effacing lesion. A non-LEE encoded effector protein, *E. coli* secreted protein F-like protein from prophage U (EspFu) cooperates with Tir to recruit host proteins and disrupt the host cytoskeleton and actin polymerization[29]. This results in accumulation of actin beneath attached bacteria, forming the characteristic pedestal-like structure. In addition to Tir, intimin can bind to host cell proteins nucleolin and possibly to β_1 integrin[30].

There are several intimin types defined by heterogenicity of the C-terminal involved in binding to Tir[31]. Differences in intimin subtypes influence the pattern of colonization and tissue tropism in the host[23]. The most common types are *α, β* and *γ.* Intimin-γ is the primary adhesin of *E. coli* O157:H7[32]. Its expression correlates with *E. coli* O157:H7 colonization of the human ileal follicle-associated epithelia of Peyer's patches[33].

The role of intimin in the adherence of EHEC has been demonstrated in cultured cells and in vitro human intestinal organ cultures[34, 35] as well as in a newborn piglet model[34]. Using the mouse ileal loop model *E. coli* O157:H7 was found to induce A/E lesions in mouse intestine[36].

EHEC strains lacking *eae* are able to bind to the host epithelial cells in vitro[37] and to cause severe disease, including HUS, in humans[37, 38]. This indicates that these strains possess other mechanisms of colonization.

Type III secretion system

The TTSS allows EHEC to deliver effector proteins into the host cells and modulate host cell signaling pathways. The structure of the TTSS resembles a "molecular syringe" as depicted in Figure 3. The TTSS enables proteins to pass through the bacterial inner and outer membranes and then through a channel and pore into the targeted cell. It is composed of a basal body onto which a "syringe" and translocon filament is assembled. The basal body is a multi-ring structure composed of the *E. coli* secreted apparatus EscQ, EscR, EscS, EscD, EscT, EscU, EscV in the inner membrane[39, 40]. EscN (a cytoplasmic ATPase) is associated with the inner membrane basal structure and provides the energy required for the TTSS transport mechanism[41]. EscC forms the outer membrane ring of the structure[39].

EscJ bridges the inner and outer membranes of the TTSS[42]. EscF forms a needle structure onto which the translocon can be assembled. The translocon is composed of EspA filaments, which form a channel, whereas EspD/B form a pore in the host cell membrane through which bacterial proteins are injected into the cell[43, 44].

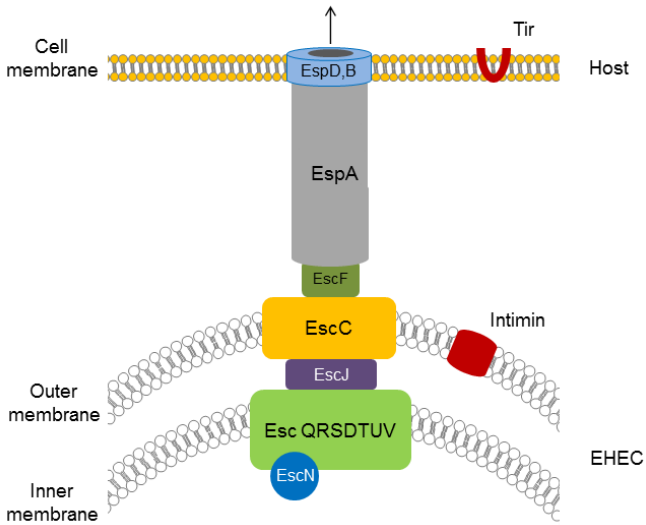

Figure 3. Schematic representation of the TSSS (modified from[27, 45])

E. coli O157:H7 (Sakai strain) encodes more than 60 effectors, of which 39 are translocated into the host cell[46]. Many of these effectors are homologous to those secreted by EPEC strains and are responsible for the establishment of an environment suitable for pathogen proliferation and the subversion of host responses. The exact function of many of these proteins is unknown but some of them have been implicated in bacterial virulence, including Tir, Map, EspF, EspG, EspH, EspB and EspZ. The effector proteins translocated through the TTSS and their predicted functions are presented in Table 1.

Table 1: TTSS effector proteins

	Effector protein	Predicted function	Ref
LEE	EspB	Translocation pore component, prevention of phagocytosis	47
	EspF	Disruption of intestinal tight barrier junctions, induction of apoptosis, modulation of the intestinal cytoskeleton and prevention of phagocytosis	48, 49
	EspG	Disruption of ion balance and H_2O absorption	47
	EspH	Disruption of the actin cytoskeletal structure and promotion of pedestal formation	50
	EspZ	Inhibition of translocation of Tir, Map and EspF and inhibition of the formation of actin pedestals	51
	Map	Effacement, formation of filopodia, disruption of mitochondrial function	47
	Tir	Translocated intimin receptor, actin pedestal formation	29
Non-LEE	NleA	Inhibition of protein trafficking and disruption of tight junctions. Upregulated by starvation stress	52, 53
	NleB	Inhibition of TNFα-mediated NF-κB activation and suppression of the host inflammatory response	54
	NleC	Zinc protease, which inhibits the NF-κB pathway in cooperation with NleE and suppresses the host inflammatory response	55, 56
	NleD	Metalloprotease, which prevents JNK-mediated pro-apoptotic signaling. In cooperation with NleC inhibits IL-8 secretion	56
	NleE	Inhibition of the NF-κB activation and suppression of the host inflammatory response. NleE activity is enhanced by NleB	57
	NleF	Inhibition of caspases -4, -8, -9 and prevention of apoptosis induction	58
	NleH	Inhibition of apoptosis by targeting BI-1, a cellular inhibitor of the anti-apoptotic protein Bax	59

LEE: locus of enterocyte effacement, Nle: non-LEE encoded, Esp: *E. coli* secreted protein, Map: mitochondrion-associated protein, NF-κB: nuclear factor-kappa B, JNK: c-Jun N-terminal kinase, BI-1: Bax inhibitor 1.

Non-LEE factors

To prevent their early elimination and apoptotic clearance, A/E pathogens EHEC and EPEC control the host response by delivering anti-inflammatory and anti-apoptotic effector proteins into the host cells. These effects are mediated by non-LEE encoded effectors (Nle) presented in Table 1. Their effects have been demonstrated in vivo. Rabbits infected with REPEC strain exhibited decreased apoptosis in the ileum and ileal Peyer's patches[60]. Bacterial suppression of the inflammatory and apoptotic response in the host may, at least in part, explain symptom-free carriage of EHEC bacteria in humans.

A number of fimbrial and nonfimbrial adhesins have been implicated in initial EHEC adherence to the host mucosa. Some of them include fimbriae and flagella. Flagella allow bacterial swimming motility and may promote adherence to mucins[61]. H7 flagella have been shown to act as adhesins to bovine intestinal epithelium[62]. H7 flagellin is able to induce proinflammatory signals in human colon epithelial cells[63]. Long polar fimbriae are associated with the adherence of EHEC to cultured epithelial cells[64] and bind to the intestinal extracellular matrix[65]. Type IV pili promote bacterial attachment to human colonic epithelial cells as well and may contribute to biofilm formation[66].

The primary virulence factors of EHEC strains are chromosomally encoded, but plasmids also play a role in the pathogenesis of EHEC strains. Hemolysin (EHEC-Hly) is a pore-forming cytolysin encoded on a plasmid pO157. Hemolysin induces lysis of human red blood cells in vitro on blood agar plates but not in vivo as it is not secreted from bacteria[67]. It was found to be toxic to human brain microvascular cells in vitro[68].

Release of toxins

EHEC is a non-invasive bacterium and does not cause bacteremia[69]. In order to cause disease bacterial virulence factors need to be delivered into the circulation and via the bloodstream to target organ cells.

Shiga toxins

Shiga toxins (Stx) are AB_5 toxins consisting of a catalytic A-subunit and a pentameric B-subunit that binds to specific glycosphingolipid receptors, particularly globotriaosylceramide (Gb3). Gb3 receptors are differently expressed on various tissues and in different species[70, 71]. When Stx bind the Gb3 receptor, it is internalized as a holotoxin by endocytosis of clathrin-coated pits and trafficked through the Golgi apparatus to the endoplasmic reticulum. In the endoplasmic

reticulum the A subunit is cleaved by the protease furin[72]. The A1-fragment cleaves a specific adenine residue from the 28S ribosomal RNA thus inhibiting host cell protein synthesis[73].

Shiga toxins are encoded on a bacteriophage inserted into the chromosome. Stx-producing bacteria may produce more than one type of Stx[74]. Two major Stx families have been identified, Stx1 and Stx2, and a number of subtypes. Stx1 is closely related to Stx produced by *Shigella dysenteriae* with a single amino acid difference in the catalytic subunit, while Stx2 shares approximately 60 % amino acid identity[75]. Epidemiological studies suggest that EHEC responsible for HUS express Stx2 more often than Stx1[76, 77]. In mouse models Stx2 is 100 times more potent than Stx1[78]. Stx2 subtypes are linked to different clinical outcomes. Stx2c is associated with HUS in humans but Stx2d and Stx2e are not[76, 77]. $Stx2d_{activatable}$ is also associated with HUS[38]. The virulence of this toxin is increased by enzymes in the intestinal mucus. Different Stx2 subtypes may display some differences in receptor preference[79].

Subtilase cytotoxin

An additional STEC AB_5 toxin termed subtilase cytotoxin (SubAB) was discovered in an *eae*-negative but Stx2-positive O113:H21 strain, which was responsible for an outbreak of HUS in Australia[80]. It has since then been detected in numerous other STEC serotypes as well as in Stx-negative *E. coli* strains[81]. SubAB is cytotoxic *in vitro* for a range of cell types, and it is more toxic for Vero cells than Stx[82]. It is also lethal for mice when injected intraperitoneally, producing microangiopathic changes resembling HUS in humans[83].

Lipopolysaccharide

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria contributing to the structural integrity of the bacteria and protecting the membrane against bile salts and lipophilic antibiotics[84]. LPS consist of lipid A, responsible for its toxic effects, a core oligosaccharide, and a polysaccharide side chain which projects from the surface of the bacteria and is referred to as the O-antigen[85]. The O-antigen is composed of repeating units of oligosaccharides. Variation of these repeating units contributes to LPS heterogenicity and is used in serotyping of bacteria. Lipid A, if released into the circulation, binds to the acute-phase serum protein LPS-binding protein (LBP). Serum antibodies to EHEC LPS suggest that HUS patients respond to LPS[86]. LPS circulates bound to blood cells in patients with HUS indicating a role in the pathogenesis[87].

Stx in the intestine

Several studies have demonstrated that Stx promotes EHEC intestinal colonization in different hosts. Mice infected with Stx-producing strains exhibited a higher bacterial burden compared to isogenic non-Stx-producing strains[88]. Toxin neutralizing antibodies reduced the bacterial burden in mice and also protected them from weight loss and death[89]. The presence of Stx may represent an advantage for bacteria in the competitive environment of the intestine.

Translocation of Stx from the intestinal lumen to bind to the underlying Gb3-enriched endothelium is an important step in the course of the infection. The mechanisms involved in this process have not been fully elucidated.

Stx was demonstrated inside ileal and colonic epithelial cells from a patient with EHEC infection indicating that it can be taken up by these cells. Toxin was also present in the lamina propria indicating that it can cross the epithelial monolayer[90]. Studies have shown that intestinal epithelial cells may express small amounts of the Gb3 receptor to which the toxin binds[91, 92]. Stx binds to the Gb3 receptor on specialized Paneth cells located at the base of colonic crypts[93]. In addition, a mechanism mediated by macropinocytosis has also been suggested for Stx uptake by intestinal epithelial cells[90].

An alternative pathway has recently been proposed for Stx translocation via bacterial uptake by M cells and underlying macrophages[94]. M cells are specialized cells overlying Peyer's patches and lymphoid follicles primarily involved in sampling intestinal antigens. They translocate antigens from the intestinal lumen to the basolateral side of the epithelium and deliver them to underlying macrophages. They lack microvilli and the thick glycocalyx present on enterocytes and express toll-like receptors (TLR) and β_1 integrin[95]. Some invasive bacteria such as *Salmonella enterica* are able to across the follicle-associated epithelium via M cells[96]. *E. coli* O157:H7 strains were shown to be able to translocate through the murine ileal epithelium overlying Peyer's patches *ex vivo* and cross M cell monolayers in vitro. Moreover, EHEC strains were able to survive and to produce Stx in macrophages, which induced cell apoptosis and Stx release[94].

Stx-mediated intestinal epithelial damage was observed in a human organ culture model[97]. Presumably Stx may be transmitted through possible "holes" in the epithelium that result from A/E lesions and Stx-mediated damage[98]. EHEC-induced gastrointestinal infection in humans is accompanied by severe inflammation and mucosal injury. It seems likely that Stx can leak through damaged epithelium at later stages of infection.

Stx may trigger different signaling pathways. It inhibits protein synthesis and may cause apoptotic cell death characterized by cell shrinkage due to cytoplasmic condensation, membrane blebbing, apoptotic body formation, chromatin condensation and DNA fragmentation[99]. In human endothelial cells Stx target nuclear DNA leading to DNA fragmentation, activation of the apoptotic program and cell death[100]. In human microvascular endothelial cells Stx activates the cleavage of caspase-3,-6,-8 and -9 and upregulation of the DNA damage-inducible protein 153[101]. Apoptosis induced by Stx can be associated with increased expression of proapoptotic proteins Bax and Bak (reviewed in[102]).

EHEC is also capable of inducing apoptotic cell death independent of Stx expression as demonstrated in human epithelial cells treated with strains that both produced and did not produce Stx[103]. Moreover, EHEC may translocate pro-apoptotic effector proteins such as EspF into intestinal epithelial cells to induce apoptosis[49]. Apoptotic cell death was demonstrated in the intestines in in vivo models, including rabbits[104] and mice[105]. The apoptosis-inducing effect may initiate an inflammatory response and neutrophil influx. Neutrophil migration towards the intestinal lumen occurs simultaneously and may enhance Stx translocation from the lumen via enterocytes in vitro[106].

Host responses

Host cells have the capacity to detect intruding bacteria by sensing pathogen-associated molecular patterns (PAMPs) such as LPS, flagellin and lipoproteins[107]. These PAMPs are recognized by TLRs. TLRs recruit a specific set of adaptor molecules such as MyD88 and TRIF and initiate downstream signaling pathways leading to the secretion of inflammatory cytokines, type I interferon, chemokines, and antimicrobial peptides[108].

The importance of TLR4, TRIF and MyD88 for the pathogenesis of EHEC infection was demonstrated in mice infected with *E. coli* O157:H7, both Stx2-producing and non-producing[109]. Only mice infected with the Stx2-producing strain developed symptoms while MyD88-knock-out mice exhibited most severe symptoms and the highest bacterial burden suggesting that the innate immune response at the intestinal mucosa was important for bacterial clearance.

Stx induces production of interleukin-8 (IL-8) and other cytokines on human colonic epithelial[110, 111] and human endothelial cells[112]. Stx-induced IL-8 expression is associated with activation of the stress-activated mitogen-activated protein (MAP) kinases JNK/SAPK and p38 in intestinal epithelial cells[113] and vascular endothelial cells[100]. These and other studies indicate that inhibited protein

synthesis associated with ribotoxic stress caused by Stx-induced rRNA injury may simultaneously promote translation of inflammatory cytokines[114].

The EHEC H7 flagellin may contribute to inflammation via the TLR5 receptor. H7 flagellin may activate nuclear factor-kappa B (NF-κB) and MAP pathways in the human intestinal epithelial cells leading to secretion of the potent neutrophil chemotactic factor IL-8[63]. Long polar fimbriae can also induce expression of proinflammatory cytokines in the intestinal epithelial cells[65]. Flagellin- and long polar fimbriae-mediated inflammation may contribute to local damage of the intestinal mucosa. Released cytokines will in turn lead to upregulation of Gb3 expression on endothelial cells suggesting that the host response might enhance toxin-induced cell-damage[115].

Gastrointestinal disease and intestinal pathology

EHEC causes a wide spectrum of intestinal manifestations ranging from asymptomatic disease to mild diarrhea and hemorrhagic colitis attributed primarily to the actions of Stx[116]. Serious complications may occur including massive bleeding, toxic dilatation of the colon, intussusception, necrosis of the bowel wall and perforation[117]. These complications may require partial or total colon resection and may to lead to colonic stricture[118].

Colonic specimens from patients with *E. coli* O157:H7-associated colitis exhibited hemorrhage, edema, mucosal necrosis and pseudomembrane formation. Fibrin-platelet microthrombi, and neutrophils focally infiltrating the lamina propria and crypts were also observed[118, 119].

Intestinal pathology demonstrated in mice included edema and erosions, congestion of the lamina propria, inflammatory infiltrates, goblet cell depletion and necrosis[109, 120].

EHEC virulence factors in the circulation

When absorbed into the bloodstream Stx will circulate bound to blood cells[87, 121] thus reaching the Gb3-enriched endothelium in target organs, such as the kidney and the brain. Stx is not cytotoxic to neutrophils, monocytes and platelets (the latter have minimal protein synthesis) and can thus circulate on these cells without decreasing their life span [122, 123.]

Stx has been detected bound to neutrophils or neutrophil-platelet complexes in patients with HUS[121, 124]. Stx binds to TLR4 on neutrophils[125]. In vitro studies have shown that Stx was transferred from the surface of neutrophils onto endothelial cells thus impairing protein synthesis and triggering production of proinflammatory cytokines[126]. Stx's interaction with neutrophils results in their activation and degranulation[124, 126]. Elevated neutrophil counts are usually observed in patients with HUS and higher levels are associated with a worse outcome[123].

Patients with HUS also exhibit Stx on monocytes and platelet-monocyte complexes[124]. Stx binds to monocytes via the Gb3 receptor and this binding is enhanced by LPS. Monocytes are activated and release cytokines IL-1β, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) in vitro[127]. In addition, Stx induces release of monocyte-derived microvesicles expressing tissue factor (TF) TF initiates the coagulation cascade by binding factor VII[128]. Its expression is enhanced when monocytes are stimulated with both Stx and LPS[124]. TF-bearing microvesicles may fuse with activated platelets and thus contribute to the pro-thrombotic process[129].

In addition to neutrophils and monocytes, Stx circulates in vivo bound to platelets during HUS[87]. It binds to activated platelets via the Gb3 receptor and an alternative glycosphingolipid receptor termed band 0.03[130]. Upon binding Stx is internalized, leading to further platelet activation, aggregation, structural changes increasing the surface area, and enhanced fibrinogen-binding capacity[131]. Furthermore, LPS may induce platelet activation and aggregation both in vitro and in vivo. O157LPS binds to platelets via a receptor complex composed of TLR4 and CD62 (P-selectin)[87]. LPS has been detected on the platelets of HUS patients suggesting that it may activate platelets in the circulation[124].

EHEC virulence factors in the kidney

Stx was demonstrated in human kidneys from patients with HUS, in both glomeruli and tubuli[132]. During HUS glomerular endothelial cells typically exhibit swelling and detachment from the basement membrane. It is unknown how Stx is transferred from blood cells to the renal endothelium in vivo but it has been proposed that this transfer may be related to a higher affinity for the Gb3 receptor on endothelial cells in the kidney[126, 133].

Subtoxic doses of Stx induce profound alterations in endothelial cells. The toxin upregulates mRNA expression and protein levels of chemokines such as IL-8 and monocyte chemoattractant protein 1 (MCP-1)[134], chemokine receptors (CXCR4 and CXCR7)[135], and cell adhesion molecules including P-selectin[136]. These effects promote leukocyte recruitment and adherence and amplify endothelial cell injury.

Stx has been demonstrated to promote platelet adhesion to human microvascular endothelial cells, particularly under conditions of high shear stress that mimic the environment in the microcirculation[136]. Stx may also increase TF activity on TNF-α stimulated glomerular endothelial cells[137]. Elevated TF levels have been detected in plasma of patients with HUS[138], on circulating platelet-derived microvesicles[124] and demonstrated in the kidney[139]. Stx may also impair ADAMTS13 cleavage of von Willebrand factor thus promoting a prothrombotic environment[140]. LPS enhances the cytotoxic effect of Stx on human vascular endothelial cells[141] and in animal HUS models[142]. Taken together, Stx and LPS favor inflammation and induce a prothrombotic phenotype on the glomerular microvascular endothelium.

Endothelial cells detach from their basement membranes, exposing subendothelial matrix proteins such as collagen, von Willebrand factor, fibrinogen and fibronectin. Adhesion of platelets is followed by thrombus formation. Narrowing or occlusion of the capillary lumen leads to reduced blood supply to the glomeruli. These lesions lead to thrombotic microangiopathy (TMA) forming predominately in the glomeruli and small renal arterioles. TMA lesions account for the cardinal manifestations of HUS such as thrombocytopenia, hemolytic anemia and kidney failure.

Hemolytic uremic syndrome

Thrombocytopenia is caused by consumption of platelets in microthrombi on the surface of damaged endothelium. Other mechanisms may also contribute, such as

activation of platelets in the circulation by Stx, cytokines, TF release leading to platelet aggregation and deposition on endothelial cells as discussed above.

Hemolytic anemia is associated with the formation of fragmented red blood cells termed schistocytes or helmet cells. It is assumed that they are produced by shear stress caused by vascular occlusion and mechanical damage to red blood cells[143]. Oxidative damage of red blood cells has also been proposed to contribute to hemolysis[144]. However, the exact mechanism by which hemolysis occurs is still unclear.

Kidney failure results from ischemic glomerular damage, secondary to the occlusion of the microvasculature, as well as direct injury to tubular, mesangial and glomerular epithelial cells, all expressing the Gb3 receptor in humans. Renal cortical biopsies from patients with HUS revealed evidence of apoptotic cell death**,** predominately in renal tubular cells[145]. In mice the Gb3 receptor is present on tubular cells but not glomerular. Thus mice develop a tubular phenotype after exposure to Stx[71]. Proximal tubular epithelial cells are highly sensitive to Stx in vitro and this susceptibility is further increased by co-incubation with LPS [146].

Kidney biopsies from HUS patients may reveal vascular lesions as well as tubular lesions. Currently it is not clear whether the toxin targets certain renal cells preferentially or the various cells are affected simultaneously. Glomerular epithelial cells (podocytes) are potent targets for Stx as well[147]. Stx has been shown to enhance the synthesis of vasoconstrictor peptide ET-1 in murine podocytes[148]. Patients with HUS may exhibit proteinuria, which could be associated with podocyte injury[149]. Mesangial expansion and mesangiolysis were found in kidney biopsies as well[150]. Stx stimulation of mesangial cells in vitro inhibits protein synthesis, an effect potentiated by IL-1 and TNF-α[151].

Laboratory investigation of EHEC infection

EHEC may be difficult to detect in feces, particularly later in the course of the disease, when complications such as HUS develop.

Polymerase chain reaction

When EHEC infection is suspected PCR detection of *stx1*, *stx2*, *eae* or *uidA* (for O serotype 157) in fecal samples can be used for screening[152]. Extracts of feces or primary cultures are used as templates.

Fecal culture and serotype

E. coli O157:H7 is easy to identify by culturing stools on sorbitol-MacConkey agar (SMAC). This serotype is usually unable to ferment sorbitol and thus forms colorless colonies in contrast to other sorbitol-fermenting intestinal *E. coli* stains that form pink colonies[153]. Suspected colonies can be further assayed for the O157 somatic antigen by latex agglutination or for the H7 flagellar antigen by immunoassays. The amount of EHEC is usually very low in human feces. For enrichment, samples may be cultured in liquid enrichment medium, or subjected to immunomagnetic separation using magnetic beads coated with an antibody to the O157 antigen [154].

SMAC fails to detect other serotypes than *E. coli* O157:H7 or O157:H$^-$ capable of fermenting sorbitol. The diagnosis of non-O157 STEC is complex and currently requires a sequential approach that entails screening for Stx or its encoding genes by PCR, followed by culture, colony identification and serotyping of the respective strain[155].

Enzyme-Linked Immunosorbent Assay

Serodiagnosis by ELISA is valuable in cases in which EHEC is no longer present in the intestine. Serum samples can detect antibodies to LPS for some of the common EHEC serotypes [156]. The assay is serotype-dependent.

Pulsed-field gel electrophoresis

PFGE is a molecular subtyping ("fingerprinting") technique of the bacterial genome that allows linking of clinical bacterial isolates to a suspected source of contamination and is used for epidemiological purposes[157].

The acquired immune response to EHEC infection

An acquired immune response develops after EHEC infection. Patients develop serum antibodies against Stx and LPS[86, 158]. Antibodies against Stx and LPS have also been detected in asymptomatic household contacts[159]. An increased prevalence of anti-Stx antibodies in rural compared to urban residents has been found. This might be due to exposure to STEC, not necessarily disease-associated EHEC, as a result of contact with farm animals[160].

EPEC is a similar pathogen causing diarrhea in developing countries[6]. In similarity to EHEC, it forms A/E lesions on the intestinal epithelium, but does not produce Stx. Antibodies against antigens common to both strains, such as intimin and *E. coli* secreted proteins A and B, have been found in human serum, saliva, colostrum

and breast-milk[161, 162]. These antibodies may have a protective effect and thus explain the low prevalence of EHEC infections in EPEC-endemic areas[163]. The protective effect of cross-immunity between EPEC and EHEC was demonstrated in a mouse model in which mice were first infected with EPEC followed by inoculation with EHEC[164]. Mice pre-challenged with EPEC developed antibodies against common antigens and were protected from symptoms as well as intestinal and renal pathology caused by EHEC infection.

Treatment

Currently, there is no specific treatment for EHEC infection or HUS. The treatment consists primarily of supportive care including rehydration[165].

Antibiotics should not be used during the diarrheal phase of EHEC infection. In vitro studies have shown that, at least for *E. coli* O157, sublethal doses of antibiotics, particularly trimethoprim, quinolones or furazolidone, promote the production and release of Shiga toxins[18]. Clinical studies have also shown that patients treated with antibiotics for hemorrhagic colitis have a higher risk of developing HUS[166]. However, patients with established HUS may benefit from antibiotic treatment as shown in a large cohort of patients from the *E. coli* O104:H4 outbreak in Germany[167].

Research is ongoing on Stx antibodies, novel peptides as well as zinc-based salts. Humanized monoclonal antibodies against Stx1 and Stx2 (Shigamabs, Thallion Pharmaceuticals Inc., Canada) are being tested in phase 2 trials.

Complement in EHEC-HUS

Activation of the complement system via the alternative pathway has been documented during EHEC-associated HUS. Low C3 levels and elevated levels of C3 degradation products were demonstrated in plasma samples during the acute phase of disease[168]. All these parameters rapidly normalized after resolution and did not correlate with the degree of renal injury. It was hypothesized that the elevated levels were secondary to Stx-triggered endothelial damage[168]. C3 deposits were demonstrated on circulating platelet-monocyte complexes as well as on platelet- and monocyte-derived microvesicles, the latter exhibited C9 deposits as well during the acute-phase, but not after recovery[169].

Stx may directly contribute to complement activation documented by C3 deposition on microvascular endothelial cells exposed to Stx and then perfused with human serum in vitro[170]. High concentrations of purified Stx2 were shown to bind to and inhibit the complement regulator factor H thus promoting activation of the alternative pathway[171].

At the time of the outbreak of *E. coli* O104:H4 in Germany in 2011, a publication in the *New England Journal of Medicine* described the use of eculizumab (Soliris, Alexion, Cheshire, Conn) in three children with HUS[172]. Eculizumab is a humanized monoclonal antibody that binds to complement C5 and blocks the terminal complement pathway and is successfully used for the treatment of complement-mediated diseases such as paroxysmal nocturnal hemoglobinuria[173] and atypical HUS[174]. This report prompted the German Society of Nephrology to recommend the use of eculizumab for the sickest patients during this outbreak. No short-term benefit was detected that could be attributed to eculizumab treatment[167]. This may be due to administration late in the course of disease to the sickest patients. Treatment with an antibody that blocks the terminal complement pathway may have deleterious effects in the intestine thus increasing the bacterial burden and prolonging bacterial survival. However all patients treated with eculizumab were also treated with antibiotics to prevent meningococcal infection. Controlled studies are needed to address the use of a complement inhibitor in STEC-HUS[175].

The alternative pathway of complement

Overview

The complement system, an essential part of the innate immune system, was discovered more than 100 years ago as a result of its "complementary" bactericidal activity and its role in phagocytosis of cellular debris. It plays a crucial role in microbial killing, clearance of apoptotic cells, handling of immune complexes and modulation of adaptive immune responses[176-178]. It is composed of more than 40 interacting soluble plasma and cell-surface proteins[179]. The complement system may be activated via three specific pathways: the classical pathway, the lectin pathway and the alternative pathway. These pathways converge in the activation of the central protein in the system, the third complement component C3. This thesis will focus on the alternative pathway due to its central role in C3 glomerulopathy and aHUS.

The alternative pathway may be initiated on activating surfaces such as on bacterial cell walls or on altered host cells. It also undergoes low-grade intrinsic activation. The alternative pathway "tick over" occurs continuously in plasma enabling a rapid response to pathogens[180] (Figure 4). C3 is activated by spontaneous hydrolysis of a reactive thioester group leading to the formation of $C3(H_2O)$ which binds factor B (CFB) to form the $C3(H_2O)B$ complex. CFB is proteolytically activated and cleaved to Bb and Ba by factor D (CFD) to generate the initial fluid phase alternative pathway convertase $C3(H_2O)Bb$. $C3(H_2O)Bb$ cleaves additional C3 molecules to C3a and C3b. The C3b may covalently attach to cell surfaces and bind CFB to form C3bBb (C3 convertase). The C3 convertase is a labile enzyme with a half-life about 90 seconds[181] which can be stabilized up to 10-fold by binding to the only positive complement regulator properdin[182]. The C3 convertase cleaves more C3 molecules and this reaction is further amplified in the alternative pathway amplification loop[183].

C3b binding to C3bBb forms the enzyme C3bBbC3b (C5 convertase) which cleaves C5 into C5a and C5b. C5b initially bound to C5 convertase binds to C6.

The C5b-C6 complex binds C7 and exposes hydrophobic sites which allow release of this complex from the convertase and its insertion into the target membrane where it serves as a receptor for C8. The C5b-C8 complex can then bind multiple molecules of C9 forming the membrane attack complex (MAC)[184, 185]. The MAC may be cytolytic, forming a pore-shaped structure in the surface membrane, which causes osmotic lysis of the target cell or, in sublytic amounts, may be associated with cell activation[186]. Alternatively, C5b-9 also termed TCC (terminal complement complex) or soluble C5b-9 can accumulate in the fluid phase as an inactive complex[187].

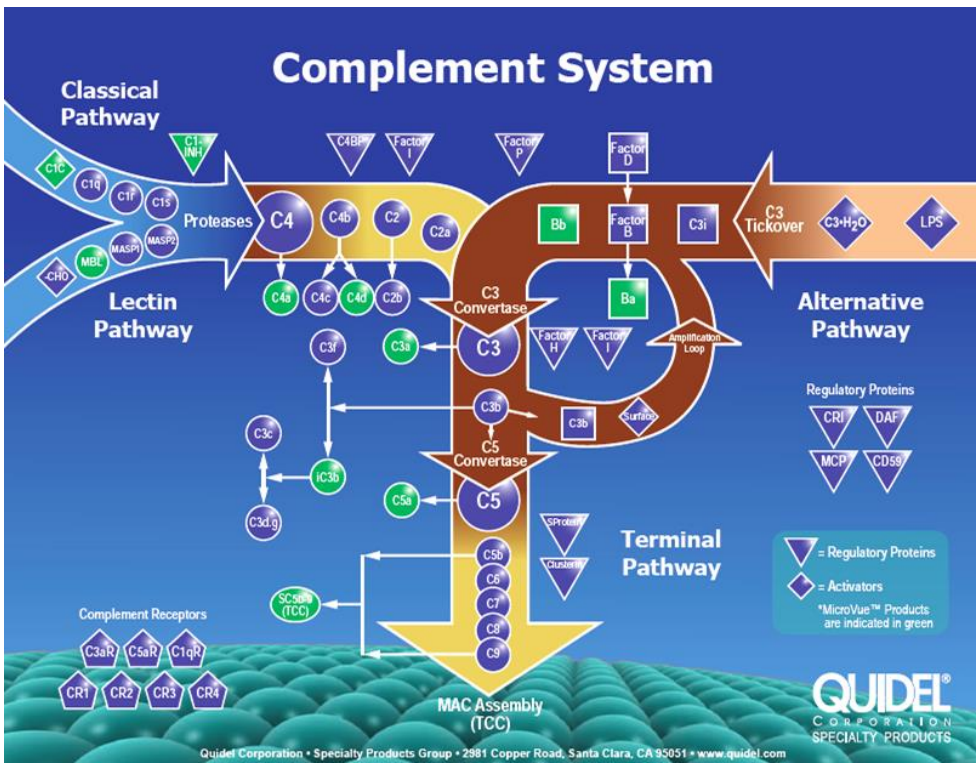

Figure 4. The complement system (reproduced with permission from Quidel)

The cleavage products C3a and C5a mediate several inflammatory responses. They are potent anaphylotoxins that stimulate histamine release from mast cells, increase vascular permeability and promote vasodilatation. C3a and especially C5a are chemoattractants that guide neutrophils, monocytes and macrophages toward sites of complement activation. They trigger proinflammatory signaling via

receptor binding (C3aR and C5aR)[178] and also possess antimicrobial properties. Excessive complement activation may result in host tissue injury and disease.

Complement proteins

C3

C3 is a large protein (185 kDa) belonging to the α2-macroglobulin family. It is composed of an α-chain (110-112 kDa) and a β chain (75 kDa) that are connected covalently by a single disulfide bond[188]. The protein is encoded by the *C3* gene located on chromosome 19[189]. C3 is formed by 13 domains including an anaphylatoxin domain (ANA) that generates C3a after cleavage, a TED domain that carries the reactive thioester, eight homologous domains termed macroglobulin (MG) domains, and others[190] as presented in Figure 5. Proteolytic activation of C3 by the C3 convertase leads to cleavage between residues 726 and 727 (Arg-Ser) and the generation of C3b (176 kDa) and C3a (9 kDa)[188].

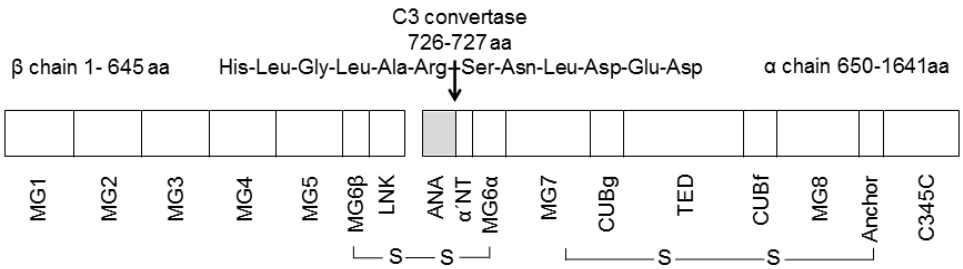

Figure 5. Structure of the C3 protein

C3 is the most abundant complement protein in human serum (concentration 1.2 g/L). The majority of C3 is synthesized in the liver[191] but the kidney contributes measurably to C3 production as cells within a transplanted human kidney contribute about 5% of the circulating C3 pool[192]. Increased C3 gene expression in the kidney occurs in vivo during renal inflammation[193, 194] and in proteinuric diseases[195]. C3 synthesis is up-regulated by inflammatory cytokines in glomerular endothelial and epithelial, mesangial and proximal tubular epithelial cells in vitro[196]. Leukocytes present in the kidney during inflammation can produce C3 as well[197, 198].

CFB

CFB is a zymogen that carries the convertase serine protease domain necessary for amplification of the alternative pathway. Upon interaction with C3b, CFB is cleaved by plasma serine protease CFD into two fragments, Ba and Bb. The Ba fragment dissociates from the complex, whereas Bb remains bound to C3b to form the active C3 convertase. Dissociation of the two components of this complex results in inactivation of the convertase and is promoted by decay accelerating factor, complement receptor 1 and factor H. Once released from C3bBb, Bb is no longer active.

The gene encoding *CFB* is located on chromosome 6p21.3. CFB is a glycoprotein consisting of 5 domains. The proenzyme CFB consists of three N-terminal short consensus repeats (SCR) domains, connected by a 45-residue linker to a von Willebrand factor-like A domain and a C-terminal serine protease domain, which carries the catalytic center for C3 convertase[199]. The SCR domains and the linker form fragment Ba. The von Willebrand factor-like A and serine protease domains form fragment Bb. The binding of CFB to C3b depends on elements in fragment Ba and the Mg^{2+}-dependent metal ion-dependent adhesion site (MIDAS) motif in the von Willebrand factor-like A domain of fragment Bb[199].

Complement regulators

Complement regulation is crucial to prevent undesirable complement activation and avoid severe damage to host tissues. Most of the alternative pathway regulators belong to the superfamily known as the regulators of complement activation (RCA) encoded within the RCA gene cluster on chromosome 1q32[200]. These proteins include fluid phase regulators such as complement factor H (CFH), CFH-related protein 5 (CFHR5) and membrane bound regulators: membrane cofactor protein (MCP), decay accelerating factor (DAF) and complement receptor 1 (CR1). The RCA family proteins share a common basic structure consisting of 60 amino acid domains termed SCRs. Another regulator of the alternative pathway is factor I (CFI) encoded elsewhere. Further down in the complement cascade the terminal pathway is regulated by CD59, clusterin and vitronectin.

CFH

CFH is essential for the regulation of the alternative pathway in the fluid-phase and on cell surfaces. CFH is a large glycoprotein (150 kDa) composed of 20

SCRs[201]. It circulates in human plasma at a concentration of 125-400 μg/ml[202]. CFH down-regulates activation of the alternative pathway by acting as a cofactor for CFI-mediated proteolytic inactivation of C3b, by competing with CFB for C3b binding and inhibiting the formation of the C3 convertase (C3bBb) and by accelerating decay (dissociation) of already formed C3 convertase[203]. These regulatory activities are mediated by the N-terminal region SCRs 1-4[204]. CFH also possesses the ability to recognize host cells and exposed basement membranes by binding to polyanions such as sialic acid and glycosoaminoglycans via SCRs 19-20 located at the C-terminal region[205, 206]. Recent studies have shown that SCR 19 contains a C3b binding site while glycosoaminoglycan binding is mediated by SCR 20 and both of these interactions are necessary for full binding of CFH to C3b on non-activating host surfaces (for target recognition)[207, 208].

CFI

CFI is an 88 kDa glycoprotein composed of two chains linked by a disulfide bond[209]. The gene encoding *CFI* is located on chromosome 4q25[210]. The light chain contains the catalytic serine protease domain while the function of the heavy chain is unclear[211]. CFI down-regulates the alternative pathway by cleaving C3b into the inactive iC3b in the presence of cofactors CFH[209] in the fluid phase or membrane-bound MCP[212] and CR1[213]. Further cleavage of iC3b by CFI with CR1 as a cofactor generates the C3c and C3dg fragments.

CFHRs

Factor H-related proteins comprise a group of five plasma proteins: CFHR1, CFHR2, CFHR3, CFHR4 and CFHR5. The *CFHR* genes are located downstream of the factor H gene and each *CFHR* gene codes one plasma protein[214] (Figure 6). According to their conserved domains, CFHRs are divided into two major groups[215]. The first group includes CFHR1, CFHR2 and CFHR5 and is characterized by their conserved N-terminal (SCR1 and SCR2), which have more than 80% sequence homology and mediate dimerization of these proteins. These proteins circulate in plasma as homo- or heterodimers and in vitro have been shown to compete with CFH for ligand binding[216, 217]. The second group includes CFHR3 and CFHR4 proteins lacking the N-terminal dimerization domains.

CFHR1 regulates the terminal pathway by inhibiting C5 convertase and TCC assembly[218].

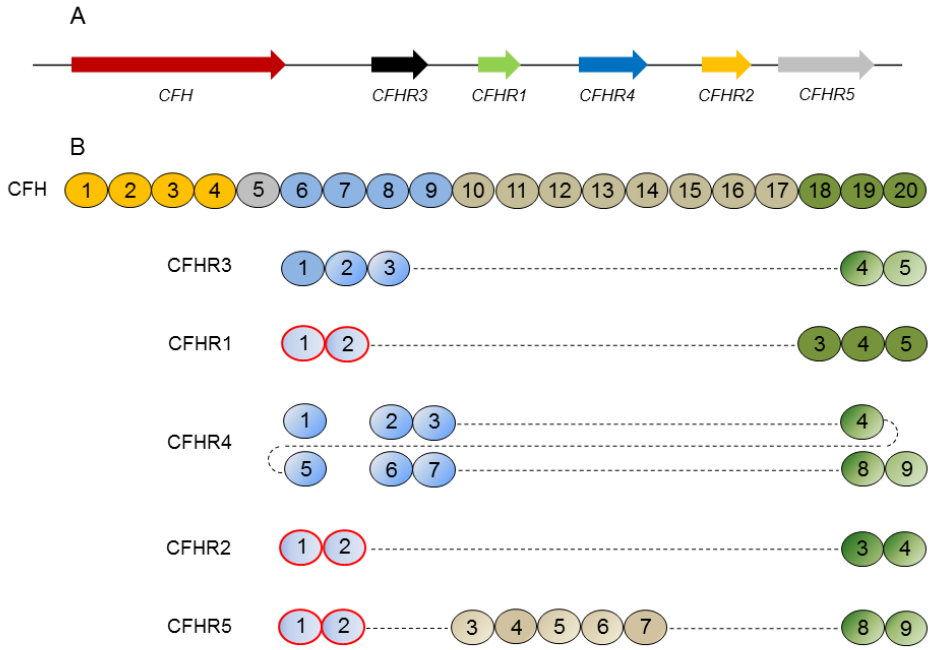

Figure 6. CFHR protein family. A. Genomic organization of *CFH* and *CFHR1-5* genes. Arrows show genes with their names. B. Structural organization of the CFH and CFHR proteins. Short consensus repeats (SCRs) are presented by ovals and are numbered from the N-terminal end. The colors of SCR domains reflect the degree of amino acid homology between CFHR and CFH domains. Dimerization domains are marked in red. CFHR4A is depicted. A 5 amino acid variant, CFHR4B, has also been described. Modified from[215, 217].

Membrane cofactor protein (CD46)

Membrane cofactor protein is a transmembrane glycoprotein present on the surface of most human cells with the exception of erythrocytes[219]. The extracellular segment consists of four SCRs, a highly glycosylated region, followed by 12 amino acids of unknown function, a transmembrane region and then one of two cytoplasmic tails[220]. The MCP protein acts as a cofactor for CFI - mediated cleavage of C3b.

Decay accelerating factor (CD55)

Decay accelerating factor consists of four SCRs and is anchored to the plasma membrane by a carboxy-terminal glycophosphatidylinositol (GPI) linkage[221]. It is expressed by most cells types and acts by accelerating decay of the C3 and C5 convertases.

Complement receptor 1 (CD35)

Complement receptor 1 is a transmembrane protein consisting of 30 SCRs expressed on many cells. It has both cofactor and decay accelerating activity[222].

Regulators of the terminal pathway

Protectin (CD59)

Protectin is a membrane-bound GPI-linked protein expressed by erythrocytes and most nucleated cells. It acts by binding to the C8/C9 components of the MAC thus preventing its insertion into the membrane[223].

Clusterin and vitronectin

Clusterin and vitronectin are fluid-phase regulators. Clusterin (apolipoprotein J) is a heterodimeric multifunctional protein expressed in a variety of tissues and cells. It forms high density lipid complexes in plasma and participates in the control of the lytic activity of the TCC. Together with vitronectin, clusterin binds to the amphiphilic C5b-9 complex, rendering it water soluble and lytically inactive[224]. Vitronectin (S-protein) is a multifunctional plasma and extracellular matrix protein binding predominantly to C5b-7, so that the newly formed SC5b-7 is unable to insert into cell membranes, and a lesser effect on the inhibition of C5b-9 lytic pore formation[225].

Complement regulators in the kidney

Complement regulatory proteins are present in the kidney. Endothelial and mesangial cells express membrane-bound regulators MCP, DAF and CD59. Podocytes express four membrane-bound regulators: MCP, DAF, CR1 and CD59. Both mesangial cells and proximal tubular cells also express and secrete soluble

CFH in cultured cells. Complement regulators in the human kidney are present in Table 2.

Glomerular basement membranes (GBM) are devoid of membrane-bound complement regulators and are thus dependent on soluble regulators. It has been proposed that the GBM attaches or absorbs regulators from plasma, such as CFH, CFHR1, clusterin and vitronectin[226]. The GBM exhibit a negative charge due to heparin sulphate proteoglycans such as perlecan and agrin containing sulfated glycosaminoglycans[227]. Glycosaminoglycans and other negatively charged macromolecules interact with positively charged recognition sites on CFH and enhance CFH binding to C3b[228]. CFH can bind to cell surface polyanionic molecules in the absence of C3b, although this binding is weak[229]. CFH was demonstrated in the GBM of normal human kidney. A transmembrane gradient across the GBM was found with maximal CFH concentrations on the vascular side and minimal on the urinary space side of the GBM[230]. CFH protection of the GBM from C3 deposition was clearly demonstrated in animal models as discussed below.

Table 2: Expression of complement regulators in human kidney

Renal cells	**Membrane-bound**				**Fluid phase**	**Ref**
	MCP (CD46)	DAF (CD55)	CR1 (CD35)	Protectin (CD59)	CFH	
Endothelial cells	+	+	-	+		231
Mesangial cells	+	+	-	+	+	231, 232
GBM	-	-	-	-	*	226, 230
Glomerular epithelial (podocytes)	+	+	+	+		231, 233
Tubular cells	+	+	-	+		231, 234

*from the plasma, GBM: glomerular basement membrane, MCP: membrane cofactor protein, DAF: decay-accelerating factor, CR1: complement receptor 1, CFH: complement factor H

As complement regulators are present in the kidney, a crucial question raised is why complement attack targets the kidney in certain kidney diseases. This question is addressed in the second part of this thesis.

C3 glomerulopathy

The pathophysiology and clinical phenotype

C3 glomerulopathy is a recently recognized clinical entity including rare types of glomerulonephritis: dense deposit disease (DDD) and C3 glomerulonephritis (C3GN). The key histological feature and the defining criterion for C3 glomerulopathies is isolated complement C3 deposition without significant immunoglobulin deposition in the glomerulus[235, 236].

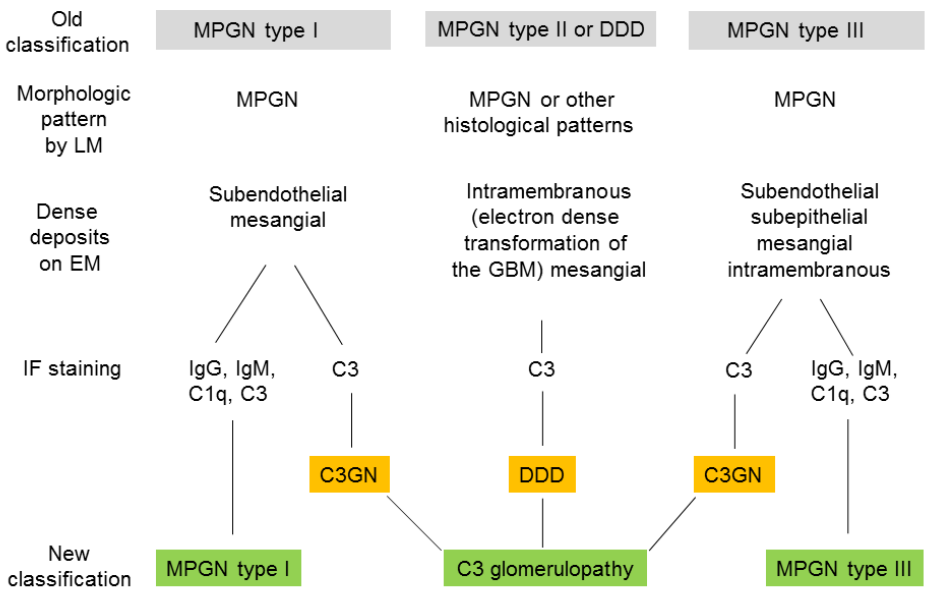

Figure 7. The evolving classification of C3 glomerulopathies (adapted from[237]). MPGN: membranoproliferative glomerulonephritis, LM: light microscopy, IF: Immunofluorescence microscopy, EM: electron microscopy, GBM: glomerular basement membrane.

Both DDD and C3GN may be associated with a membranoproliferative glomerulonephritis (MPGN). The membranoproliferative histopathological lesion is characterized by glomerular hypercellularity, increased mesangial matrix and thickening of the glomerular basement membrane with formation of double contours and subsequent thickening of the capillary wall[236]. These changes result from the deposition of immunoglobulins, complement factors and cellular debris. Based on electron microscopy and immunofluorescence findings membranoproliferative glomerulonephritis was, until recently, classified into three types: MPGN types I-III[236, 238] as depicted in Figure 7. MPGN I was characterized by subendothelial deposits, MPGN II by deposits within the glomerular basement membrane (GBM) and MPGN III by both subendothelial and subepithelial deposits. DDD was previously termed MPGN II but paucity or complete lack of immunoglobulin deposition, and the finding that the light microscopic pattern of the glomerular injury is not always membranoproliferative, have separated DDD from the immune complex-mediated diseases MPGN 1 and MPGN III. In MPGN I and MPGN III (with activation of the classical pathway of complement) glomerular C3 as well as immunoglobulin deposition are typical[236]. Glomerular lesions resembling MPGN I and MPGN III with isolated C3 deposits acquired the name C3 glomerulonephritis. Figure 7 depicts the old and the new classification.

The unifying pathophysiology of C3 glomerulopathies is dysregulation of the alternative pathway of complement. Dysregulation includes autoantibodies that promote excessive C3 convertase activity such as C3 nephritic factor (C3NeF), antibodies to CFH, CFB, as well as mutations in complement regulators or proteins CFH, CFI, MCP, C3 and genetic rearrangements in CFHR proteins[236] described below. These abnormalities trigger excessive C3 activation and increased generation of C3 breakdown products that deposit in the glomerular capillary wall[236].

Immunofluorescence microscopy shows intense staining for C3 in the glomerular capillary walls and in the mesangium in all forms of C3 glomerulopathies[236]. The histological glomerular lesion seen by light microscopy is heterogeneous as presented in Table 3[239].

Electron microscopy enables discriminating DDD from the other subtypes of C3 glomerulopathy. The essential diagnostic feature of DDD is electron dense transformation of the GBM containing dark ribbon-like deposits typically located within the lamina densa [236, 240]. The deposits may also be visualized in the mesangium, tubular basement membrane and Bowman's capsule. In contrast, in C3GN the electron dense deposits are present in the mesangium and/or in the subendothelial or subepithelial areas of the GBM[236]. Discontinuous

intramembranous deposits may be occasionally seen but without a ribbon-like appearance.

The composition of deposits was analyzed by mass spectrometry to identify the proteins in laser micro-dissected glomeruli from patients with DDD[241] and C3GN[242, 243]. These deposits contained components of both the alternative pathway and the terminal complement complex. C3, C9 and two fluid-phase regulators of the terminal complement complex, clusterin and vitronectin, were most extensively present in all glomeruli. C5, C6, C7, C8, CFHR1, CFHR5 were present as well but immunoglobulins were not. Using gold-conjugated antibodies immunelectron microscopy demonstrated the presence of C9 within intramembranous deposits and C3 within subendothelial deposits [235].

Table 3: Histological patterns of C3 glomerulopathy[236, 239]

Histolological pattern	Light microscopy features
Membranoproliferative	Mesangial hypercellularity, endocapillary proliferation (swelling of endothelium toward the capillary lumen) and capillary wall remodeling with mesangial interposition and duplication of glomerular basement membranes, lobular attenuation of the glomerular tufts
Mesangial proliferative	Focal segmental mesangial hypercellularity
Crescentic	Crescents involving > 50% glomeruli
Acute proliferative and exudative	Endocapillary proliferation with prominent infiltration of neutrophils

These diseases present clinically with symptoms of glomerulonephritis: proteinuria (sometimes nephrotic-range), hematuria, hypertension and renal impairment[242, 244]. Depleted plasma C3 levels are common findings due to C3 consumption while the C3 breakdown product C3dg is elevated[245]. In some patients elevated levels of TCC have been demonstrated[243, 246].

Patients with C3 glomerulopathy may also exhibit ocular drusen, a lipoproteinaceous deposition of complement containing debris, between the Bruch's membrane and the retinal pigmental epithelial cells[247, 248]. Drusen in DDD patients are often detectable in the second decade of life and lead to the long-term risk for visual problems in approximately 10% of cases[244].

Acquired causes of DDD

C3 nephritic factors are very common in patients with DDD. C3NeF is an autoantibody directed against neoepitopes on the newly formed C3 convertase[249, 250]. C3NeF stabilizes fluid-phase and cell-bound C3 convertase against spontaneous- and CFH-mediated decay by prolonging its half-life by approximately 10-fold[250]. The lack of glomerular immunoglobulin staining in C3 glomerulopathies indicates that C3NeFs are not deposited in the kidney but rather act in plasma. C3NeFs are a heterogeneous group of antibodies not entirely unique for C3 glomerulopathies. They are present in up to one half of patients with MPGN I and III[245, 251], in non-glomerular diseases such as acquired partial lipodystrophy[252] and have been detected in healthy individuals as well[246]. Their causative role in the pathogenesis remains unclear. C3NeFs may be detected in patients with normal C3 levels[245, 251, 253] and their fluctuating levels do not correlate with the course of glomerulonephritis[245, 254]. C3NeFs have also been detected in patients with mutations in complement genes[251].

An autoantibody against CFB has been described in one DDD patient[255]. Two patients have been reported lacking C3NeF with autoantibodies to individual components of the C3 convertase (both to C3b and CFB)[256]. Enhanced C3 convertase activity was demonstrated by higher levels of Ba and C3a after addition of purified immunoglobulins from the patients to normal human serum or by assembly of the C3 convertase in vitro.

Patients with DDD may have autoantibodies against CFH[246]. One such antibody was able to bind to CFH and cause activation of the alternative pathway in the fluid phase[257]. The binding site of this antibody was localized to SCR3 of CFH preventing the interaction of CFH with C3b[258].

Genetic causes of DDD

CFH mutations have been identified in patients with DDD[251, 259]. These mutations are homozygous or compound heterozygous and cluster at the N-terminus of CFH. They are localized in conserved amino acid residues that include cysteines forming disulfide-bridges, important for maintenance of the tertiary structure of the SCR module. Mutations result in an expressed but non-secreted protein which is accumulated intracellularly[260]. In a recent French study CFH mutations were identified in five of 29 patients with DDD[251]. Four patients with heterozygous mutations were positive for C3NeF.

SNPs (single nucleotide polymorphisms) and haplotype blocks act as susceptibility factors for the development of disease. The CFH-H1 haplotype *CFHcgcag* (-332C, c.184G, c.1204C, c.2016A, c. 2808G) is particularly associated with DDD[261, 262].

A C3 mutation has recently been described in familial DDD[263]. A heterozygous deletion of two amino acids in the *C3* gene resulted in a hyperfunctional C3 convertase resistant to CFH-mediated regulation. The C3b mutant was resistant to proteolysis by CFI in the presence of CFH, but was efficiently inactivated by CFI in the presence of MCP indicating fluid-phase alternative pathway dysregulation.

C3 is expressed as different allotypes at amino acid residue 102 termed C3S (slow running in agarose-gel electrophoresis) or C3F (fast running). The molecular basis of the S/F polymorphism is a single base change at the DNA level resulting in a single amino acid substitution at the protein level[264]. $C3_{R102G}$ has been shown to enhance activation of the alternative pathway by influencing CFH regulation[265]. A complotype (a combination haplotype) *CFH* p.V62I *CFH* p.Y402H, *C3* p.R102G, *C3* p.P314L has also been associated with DDD [266].

Animal models of DDD

Animal models have provided valuable insight into mechanisms of uncontrolled alternative pathway activation. The importance of CFH was first demonstrated in piglets (Yorkshire breed) that developed spontaneous DDD due to inherent CFH deficiency[267, 268]. Kidney lesions in affected piglets exhibited thickening of the glomerular capillary walls, mesangial cell proliferation and intramembranous dense deposits within GBM morphologically analogous to human DDD. Porcine DDD was caused by CFH deficiency due to a spontaneous homozygous I1166R mutation within SCR20 of *CFH* leading to intracellular protein retention[269]. Excessive complement activation was demonstrated by low C3 and high TCC levels in plasma and massive glomerular deposits of C3 and C5b-9 without immunoglobulins[270]. Affected piglets developed kidney failure leading to death. Animals treated with weekly plasma infusions exhibited improved survival.

Mouse models have been developed for the study of DDD. C*fh*$^{-/-}$ mice developed profound plasma C3 depletion and spontaneous murine DDD characterized by deposition of C3 and C9 on glomerular capillary walls, followed by the appearance of subendothelial electron dense deposits along the GBM which in turn preceded light microscopy changes typical of membranoproliferative

glomerulonephritis[271]. The chronology of the renal lesion suggests that uncontrolled C3 activation is the primary event leading to morphological lesions and renal disease in these mice. Administration of purified murine CFH to C*fh*$^{-/-}$ mice resulted in restored control of C3 activation in the fluid phase and reduction of C3 staining along the GBM within 24 hours. Moreover, the C3 degradation fragment iC3b was shown be the fragment of C3 that was deposited along murine GBM in glomeruli isolated using laser dissection microscopy[272].

In mice renal disease did not develop if C3 activation was blocked. This was demonstrated by introducing a second mutation in CFB. Normal C3 levels and a normal renal phenotype were observed in the *Cfh*$^{-/-}$*B*$^{-/-}$mice[271]. Furthermore, mice with combined CFH and CFI deficiency *Cfh*$^{-/-}$*fi*$^{-/-}$, despite uncontrolled complement activation, did not develop DDD because they could not degrade C3b by CFI. Administration of mouse CFI released C3 fragments in plasma and induced capillary wall C3 deposition. In support of animal data DDD has not been reported in humans deficient in CFI[273]. Renal transplantation experiments demonstrated that both wild-type donor kidneys and donor kidneys from mice with combined C3 and CFH deficiency developed capillary wall C3 staining when transplanted into the C*fh*$^{-/-}$ mice confirming that C3 deposits were derived from the circulation[274].

Murine DDD may develop when C5 activation is blocked. Mice deficient in both CFH and C5 and thus unable to activate the terminal complement pathway still develop disease albeit with less glomerular inflammation and reduced mortality[275].

Acquired causes of C3GN

Patients with C3GN may have circulatory autoantibodies with an underlying role in the pathogenesis. About a half of patients exhibits C3NeFs. Anti-CFH antibodies have also been reported[243].

Genetic causes of C3GN

Various mutations in complement regulators have been described in C3GN. These include mutations in the *CFH*, *CFHR* and *MCP* genes. In CFH a homozygous deletion (Δ_{K224}) of a lysine residue in SCR4 was reported[276]. The mutant protein exhibited reduced C3b binding and severely reduced cofactor and decay-accelerating activity[276]. Heterozygous mutations were reported as well[251,277]. Some

mutations were common between C3GN and aHUS suggesting that mutations in genes encoding regulators of the alternative pathway do not solely determine the disease phenotype.

A high degree of sequence homology between *CFHR* genes predisposes to genetic rearrangements such as duplications, deletions and the formation of hybrid genes. C3GN is associated with mutations and rearrangements in CFHRs or *CFHR* hybrid genes. CFHR5 nephropathy is a subtype of C3GN described in over 100 individuals of Greek Cypriot ancestry[278]. CFHR5 nephropathy is inherited in an autosomal dominant manner with a heterozygous mutation resulting in duplication of exon 2 and exon 3 in the *CFHR5* gene[279] (Figure 6). This may result in abnormal interaction with CFHR1, CFHR2 or wild-type CFHR5, competing with CFH for cell-bound C3b and impairing the cell surface protection from complement attack[216]. Clinical features differ from other C3 glomerulopathies and resemble those in IgA nephropathy. Chronic renal failure develops in about half of patients, predominantly in men. In contrast to other C3 glomerulopathies nephrotic-range proteinuria or low C3 levels have not been reported.

Likewise patients may have a CFHR1 mutation[217] affecting binding to C3b, iC3b and C3dg as well as enhanced competition with CFH for binding to C3b and iC3b. A CFHR3-1 rearrangement has been identified resulting in a *CFHR3-CFHR1* hybrid gene comprising exons 1, 2 and 3 from CFHR3 and exons 2,3,4,5 and 6 from CFHR1[280]. The hybrid protein was more potent in dysregulation of the alternative pathway[216].

CFI mutations have also been identified in patients with C3GN[251]. These mutations were previously reported in aHUS, one of them resulted in impaired secretion of the mutated protein and decreased cleavage of cell bound C3b[281].

An *MCP* gene mutation has been reported but the functional consequences have not been characterized[282]. A risk-associated MCP1 haplotype *MCPaaggt* (-652A, -366A, IVS9-78G, IVS12+638G, c.4070T) has been reported in C3GN[251].

Treatment of C3 glomerulopathies

Eculizumab has been proposed to be beneficial in C3 glomerulopathies based on dysregulation of the alternative pathway, the identification of C5b-9 in glomerular deposits and demonstrated amelioration of murine glomerulonephritis by prevented C5 activation[275].

In the largest published study 6 adult patients, 3 with DDD and 3 with C3GN were treated with eculizumab for a 1 year period[282]. Repeated kidney biopsies were performed in all patients. An improvement of clinical and/or histological parameters was observed in four patients, including three patients treated with eculizumab for recurrent disease after renal transplantation. Two patients (1 DDD and 1 C3GN) developed declining renal function during this treatment.

The follow-up renal biopsies showed reduced active glomerular proliferation and neutrophil infiltration in patients indicating effective C5 blockade which prevents generation of the chemoattractant C5a[282]. Deposition of C3 and C5b-9 were similar or reduced in the pretreatment and post-treatment biopsies[283]. An unexpected finding in biopsies after eculizumab treatment was strong staining for IgG not present prior the treatment. The presence of staining exclusively for IgG2, IgG4 and κ light chain, specific regions for eculizumab construct, suggested eculizumab binding and deposition in glomerular tissue with unclear clinical significance for long-term use.

The best response was seen in a patient with marked elevation of soluble sC5b-9 prior to initiating the therapy[282]. Other case reports confirmed that eculizumab may be more effective in a subset of patients with elevated sC5b-9[284].

Thus to summarize, lack of response to eculizumab in some patients indicates that prevention of C5 activation may not always be efficacious. Not even patients who respond to eculizumab treatment show uniform effects. For these conditions here may be a need for anti-complement therapies to prevent complement activation at the C3 level.

Soluble CR1 therapy in a child with DDD and end stage renal disease was shown to improve serum C3 and normalize sC5b-9 during short-term use. C3 dropped to pretreatment levels and sC5b-9 levels rose following withdrawal of therapy. In vitro experiments using serum from DDD patients showed that soluble CR1 prevented dysregulation of the C3 convertase even in the presence of C3NeFs. *Cfh*$^{-/-}$ mice transgenic for human CR1 treated with soluble CR1 exhibited normalized serum C3 levels and reduced C3 deposition in the kidneys indicating that regulation of the C3 convertase was restored in these mice[285].

Nonspecific treatment with renin-angiotensin system inhibitors to reduce proteinuria or immunomodulatory therapies such as corticosteroids, alkylating agents, calcineurin inhibitors as well as plasma exchange to remove autoantibodies or mutated complement proteins have been used with inconsistent results[244]. Most trials include patients with all types of MPGN making the analysis of treatment results more difficult to interpret. The monoclonal anti-CD20 antibody rituximab

has been used in attempt to deplete B lymphocytes for reduction of autoantibodies[256]. Immunosuppression with mycophenolate mofetil (MMF) has been attempted as well to reduce differentiation and maturation and of B lymphocytes[286]. Long-term plasma infusion has been reported as successful treatment in two siblings with familial C3GN related to circulating mutant CFH[287].

Atypical hemolytic uremic syndrome

The pathophysiology and clinical phenotype

The key histolopathological feature of aHUS is TMA in arterioles and capillaries predominantly in the kidney. Vessel walls are thickened due to swelling and detachment of endothelial cells from the basement membrane and accumulation of proteins and cellular debris in the subendothelial space. Vessel lumina are narrowed or occluded due to intracapillary thrombosis and congestion[143, 238]. C3 deposits along the GBM can be seen[238].

aHUS is associated with dysregulation of the alternative pathway of complement due to mutations in complement regulators CFH, CFI, MCP, clusterin, thrombomodulin, or complement factors C3 and CFB, genetic rearrangements in CFHR proteins or autoantibodies to CFH[288, 289]. Abnormalities of the alternative pathway have been reported in about 70% of cases[290]. They result in defective protection of endothelial cells and platelets against complement activation, amplified generation of the C3 convertase and secondary generation of the C5 convertase with activation of the terminal complement pathway. Increased generation of C3a, C5a and MAC at the endothelial cell surface cause cell damage with exposure of the subendothelial matrix and thrombus formation.[291]

Recently mutations not involved in the complement system have been described in the DGKE gene (encoding diacylglycerol kinase ε) indicating a new pathophysiologic mechanism of aHUS [292].

aHUS primarily affects children and young adults but it can present at any stage of life including newborns[290, 293]. In a recently published French series the onset of aHUS occurred frequently during adulthood [294].

Many patients exhibit a relapsing course of disease[288]. Hematological manifestations (hemolytic anemia and thrombocytopenia) cease to occur as renal failure progresses but recur upon renal transplantation, indicating that viable renal tissue contributes to disease activity. It is as yet unclear why the kidney is a target organ in this condition and why disease activity correlates to renal function.

Arterial hypertension is common and often severe. It has been proposed that arterial hypertension occurs due to vascular damage[295]. Patients may develop malignant hypertension leading to seizures, cerebral infarction eventually necessitating bilateral nephrectomy if uncontrolled.[296, 297]. Extrarenal, particularly neurovascular and cardiovascular manifestations, may occur including cerebral TMA[298] and dilated cardiomyopathy[299]. Most importantly, extrarenal manifestations have been reported in patients after bilateral nephrectomy indicating that low-grade complement activation may occur after removal of the primary target for microangiopathic lesions[297, 300]. Involvement of arteries has also been described suggesting that not only the microvasculature but also large vessels may be affected in aHUS patients[297, 301].

Genetic causes

aHUS is a complex disease that may involve a combination of multiple genetic and environmental risk factors. Patients may have more than one mutation in genes encoding complement factors[288,302]. Mutations in CFH, CFHR, CFI, MCP, clusterin, C3, CFB, and thrombomodulin genes have been described in aHUS. Some have exhibited phenotypic consequences with loss-of-function in complement regulators or gain of function in complement factors. In addition to mutations patients may have disease-associated polymorphisms in complement regulators. Disease penetrance in mutation carriers is approximately 50 %. Thus environmental factors, such as infections or pregnancy, may trigger the disease[290, 303].

The most thoroughly studied genetic variations were described in the *CFH* gene[304] in about 40 % of familial aHUS cases and in 10-20% of sporadic cases[291]. They are most often clustered at the C-terminal region of CFH, particularly in SCR 20[305]. These mutations impair CFH-mediated protection of cell surfaces against complement-mediated injury whereas fluid-phase regulation is unaltered. The C-terminal mutations exhibit reduced binding to C3b, heparin, endothelial cells and platelets[306, 307] resulting in complement activation on the endothelium and platelets[308]. Less commonly, aHUS-associated mutations affect the N-terminal region leading to defective binding to C3b or reduced cofactor activity for CFI[309]. Decreased plasma C3 levels are found in about one half of patients with heterozygous CFH mutations[288]. Some mutations are associated with decreased CFH levels, but many, including the majority of mutations in SCRs19 and 20, are associated with normal CFH levels in plasma indicating an isolated functional defect[288]. Most of the CFH mutations are heterozygous indicating that the healthy

allele is not sufficient to counteract the dysfunction of the mutated allele. In addition to mutations the CFH-H3 haplotype *CFHtgtgt* (-332T, c.184G, c.1204T, c.2016G, c.2808T) is significantly increased in aHUS patients[262].

Rearrangements or mutations in the *CFH/CFHR* gene cluster in aHUS may result in the loss of CFH functions on the cell surfaces. These include a hybrid *CFH/CFHR1*[310] gene and *CFH/CFHR3* gene[311]. Few *CFHR5* mutations and genetic variants have also been identified[312].

Mutations in *CFI* have been reported affecting 4-10 % of aHUS patients[290, 313]. All mutations are heterozygous, most commonly found in the serine protease domain. These mutations result in a quantitative deficiency of CFI or reduced capacity to degrade C3b in the fluid phase or on cell surfaces[314]. However, analysis of some CFI variants failed to demonstrate any functional consequence[281].

Mutations in the *MCP* gene, mostly heterozygous, account for 5-15 % of all mutations in aHUS[288]. Most *MCP* mutations cluster in the four extracellular SCRs domains that are critical for complement regulation[219]. Many patients have decreased MCP expression on white blood cells and quantitative deficiency in complement regulation. The MCP mutants exhibit low C3b-binding and decreased cofactor activity[219, 315]. One aHUS patient with an MCP mutation as well as a loss-of function mutation in the clusterin gene was described[316]. In addition, the MCP2 haplotype *MCPggaac* (-652G, -366G, IVS9-78A, IVS12+638A, c.4070C) is associated with an increased risk of aHUS[317].

Heterozygous mutations in the *C3* gene, usually with low C3 levels, occur in approximately 10% of aHUS patients[318]. Mutations may lead to gain-of-function of the C3 convertase by enhanced binding to CFB or indirectly by decreased binding to MCP[299, 319].

Mutations in *CFB* occur in 1-4 % of aHUS patients, all with low C3 levels[288]. Some of these mutations are heterozygous gain-of-function mutations leading to a hyperfunctional C3 convertase or increased resistance to inactivation by complement regulators[320, 321]. In addition, certain mutants had the unique ability to bind to iC3b and form iC3bBb mutant convertase that was functionally active[321]. The described mutations cluster in the VWA domain of CFB near the Mg^{2+} adhesion site termed MIDAS. This domain is critical for the interaction between CFB and C3b. One patient with a combined CFB and CFI mutation has been reported[302]. In addition to mutations, several polymorphic variants of CFB have been identified[290].

Thrombomodulin is an endothelial membrane-bound anticoagulant glycoprotein that accelerates CFI-mediated inactivation of C3b in the presence of CFH and indirectly inactivates the complement by-products C3a and C5a[322]. *THBD* gene mutations have been found in 3-5% of aHUS patients[290, 323] resulting in a loss of cofactor activity[322].

Disease prognosis varies depending on the type of mutation present. Patients with CFH mutations have the highest rate of ESRD or death during the first year after disease onset (50-70%) and for recurrence after renal transplantation (75-90%). Patients with MCP mutations have the best outcome with the rate of mortality and ESRD after the first disease episode being 0-6 % and the risk of recurrence after renal transplantation being <20%[288].

Animal models in the study of aHUS mutations

A mouse expressing mutant CFH protein lacking the C-terminal surface recognition domains FHΔ16-20 was designed to mimic the mutations commonly seen in aHUS[324]. These mice developed spontaneous HUS and renal pathology characteristic of TMA providing evidence that CFH mutations specifically impairing surface recognition result in HUS. The FHΔ16-20 mutant protein demonstrated impaired binding to heparin and endothelial cells but retained normal cofactor activity in vitro. CFH deficient mice expressing mutant FHΔ16-20 at approximately 30 % of wild-type mouse levels demonstrated significantly higher C3 levels in plasma compared to unmanipulated CFH deficient mice indicating that FHΔ16-20 protein lacking the C terminal was capable of regulating plasma C3 levels in vivo. The histopathology in these animals was characteristic of HUS not DDD. An expression of very low FHΔ16-20 protein (2% of wild-type levels) was insufficient to allow HUS to develop but sufficient to ameliorate DDD phenotype.

A critical role of C5 activation in the aHUS murine model was demonstrated when *Cfh*$^{-/-}$FHΔ16-20 mice were backcrossed with *C5*$^{-/-}$ mice. These mice they did not develop spontaneous HUS and showed normal renal histology suggesting that the absence of C5 protected them from development of aHUS[325]

Acquired causes

aHUS patients have circulating anti-CFH autoantibodies (5-10% of cases) and these represent a subtype of aHUS also called DEAP-HUS (*De*ficiency of CFHRs and *A*utoantibody *P*ositive form of *H*emolytic *U*remic *S*yndrome)[326-328]. These antibodies have been shown to block the C-terminal recognition domain of CFH (SCRs19-20) with similar consequences to those produced by CFH mutations[326, 327]. The majority of patients with anti-CFH antibodies have a complete deficiency of CFHR3-1 proteins secondary to a homozygous deletion Δ*CFHR3-1*[329, 330]. This deletion is also observed in the healthy population[331]. Thus a second trigger in addition to genetic predisposition is required in order to develop DEAP-HUS. Patients with anti-CFH antibodies may also have mutations in the complement genes summarized above[290, 328].

Treatment

Activation of C5 seems to be essential for the development of aHUS based on effective treatment with eculizumab. Blockade of the terminal complement complex with eculizumab prevents recurrence and promotes remission in the majority of treated patients[174, 332]. The beneficial response is observed in patients regardless of the type of mutation and even in patients without identified mutations in complement proteins. The preemptive use of eculizumab enables transplantation without recurrence in the majority of aHUS patients[333].

Plasma exchange is the first line treatment in patients with anti-CFH antibodies together with immunosuppressive drugs[288].

A human plasma-derived CFH concentrate[288] or recombinant CFH may be available in the future[334].

The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) plays a major role in control of blood pressure, fluid and salt balance as well as in the pathogenesis of several diseases including hypertension[335, 336]. Renin release is the first and rate-limiting step in the RAAS cascade[337]. Renin is secreted from the juxtaglomerular (JG) cells of the kidneys in response to reduction in blood pressure and renal perfusion, reduced plasma sodium levels, or upon β-adrenergic receptor stimulation[335]. The only known physiological substrate for renin is angiotensinogen. Renin cleaves angiotensinogen into the inactive peptide angiotensin I (Ang I) (Figure 8). Ang I is further activated in the circulation and tissues by angiotensin-converting enzyme (ACE) to form biologically active peptide hormones of which angiotensin II (Ang II) is the major effector of the RAAS system[338]. Ang II exerts its effects mainly via type-1 angiotensin (AT1) receptors and less via type-2 angiotensin (AT2) receptors. Ang II interacts with AT1 receptors inducing a number of rapid effects aimed at increasing blood volume in order to maintain blood pressure. These include vasoconstriction, increased aldosterone secretion and renal sodium reabsorption as well as sympathetic nervous system stimulation[339]. Ang II can, however, cause hypertension and tissue damage after prolonged stimulation, by inducing cell growth and proliferation, resulting in cardiac hypertrophy, vascular thickening, atherosclerosis, and glomerulosclerosis[340]. Activation of AT2 receptors mediates opposing effects such as vasodilatation and inhibition of growth.

Renin

Renin is synthesized by the JG cells of the renal afferent arterioles located at the vascular pole of the glomerular capillary network[341]. Renin is encoded by a single gene found on chromosome 1q42[342]. Renin mRNA is first translated into preprorenin containing 401 amino acids[335]. In the endoplasmic reticulum of the JG cell a 20-amino-acid signal peptide is cleaved from preprorenin, generating prorenin, which is packaged into secretory granules in the Golgi apparatus[343]. Prorenin is glycosylated and sorted to both the regulated and constitutive secretory

pathways. In the regulated pathway the inactive prorenin is converted to active renin in the dense secretory granules by release of the 43 amino acid N-terminal prosegment. Mature active renin is a glycosylated aspartyl protease with a molecular weight of approximately 44 kDa. Renin is released from the JG cells in response to cellular stimuli[335]. JG cells possess AT1 and AT2 receptors and circulating Ang II participates in a negative feedback mechanism to inhibit renin secretion by binding to these receptors[344] (Figure 8). Prorenin is secreted into the circulation constitutively.

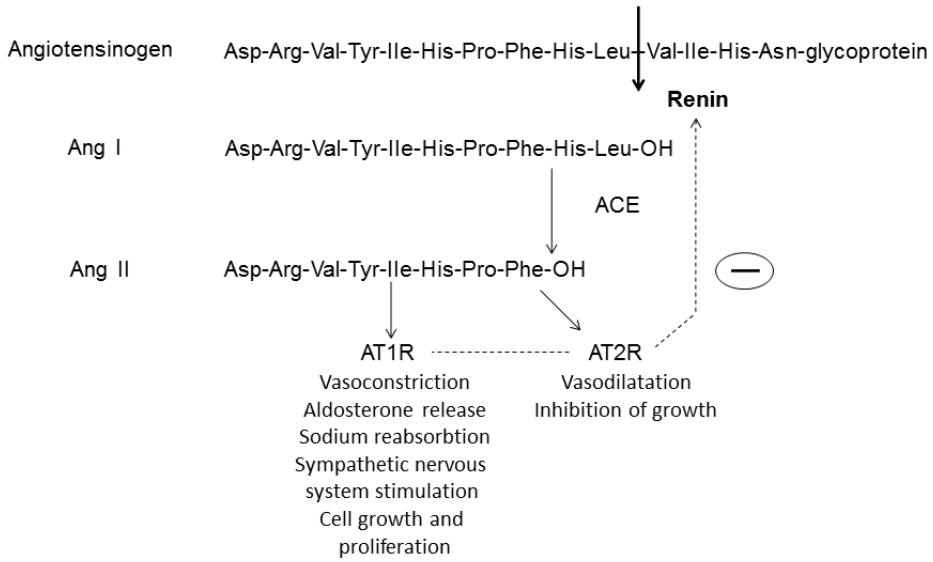

Figure 8. The RAAS system

Both renin and prorenin have been shown to bind to the (pro) renin receptor (P)RR[345] that enhances renin's catalytic activity and activates prorenin. When bound to (P)RR both renin and prorenin induce angiotensin-independent intracellular signaling pathways resulting in the production of profibrotic mediators[345, 346]. However, the role of (P)RR has been challenged recently, as most studies failed to demonstrate the role of (P)RR in cardiovascular and renal diseases or to identify it as a therapeutic target to optimize RAAS blockade[347, 348].

The half-life of renin in the circulation is from 30 to 90 min, metabolism and excretion occur via the liver with a minor degree occurring via the kidneys[335].

Complement effects on RAAS have been studied in obstructed kidneys in wild-type and C3-deficient mice[349]. In these mice increased renin expression was demonstrated in the tubules of wild-type but not in C3-deficient mice. C3a induced epithelial-to-mesenchymal transition of mouse renal tubular epithelial cells and subsequent renin expression in vitro. These results indicate that excessive C3 might be involved in the activation of the intrarenal renin-angiotensin system that induces injury and fibrosis.

Renin inhibitors

Renin is an enzyme belonging to the family of aspartyl proteases which includes pepsin, cathepsins D and E, and chymosin[350]. Each enzyme has two aspartic acid residues in the active site necessary for catalytic activity. Based on x-ray crystal structures the renin molecule consists of 2 homologous lobes with the active site located in the cleft between the lobes[351]. In human renin two aspartic acid residues, Asp32 and Asp215, each located in one lobe of renin, cleave the Leu-Val peptide bond within angiotensinogen to generate Ang I[352] (Figure 8). The active site of human renin contains a distinct sub-pocket S3sp not found in other aspartyl proteases or other species of renin[351].

Aliskiren

Aliskiren is a low-molecular-weight hydrophilic non-peptide that exerts a competitive inhibition on renin. Binding to the S3sp sub-pocket makes it a potent and specific renin inhibitor in primates[353]. The IC_{50} (the concentration at which it can inhibit 50% of renin activity) is 0.6 nM. Aliskiren has 10 000-fold higher affinity for renin than for other aspartyl proteases.

Aliskiren (Rasilez®, Novartis, Basel, Switzerland) is an orally active renin inhibitor approved for the treatment of hypertension[354]. It has also been successfully used to reduce proteinuria in diabetic[355] and non-diabetic nephropathy[356] as well as for the control of pediatric hypertension[357]. Renin inhibition as well as ACE blockade and Ang II receptor inhibition result in a reactive increase of plasma renin concentrations due to the removal of the normal feedback inhibition of Ang II on renin release (Figure 8). In contrast to ACE blockade and Ang II receptor inhibition plasma renin activity remains suppressed during treatment with aliskiren because cleavage of angiotensinogen is blocked[358]. Moreover, aliskiren is retained in the kidney for a prolonged period of time demonstrated by a sustained decrease in blood pressure after withdrawal of

aliskiren[359]. This observation has been confirmed in animal studies showing aliskiren accumulation in the kidney[360, 361].

Complement activation was studied in Ang II-induced vasculopathy in double transgenic rats harboring human renin and angiotensinogen genes (dTGR). These rats have high Ang II levels in the circulation and in the tissues and develop spontaneous hypertension, proteinuria and renal fibrosis. The study found that C3 and C5b-9 expression in the small vessels, glomeruli and tubular epithelial cells as well as the inflammatory IL-6 response preceded proteinuria in untreated dTGR rats. All these effects were reduced in animals treated with aliskiren. The authors concluded that the complement system was most likely activated by an indirect mechanism as Ang II triggered TNF-α, which then promoted the expression of complement components[362].

Pepstatin

Pepstatin is an inhibitor of aspartyl proteases originally isolated from cultures of various Actinomyces species[363]. Pepstatin is relatively weak inhibitor of renin with an IC_{50} of 320 nM against purified human renin[364]. It has been shown to lower blood pressure in monkeys[365] but no human studies have been reported.

Thrombotic microangiopathy and malignant hypertension

TMA may be associated with severe hypertension[295]. Vascular luminal narrowing with consequent renal hypoperfusion activates the RAAS, followed by vasoconstriction, an increase in arterial blood pressure and further exacerbation of renal ischemia. Chronic RAAS activation will lead to sustained endothelial injury and platelet aggregation thus worsening the TMA lesion[295]. Plasma renin activity may be high in patients with HUS[295]. High levels of renin-containing cells were found in the juxtaglomerular apparatus in renal biopsies from patients with TMA[366].

Malignant hypertension is characterized by severe hypertension and organ damage including hypertensive retinopathy[367]. Malignant hypertension may be complicated with TMA. Microangiopathic hemolytic anemia (MAHA) may occur in up to one third of patients[368, 369]. Malignant vascular disease of the kidney is characterized by endothelial cell injury, arteriolar involvement and glomerular ischemia[295]. The endothelial injury manifests with swelling and detachment of the cells from the GBM with subendothelial accumulation of amorphous material. The arteriolar injury exhibits intimal proliferation and hyperplasia leading to luminal narrowing or obliteration. In vascular walls areas of necrosis or thrombosis are present. Collagen deposition and elongated myointimal cells arranged in concentric whorled patterns give characteristics of an “onion skin”-like configuration leading to irreversible narrowing of the vascular lumina. Preglomerular arterioles are predominantly involved. Secondary glomerular ischemia may result in retraction of the tuft with thickening and wrinkling of the capillary wall[295].

Although the exact mechanism by which malignant hypertension induces the TMA lesion has not yet been fully elucidated, endothelial cell damage with narrowed microvasculature and enhanced shear stress may play a role[295, 370]. In the acute phase RAAS is activated in most patients with malignant hypertension[371, 372]. The importance of RAAS in malignant hypertension was demonstrated in experimental animal models. In stroke-prone spontaneously hypertensive rats RAAS was activated with salt-load and accompanied TMA with fibrinoid necrosis in renal small arteries and arterioles[373]. ACE inhibitors were shown to ameliorate

this effect[374]. A renal model of TMA was developed in transgenic mice over-expressing human renin and human angiotensin in addition to a high salt diet and/or the nitric oxide synthetase inhibitor. These mice developed severe vascular and glomerular lesions similar to those found in patients with malignant hypertension[375].

The present investigation

Aims

- To investigate the presence of colonic injury and apoptotic cell death in HUS patients and to correlate intestinal cell death to the presence of Shiga toxin as well as intimin, Tir, Esps A, B, and D in a mouse model

- To determine the humoral response to EHEC intimin, EspA and EspB in patients with EHEC infection and their household members and to develop a serological assay that was not serotype-dependent

- To investigate renin cleavage of C3 as a kidney-specific mechanism of complement activation and attempt to inhibit this in patients with complement-mediated kidney disease

- To describe ongoing vascular damage in an anephric aHUS patient and the possibility that complement-mediated cerebrovascular disease occurred in the absence of HUS recurrences

Experimental conditions and results

Paper I: Intestinal damage in enterohemorrhagic Escherichia coli infection

The main questions investigated in this study were if colonic cell death due to EHEC occurs in patients with HUS and which EHEC virulence factors cause intestinal cell death and symptoms in a mouse model.

Colonic tissues from two children with hemorrhagic colitis and HUS and pediatric controls were studied by light microscopy and TUNEL assay for detection of apoptotic cell death. Both patients developed colonic perforation and underwent partial sigmoidectomy. One pediatric control underwent resection of a part of the

sigmoid colon and two pediatric controls underwent colonic biopsies. Sigmoid tissue from the HUS patients exhibited focal inflammatory infiltrates, hemorrhages, focal necrosis with cellular granulation tissue penetrating the muscularis propria as well as abundant TUNEL-positive cells in the lamina propria. Control tissues revealed normal morphology and few apoptotic cells. In order to determine which EHEC virulence factors contribute to intestinal damage and cell death we used a previously described mouse model of EHEC infection[120].

C3H/HeN mice were infected with Stx-producing wild-type *E. coli* O157:H7 strain (86-24), an isogenic non-Stx producing *E. coli* O157:H7 strain (87-23), and two mutant strains on the 86-24 background, an *eae* mutant lacking intimin (UMD619) and an *escN* mutant lacking Tir and *E. coli* secreted proteins A, B, and D due to a defective TTSS. In addition two strains complemented with the deficient genes (*eae* and *escN*, respectively) on a plasmid were used. Wild-type strains 86-24 and 87-23 were not antibiotic-resistant and antibiotics were not given to mice in this study to allow the presence of the commensal flora in the intestine. All strains were passaged through mice to enhance virulence. Mice were monitored for symptoms. Fecal samples were collected to evaluate the bacterial burden. Colons were collected for histopathological analysis and detection of cell death was carried out by a TUNEL and caspase-3 assay.

The most severe symptoms occurred in mice infected with the Stx-producing wild-type strain, followed by the non-Stx producing strain. Fewer mice infected with the *eae* mutant strain and no mice infected with the *escN* mutant strain developed severe symptoms. Mice infected with strains complemented with the deficient genes developed symptoms comparable to the wild-type strains. The highest bacterial burden was found in mice infected with the Stx-producing wild-type strain (86-24), followed by the non-Stx producing strain (87-23) whereas mutant strains caused minimal colonization. Mice inoculated with the Stx-producing wild-type strain developed significantly more intestinal cell death compared to mice inoculated with the other strains.

Paper II: Antibodies to intimin and Escherichia coli secreted proteins A and B in patients with enterohemorrhagic Escherichia coli infections

The main question investigated in this study was if patients with EHEC infection develop a humoral response to specific LEE- associated EHEC adhesins regardless of serotype and if these antibodies could be used for serodiagnostics

We investigated the serum antibody responses to *E. coli* O157:H7 antigens intimin and *E. coli* secreted proteins (Esps) A and B. Intimin, EspA and EspB were

recombinantly expressed. Serum IgA, IgG, and IgM antibodies against intimin, EspA and EspB were assayed by immunoblotting. Canadian patients with *E. coli* O157:H7 infection (n=10), Swedish patients with *E. coli* O157:H7 (n=21), non-O157 (n=18), or infection from which the *E. coli* serotype was not available (n=3), and asymptomatic household members (n=25) were studied and compared with Canadian (n=20) and Swedish controls (n=52). Non-O157 serotypes included *E. coli* O145, O26, O121, O103 and O8. The patients' diagnoses comprised HUS (n=40), hemorrhagic colitis (n=11) and diarrhea (n=1).

We demonstrated that Swedish and Canadian children infected with EHEC (n=52) mounted an antibody response to recombinant intimin, EspA and EspB indicating that these antigens are expressed during infection in vivo. Significantly more patients developed an acute response to EspB compared with controls. IgA antibodies to EspB showed a 100% specificity and 57% sensitivity in the Swedish cohort. Antibodies were even found in asymptomatic household members of the patients.

Paper III: Renin cleavage of C3: a kidney-specific mechanism of complement activation

The main question investigated in this study was if renin released in the kidney could trigger activation of the alternative pathway explaining the propensity of certain complement mediated-diseases for the kidney.

Two patients with dense deposit disease (DDD) associated with activation of the alternative pathway of complement (with circulating C3 nephritic factor and low C3 levels) were treated with the renin inhibitor aliskiren (Rasilez). Clinical improvement was documented as well as a stabilizing effect on C3 levels. In addition, renal biopsy from one of the patients showed considerably less C5b-9 deposition in glomerular capillary walls and reduced thickness of the glomerular basement membrane compared to a biopsy taken before the start of Rasilez treatment. The beneficial effect of aliskiren lead to the hypothesis that renin, a kidney specific enzyme, activates the complement system in the kidney.

Incubating C3 with renin obtained from three separate sources (plasma, kidney and recombinant) resulted in C3 cleavage into C3b and C3a. C3b was detected after SDS-PAGE by silver or Coomassie staining and immunoblotting. The release of C3a was demonstrated by ELISA.

A plasma sample was available from an anephric patient with a very low renin and normal C3 levels and was incubated with renin. Using immunoblotting no C3

cleavage by renin was detected in this patient and a healthy control suggesting that the cleavage was prevented in the presence of plasma. Furthermore, cathepsin D, which in similarity to renin is an aspartyl protease inhibited by aliskiren, did not cleave C3.

N terminal sequencing of the cleaved product confirmed that renin cleavage of C3 occurred at the same site as C3 convertase-induced cleavage because the N terminal nine amino acids of C3b were identical.

We demonstrated that C3 cleavage by renin could be blocked using three renin inhibitors, the specific renin inhibitor aliskiren, an inhibitor of aspartate proteases pepstatin and zinc. The inhibitory experiments were assayed by SDS-PAGE followed by silver staining or immunoblotting.

Functional analysis of the C3 interaction with renin was investigated by surface plasmon resonance (BIAcore) technology. Anti-C3c antibody was first immobilized on a sensorchip flow chamber. The first experiment was designed to determine whether C3b and C3a were formed after C3 cleavage by renin. Injecting an anti-C3b antibody, that specifically recognizes the C3b neoepitope formed after C3 cleavage, binding was detected to the C3- cleavage product captured on the sensorchip but not to C3 alone. The C3a antibody did not bind to the cleavage product confirming that C3a was released and washed away. The second experiment was designed to determine if the C3-cleavage product was capable of binding CFB. Increased CFB binding was demonstrated to the renin cleavage product compared to uncleaved C3. CFD could recognize the CFB in the C3bB complex and cleaved it. This interaction was confirmed by ELISA showing that C3 cleaved by renin bound to CFB and factor Ba was released in the presence of CFD.

Paper IV: Eculizumab in an anephric patient with atypical hemolytic syndrome and advanced vascular lesions

The main question investigated in this study was if complement-mediated vascular damage could occur in aHUS in the absence of kidneys and of disease recurrences and if eculizumab could stabilize the clinical course.

We described a currently 12-year-old girl who presented with aHUS at 20 months of age, developed renal failure 3 months later, underwent bilateral nephrectomy and renal transplantation but lost the graft due to recurrences of aHUS. HUS recurrences post-transplantation were associated with malignant hypertension resulting in removal of the transplant at 4 years of age. She was thereafter on

hemodialysis for 7 years. Treatment with plasma exchange and infusions was terminated after transplant nephrectomy and no hematological recurrences of HUS (hemolytic anemia and thrombocytopenia) occurred during this period.

At 10 years of age she developed a transient ischemic attack (TIA). Imaging of her carotid arteries demonstrated total occlusion of the right carotid artery and near-occlusion of the left carotid artery. Ongoing complement activation led us to the hypothesis that complement activation was inducing the vascular injury and prompted treatment with eculizumab in the absence of HUS manifestations and viable renal tissue. On this treatment she underwent a successful kidney transplant 11 months after the TIA without recurrence of HUS more than one year later. No progression of vascular occlusion was noted by repeated imaging during this period.

Genetic sequencing revealed two complement mutations and the *CFH* H3 disease-associated haplotype. The heterozygous G261D mutation in the *CFI* gene has been previously described. A novel heterozygous mutation was found in CFB, L433S. The L433S mutation was further characterized in this study.

Recombinant CFB L433S mutant was expressed in human embryonic kidney (HEK-293T) cells and shown to be secreted at the same level as the wild-type protein. The L433S mutation is located in proximity to other previously described gain-of-function aHUS CFB mutations. However, this mutation did not exhibit increased binding to C3b as assayed by ELISA and surface plasmon resonance. L433S did not induce increased C3 deposition on cytokine-activated human umbilical cord veins cells (HUVECs). In conclusion, no functional defect could be demonstrated for this mutation.

Discussion

Intestinal injury is a primary event in EHEC infection allowing the systemic spread of bacterial virulence factors in spite of lack of bacteremia.
In Paper I we demonstrated abundant cell death assayed by the TUNEL technique in two pediatric patients with HUS who had a prolonged course of disease and developed colonic perforation. In an attempt to define which EHEC virulence factors contributed to intestinal cell death we infected mice with a Shiga toxin-producing wild-type strain and non-Stx producing strain as well as mutants not expressing intimin or TTSS.

The most pronounced colonic cell death was observed in mice infected with the Stx-producing wild-type strain, which revealed the highest bacterial burden. Less cell death was found in colons of mice infected with mutant strains that exhibited minimal colonization. Lesser colonization could have reduced exposure to Stx in these mice.

Stx-producing and non-producing wild-type strains exhibited better colonic persistence and more symptoms, presumably due to the presence of intimin and TTSS effectors. The observation that the non Stx-producing strain also exhibit virulence has been described previously[120] and indicates that *E. coli* O157:H7 possesses multiple virulence factors.

A difference in the localization of cell death was noted in human and murine intestines. In the human sigmoid colon TUNEL-positive cells were predominantly found in the lamina propria and in the vasculature. In the mouse colon they were most frequently observed in the epithelial cells and mucosa-associated lymphatic tissue. These differences could be related to Stx-receptor localization, as the human intestinal microvascular endothelial cells express high levels of the receptor. Moreover, differences could be related to expression of symptoms in humans and mice. Mice did not develop hemorrhagic colitis whereas patients exhibited severe and prolonged hemorrhagic colitis with presumably an increased cytokine response, which might have contribute to the extensive injury observed in their sigmoid colon.

In summary, we demonstrated abundant cell death, at least in part, due to apoptosis, in colons of HUS patients. We suggested that profound injury to the intestinal mucosa may allow bacterial virulence factors to gain access to the circulation. In a mouse model we showed that Shiga toxin promoted bacterial colonization and intestinal cell death and contributed to severe symptoms. Intimin and intact TTSS were necessary for intestinal colonization and virulence in this mouse model. We proposed that *E. coli* O157:H7 adhesins and secreted proteins could be targeted as potential vaccines against bacterial colonization and intestinal injury.

The diagnosis of EHEC infection is based on isolation of the bacterial strain from the feces but in some patients this is not possible due to antibiotic treatment or if fecal cultures are taken long after the onset of symptoms. Serological detection enables the identification of the main serotype *E. coli* O157:H7 and only a limited number of non-O157:H7 strains. Over 100 different serotypes relevant to human disease have been identified. Thus, a serologic diagnostic assay, which would permit detection of EHEC infection regardless of serotype, is required.

In paper II we demonstrated that patients with EHEC infection, including hemorrhagic colitis and HUS, developed an antibody response to EHEC antigens intimin, EspA and EspB regardless of the infecting serotype. EspB appeared to be the most appropriate assay for the detection of recent EHEC infection based on statistical comparison, specificity and positive predictive value.

Person-to-person transmission has been described and household members of a patient with EHEC infection may be infected as well. The majority of asymptomatic household members in our study developed an antibody response to EspA and EspB indicating that antigen expression does not necessary lead to clinical symptoms.

The presence of IgA antibodies to EspB in HUS patients correlated with the severity of intestinal and renal disease. We assume that IgA is generated in response to release of EspB in the intestine and a higher antigenic dose may stimulate a greater immune response. The intestinal inflammatory response may trigger the systemic host response, which together with bacterial virulence factors may contribute to a more severe disease. In asymptomatic household members other defense mechanisms are presumably involved inhibiting the development of symptomatic infection.

In summary, we developed an assay that allows detection of EHEC infection regardless of serotype. This assay may be useful for diagnostic purposes when a fecal strain has not been isolated or during epidemics.

Certain kidney diseases such as DDD and aHUS are associated with activation of the alternative pathway of complement. The factor triggering complement activation in the kidney is unknown. Why is the kidney a target organ in these conditions?

In paper III we demonstrated, for the first time, that the kidney specific enzyme renin cleaved the main complement protein C3 in vitro. We also demonstrated that C3 cleavage by renin was blocked by renin inhibitors. Furthermore, the renin inhibitor aliskiren exerted a stabilizing effect on complement activation in two patients with DDD. Thus we suggest that renin may trigger complement activation in the kidney.

To date the only known substrate of renin is angiotensinogen. In this study we identified C3 as a novel substrate of renin and showed that renin-mediated C3 cleavage products were identical to those induced by the C3 convertase in vitro. We demonstrated that renin cleaves C3 into its physiological cleavage products C3a and C3b, and that the formed C3b is capable of binding CFB, thus forming

the C3 convertase which could activate the amplification loop of the alternative pathway.

We propose that complement activation could be triggered in the kidney by renin cleavage of C3 and amplified via the alternative pathway, which would proceed uninhibited when complement is dysregulated due to mutations or circulating antibodies, thus explaining why certain complement-mediated diseases affect the kidney. This mechanism initiates complement activation independently, with specific tropism for the kidney, as renin concentrations are higher in the kidney than in the systemic circulation[376].

Malignant hypertension with high renin levels may cause TMA. We hypothesize that elevated renin levels could trigger complement activation on damaged endothelial surfaces. In the presence of normally functioning complement factors and inhibitors this activation will be hindered, but will be able to proceed when complement regulation is dysfunctional, such as in the presence of C3 nephritic factor or mutations.

The risk for recurrence of DDD after renal transplantation is very high and thus the development of specific treatment is crucial. The complement inhibitor eculizumab has been used in certain patients and its beneficial effect may be related to blocking complement activation at the level of C5 although it has not been effective in all cases[282]. In a mouse model prevention of C5 activation ameliorated but did not abrogate the disease[275]. For DDD patients there is an obvious need for a treatment that offers complement control at the C3 level. We suggest that the renin inhibitor aliskiren provided a beneficial effect on complement activation. Animal studies have shown that aliskiren accumulates in the kidney, which could be favorable for prevention of local complement activation. This effect on complement activation was previously demonstrated in rats harboring the human renin gene[362] although the mechanism was not associated with a direct effect of renin.

In our study renin cleavage of C3 did not occur in the presence of plasma. Plasma contains multiple inhibitors, and even the C3 convertase is inhibited by multiple regulators. The exact mechanism by which renin is inhibited in plasma is unknown but the regulation of renin's effect on C3 in the systemic circulation should be advantageous to prevent excess complement activation.

In summary, we demonstrated a novel kidney-specific mechanism of complement activation and propose that renin inhibition may reduce complement activation and improve the course of disease in certain cases of complement-mediated renal disease.

In paper IV we demonstrated that a patient with aHUS developed severe systemic vascular damage in the absence of kidneys and HUS recurrences. The patient was treated with eculizumab in order to prevent further progression of severe vascular lesions, and this is, to our knowledge, the first aHUS patient treated with eculizumab in the absence of renal tissue.

Patients with aHUS develop symptomatic disease and recurrences as long as there is residual renal function as well as after renal transplantation, indicating that viable renal tissue triggers disease activity. We present evidence of cerebrovascular occlusive disease occurring in an anephric patient and challenge the dogma that complement activation in aHUS occurs only as long as there is viable native or graft renal tissue. Moreover, we show that aHUS is not only a disease of the microvasculature and that involvement of large arteries may also occur. One other aHUS case with involvement of large arteries in an anephric patient has been previously described[297]. Our patient exhibited low C3 and elevated C3dg levels at presentation, after removal of all renal tissue, before and during eculizumab treatment while the patient was on hemodialysis. We suggest that the advanced occlusion and stenosis in the patient's carotid arteries was due to low-grade ongoing complement activation progressively injuring the vascular endothelium. Other risk factors such as prolonged hemodialysis treatment and uremia-related risk factors may contribute to the development of vascular damage in aHUS patients. There has so far been no evidence for disease activation in anephric patients and therefore eculizumab has only been given to patients with residual renal function. We propose that even patients with reduced, or lack of, renal function could benefit from complement inhibition treatment.

In addition we describe a novel CFB mutation and its characterization. Few CFB mutations have been described in aHUS and these were demonstrated to lead to gain-of-function and excess complement activation. To-date no mutations in CFB have been reported that do not lead to gain-of-function. Very few publications have addressed the issue of mutations that do not alter the in vitro function of the derived mutant, which may represent a publication bias.

In summary, we suggest a pathogenic mechanism whereby low-grade constant complement activation will lead to vascular damage in aHUS patients. We propose that extra-renal vascular lesions may progress even during symptom-free intervals and that imaging for vascular changes, particularly cerebral, should be monitored. For this reason performing transplantation should be considered as early as possible in aHUS patients with end-stage renal failure. Eculizumab protection, during periods of dialysis and after renal transplantation, might prevent serious vascular damage in patients with aHUS.

Conclusions

- Abundant apoptotic cell death in the intestine occurs in patients during EHEC infection.

- Intimin and intact type III secretion system are required for effective colonization and virulence of EHEC, Shiga toxin promotes colonization, intestinal apoptotic cell death and severe symptoms in mice.

- Patients develop antibody response to EHEC antigens intimin, EspA and EspB regardless of the infecting serotype. Antibodies against EspB are the most specific for detection of recent EHEC infection.

- Renin inhibition reduced complement activation and stabilized the clinical cause in two patients with DDD.

- Renin cleaves C3 into C3a and C3b in a manner identical to C3 convertase in vitro and this cleavage is inhibited by the renin inhibitor aliskiren suggesting a novel kidney-specific mechanism of complement activation.

- Low-grade constant complement activation may lead to vascular damage in aHUS in the absence of kidneys and aHUS recurrences. These patients may benefit from complement inhibition treatment.

Populärvetenskaplig sammanfattning

Hemolytiskt uremiskt syndrom (HUS) är den vanligaste orsaken till njursvikt bland barn i västvärlden. HUS kännetecknas av hemolytisk anemi (sönderfall av och brist på röda blodkroppar), trombocytopeni (brist på blodplättar) och akut njursvikt.

Den vanligaste formen av sjukdomen, typisk HUS orsakas av Shigatoxinproducerande bakterier, främst olika stammar av Escherichia coli (E. coli) som kallas enterohemorrhagiska E. coli (EHEC). Över hundra olika varianter (serotyper) av EHEC har isolerats från patienter med HUS, men den vanligast förekommande serotypen är E. coli O157:H7. EHEC-infektioner kan förekomma sporadiskt eller i utbrott. När någon smittas av EHEC-bakterien ”bosätter” den sig i tarmslemhinnan. För att binda till celler i tarmen producerar bakterien olika bindningsstrukturer (adhesiner) som t.ex. intimin och så kallade E. coli secreted proteins (Esp). EHEC invaderar inte tarmcellerna, utan binder endast till dem. När bakterien har koloniserat tarmen frisätter den ett gift som kallas Shiga-toxin. Shigatoxinet är den viktigaste faktorn som skadar tarmcellerna och orsakar blodig diarré vilket ger toxinet tillträde till blodbanan. Bundet till blodceller når Shigatoxinet sina målorgan. Cellreceptorn för toxinet hos människor finns i de små blodkärlen (kapillärer), i framför allt i njuren och i hjärnan. Shigatoxinet kan aktivera blodplättar som då har lättare att klumpa sig. Tillsammans med skadan i kapillärväggen leder processen till bildning av små blodproppar. Minskat eller blockerat blodflöde resulterar i organsvikt.

En mindre vanlig form av HUS kallas atypiskt HUS (aHUS). Den är förknippad med gendefekter (mutationer) i speciella äggviteämnen (proteiner) i den del av vårt immunförsvar som kallas komplementsystemet. Komplementsystemet är en grupp av proteiner som aktiveras via klassika vägen, lektinvägen eller alternativa vägen i en kaskadliknande reaktion med olika biologiska effekter som bidrar till immunförsvaret och avdödning av bakterier. Vid aHUS är komplementsystemets alternativa väg aktiverad. Överdriven komplementaktivering kan leda till skador på celler/vävnader p.g.a. ökad inflammation. Normalt regleras komplementsystemet av kontrollproteiner som kallas komplementhämmare som finns bundna till cellytor eller cirkulerar lösliga i blodet. Genförändringar i dessa

proteiner eller förvärvade antikroppar kan leda till ohämmad komplementaktivering och aHUS.

Mitt intresse fokuseras på: hur uppstår tarmskadan vid EHEC-infektion och hur leder den till njurskador? Varför drabbas just njuren vid komplementförmedlade njursjukdomar? Vid avsaknad av njuren vilka systemiska skador uppkommer då? Ökad förståelse och kartläggning av dessa skeenden kan i bästa fall medföra nya behandlingsmöjligheter för patienterna.

I den första studien undersökte vi olika virulensfaktorer från EHEC-bakterien som kan orsaka tarmskada. Vi påvisade utbredd celldöd i tarmen i prov från två barn med HUS. För att undersöka vilka bakteriella virulensfaktorer som bidrog mest till tarmskadan användes en musmodell. Mössen infekterades med EHEC (vildtyp) samt mutanter som saknar Shigatoxin, typ III sekretionssystem (som inte kan producera Esp A, B, D) eller intimin. Stammar som saknade förmågan att binda till celler i tarmen gav betydligt mindre symptom och tarmskada. Shigatoxin var det viktigaste virulensfaktor för uppkomst av fullständig sjukdomsbild och celldöd i tarmen.

I den andra studien undersökte vi antikroppsvar hos patienter med blodig diarré och HUS. Diagnostik av EHEC infektioner baseras på isolering av stammen från avföringsprov. Hos vissa patienter är detta emellertid inte möjligt, antigen för att det har gått för lång tid sedan insjuknandet eller på grund av antibiotikabehandling. Serologisk diagnostik är i så fall en möjlighet, riktad mot lipopolysackarider (LPS) på ytan av bakteriestammen. Denna analys är serotypspecifik. En serologisk diagnostisk metod som inte är serotypspecifik har utvecklats i detta delarbete.

I den tredje studien har vi undersökt om ett njurspecifikt ämne kan aktivera komplementsystemet. Resultaten är under publicering.

I den fjärde studien beskrev vi en flicka med aHUS och genförändringar i flera komplementfaktorer. Genförändringen i faktor B var tidigare okänd. Vi genomförde funktionsstudier, men någon defekt funktion kunde inte påvisas. Patientens njurar var bortopererade och under dessa betingelser anses det att sjukdomsaktiviteten borde vara latent. Vi visade att patienten utvecklade, under lång tid, uttalade kärlskador i stora kärl som ledde till neurologiska symtom. Behandling med eculizumab, en antikropp som blockerar den terminala komplementvägen, stabiliserade patientens tillstånd inför njurtransplantation. Detta är den första beskrivningen av behandling med eculizumab på en patient som saknar njurar. Vi föreslår att man följer patienter med aHUS i avseende på kärlskador och behandlar mot komplementaktivering även i avsaknad av njurar.

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